

**CYANIDE DEGRADATION BY *Bacillus pumilus* C1:  
CELLULAR AND MOLECULAR  
CHARACTERIZATION**

by

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A thesis submitted in partial fulfillment of the requirements for the degree of  
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## ABSTRACT

A cyanide-degrading, Gram-positive, aerobic, endospore-forming bacterium was isolated from a cyanide wastewater dam by an enrichment technique and was identified as a strain of *Bacillus pumilus*. The bacterium was routinely cultured in Oxoid nutrient broth and rapidly degraded 100 mg/l of free cyanide in the absence of added inorganic and organic substances. The ability to degrade cyanide was linked to the growth phase and was not exhibited before late-exponential/early-stationary phase. Cyanide-degrading activity could not be induced in early exponential phase by the addition of cyanide or acetonitrile to 20 mg/l. Production of the cyanide-degrading activity required at least 0.01 mg  $Mn^{2+}$ /l in the growth medium (lower concentrations prevented the development of strong cyanide-degrading activity and also resulted in poor growth of the organism). No induction of cyanide-degrading activity occurred when  $Mn^{2+}$  ions were added to late-exponential-phase cells (or a cell-free extract from these cells) which had been grown with a low endogenous concentration of  $Mn^{2+}$ . However,  $Mn^{2+}$  ions could be added to cultures growing in low- $Mn^{2+}$  broth as late as the mid-exponential phase of growth with no apparent reduction of the cyanide-degrading activity exhibited in stationary phase. Culturing the organism in Difco nutrient broth resulted in poor growth and very low levels of cyanide-degrading activity; addition of  $Mn^{2+}$  to this medium did not significantly increase the levels of activity. Production of the cyanide-degrading activity required *de novo* transcription and translation. Cyanide-degrading activity was located

intracellularly and cell-free extracts rapidly degraded cyanide ( $0.27 \pm 0.08$   $\mu$ mole cyanide/min/mg protein at 30 °C in pH 7.4 phosphate buffer). Eight strains of cyanide-utilizing fluorescent pseudomonads were also isolated from activated sewage sludge by an enrichment technique and tentatively identified as strains of *P. fluorescens* and *P. putida*. These isolates could not degrade cyanide rapidly.

A cyanide-degrading enzyme was purified from *B. pumilus* C1 by column chromatography and sucrose-gradient centrifugation, and characterized with respect to physico-chemical and kinetic properties. The enzyme consisted of three polypeptides of 41.2, 44.6 and 45.6 kDa; the molecular mass of the active enzyme by gel filtration was 417 kDa. Electron microscopy revealed a multimeric, rod-shaped protein of approximately  $9 \times 50$  nm in addition to many shorter structures. The enzyme rapidly degraded cyanide to formate and ammonia thereby exhibiting nitrilase-like activity. Activity was reduced under anaerobic conditions. Enzyme activity was optimal at 37 °C and pH 7.8 - 8.0. Activity was enhanced by azide, thiocyanate, formate, and acetate. Enhancement by azide was reversible, concentration dependent (with maximal enhancement at 4.5 mM azide), and increased with time. The enhancing effect of azide was not manifested under anaerobic conditions. Enzymatic activity was also enhanced by  $\text{Sc}^{3+}$ ,  $\text{Cr}^{3+}$ ,  $\text{Fe}^{3+}$  and  $\text{Tb}^{3+}$ ; enhancement was independent of metal-ion concentration above 5  $\mu$ M. Radiolabelling experiments with  $^{51}\text{Cr}^{3+}$  failed to demonstrate the binding of metal to the active enzyme. No activity was recorded with the cyanide substrate analogues  $\text{CNO}^-$ ,  $\text{SCN}^-$ ,  $\text{CH}_3\text{CN}$  and  $\text{N}_3^-$  and the possible degradation intermediate,  $\text{HCONH}_2$ . Kinetic studies indicated a  $K_m$  of  $2.56 \pm 0.48$  mM for cyanide and a  $V_{\max}$  of  $88.03 \pm 4.67$  mmoles cyanide/min/mg/l. The  $K_m$  increased approximately two-fold in the presence of 10  $\mu$ M  $\text{Cr}^{3+}$  to  $5.28 \pm 0.38$  mM for cyanide and the  $V_{\max}$  to  $197.11 \pm 8.51$  mmoles cyanide/min/mg/l. The enzyme was tentatively named cyanide dihydratase.

A genomic library of *B. pumilus* C1 chromosomal DNA in the vector, pEcoR251, was screened in *E. coli* strain LK111 by a colony-Western-blot technique using antibodies to cyanide dihydratase. Failure to isolate a cyanide-degrading *E. coli* clone prompted the construction of a second genomic library of *B. pumilus* C1 DNA in the *E. coli*-*Bacillus subtilis* shuttle vector, pEB1. The pEB1 genomic library was screened in *E. coli* strain LK111 by two different procedures. The first involved a differential-growth method in which the fastest-growing *E. coli* genomic-library transformants, under cyanide-containing sloppy-agar overlays, were selected and assayed for cyanide-degrading activity. The second screening

procedure explored the hypothesis that cyanide-degrading *E. coli* clones would be able to reduce 2,3,5-triphenyltetrazolium chloride (TTC) in the presence of cyanide. This latter method involved plating transformants on a cyanide-containing indicator medium containing TTC. Neither of these screening procedures produced cyanide-degrading *E. coli* clones, suggesting that the cyanide dihydratase gene, or genes, are not expressed in *E. coli*, or that the genes are not linked on the *B. pumilus* C1 chromosome and could not be cloned as a unit by the cloning strategy employed. A Southern-hybridization technique was attempted using a synthetic oligomeric DNA probe corresponding to the predicted DNA coding region for the NH<sub>2</sub>-terminal amino-acid sequence of cyanide dihydratase. Initial experiments, however, showed that the oligonucleotide probe was not sufficiently specific to *B. pumilus* C1 chromosomal DNA to warrant screening the pEB1 genomic library by a colony-Southern-hybridization procedure.

## ABBREVIATIONS

|                |                                                               |
|----------------|---------------------------------------------------------------|
| A              | adenine                                                       |
| ABS.           | absorbance                                                    |
| Ala            | L-alanine (A)                                                 |
| Ap             | ampicillin                                                    |
| ATCC           | American Type Culture Collection                              |
| atm            | atmospheric pressure (101 325 Pa)                             |
| ATP            | adenosine 5'-triphosphate                                     |
|                |                                                               |
| BCIP           | 5-bromo-4-chloro-3-indolyl phosphate                          |
| BSA            | bovine serum albumin                                          |
|                |                                                               |
| C              | cytosine                                                      |
| Ci             | Curie                                                         |
| Cm             | chloramphenicol                                               |
| CPM            | counts per minute                                             |
| CsCl           | cesium chloride                                               |
| Cys            | L-cysteine (C)                                                |
|                |                                                               |
| Da             | dalton                                                        |
| DMSO           | dimethyl sulphoxide                                           |
| DNA            | deoxyribonucleic acid                                         |
| DTPA           | diethylenetriaminepenta-acetic acid                           |
|                |                                                               |
| EDTA           | ethylenediaminetetra-acetic acid                              |
| ESR            | electron spin resonance                                       |
|                |                                                               |
| FAD            | flavin adenine dinucleotide                                   |
|                |                                                               |
| g              | standard gravitational acceleration (9.81 m.s <sup>-2</sup> ) |
| G              | guanine                                                       |
|                |                                                               |
| h              | hour(s)                                                       |
| HPLC           | high-performance liquid chromatography                        |
|                |                                                               |
| I              | inosine                                                       |
| I              | ionic strength (moles/l)                                      |
| Ile            | L-isoleucine (I)                                              |
|                |                                                               |
| k              | kilo                                                          |
| K <sub>m</sub> | Michaelis constant                                            |
| kb             | kilobase pairs                                                |
| kDa            | kilodaltons                                                   |
|                |                                                               |
| Leu            | L-leucine (L)                                                 |

|                     |                                                         |
|---------------------|---------------------------------------------------------|
| Lys                 | L-lysine (K)                                            |
| min                 | minute(s)                                               |
| $M^{n+}$            | metal cation                                            |
| MgATP               | magnesium ATP                                           |
| MIC                 | minimum inhibitory concentration                        |
| $M_r$               | relative molecular mass                                 |
| mRNA                | messenger RNA                                           |
| NAD(P)              | nicotinamide adenine dinucleotide (phosphate); oxidized |
| NAD(P)H             | nicotinamide adenine dinucleotide (phosphate); reduced  |
| NBT                 | nitro blue tetrazolium                                  |
| nm                  | nanometers                                              |
| OD <sub>600nm</sub> | optical density at 600 nm                               |
| p                   | plasmid                                                 |
| ppm                 | parts per million (mg/l)                                |
| PAGE                | polyacrylamide gel electrophoresis                      |
| Phe                 | phenylalanine (F)                                       |
| $P_R$               | bacteriophage- $\lambda$ rightward promoter             |
| Pro                 | L-proline (P)                                           |
| RNA                 | ribonucleic acid                                        |
| RNase               | ribonuclease                                            |
| rpm                 | revolutions per minute                                  |
| s                   | second(s)                                               |
| SDS                 | sodium dodecyl sulphate                                 |
| Ser                 | L-serine (S)                                            |
| sp(p).              | species                                                 |
| T                   | thymine                                                 |
| TBE                 | Tris-borate-EDTA buffer                                 |
| TEMED               | N,N,N',N'-tetramethylethylenediamine                    |
| Thr                 | L-threonine (T)                                         |
| Tris                | tris(hydroxymethyl)aminomethane                         |
| Tris-HCl            | tris(hydroxymethyl)aminomethane hydrochloride           |
| Tyr                 | L-tyrosine (Y)                                          |
| U                   | unit of enzymatic activity                              |
| $V_{max}$           | maximum velocity of an enzymatic reaction               |

|            |                                                   |
|------------|---------------------------------------------------|
| X          | unidentified amino acid                           |
| $\alpha$   | alpha                                             |
| $\beta$    | beta                                              |
| $\Delta$   | delta (indicates a change in a measured quantity) |
| $\epsilon$ | epsilon (extinction co-efficient)                 |
| $\lambda$  | lambda                                            |
| $\mu$      | mu (abbreviation for micro; $10^{-6}$ )           |
| $\sigma$   | sigma                                             |

## CHAPTER 1

### GENERAL INTRODUCTION

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## 1.1 GENERAL INTRODUCTION

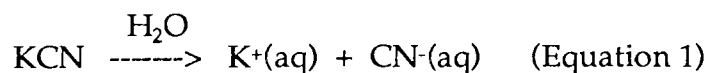
Cyanide is a notoriously toxic substance whose chemical reactivity gives it its versatility as a metabolic poison. Its toxicity to cellular processes such as respiration has been well documented and has been recorded in plants, animals and also in the microbiota. Despite this toxicity to living organisms, it is now appreciated that cyanide is a common metabolite in certain plants and microorganisms, even though its biological function in these organisms is not always fully understood.

The study of cyanide utilization and degradation in living cells as well as its effects on metabolism includes a number of biological disciplines. Over 2000 species of plants, as well as many fungi, some bacteria and a few insects are known to synthesize cyanide in a process known as cyanogenesis (Knowles, 1976; Castric, 1981; Conn, 1981; Duffey, 1981; Knowles and Bunch, 1986; Halkier *et al.*, 1988; Nahrstedt, 1988; Poulton, 1988). The biosynthetic pathway is similar in plants and insects, but is different from the pathway which bacteria and fungi appear to have in common. Most of the research into microbial cyanide metabolism has concentrated on the study of cyanogenesis in bacteria and fungi and the literature thus emphasises this aspect of cyanide metabolism over microbial cyanide degradation.

### 1.1.1 CYANIDE CHEMISTRY

Hydrocyanic acid, or hydrogen cyanide, (HCN) is a colourless, extremely toxic gas with an odour similar to that of bitter almonds. The gas is very soluble in water, but is readily lost to the surrounding air at normal temperatures (the vapour pressure of HCN at 26 °C is 100 kPa i.e. atmospheric pressure).

Simple cyanide compounds, such as KCN and Ca(CN)<sub>2</sub>, are the salts of hydrocyanic acid and dissolve in water to yield the solvated ionic species:



However, the cyanide anion, CN<sup>-</sup>, is the strong conjugate base of the weak acid, HCN, and in solution abstracts a proton from water to form the molecular (acidic) species of cyanide:



For this reason, unbuffered solutions of simple cyanide compounds have pH values in the basic range. The dissociation constant for the ionization of HCN to its ionic components is small ( $K_a = 2.03 \times 10^{-10}$  at 20 °C; Mudder, 1989) and the  $pK_a$  value for the reaction is large ( $pK_a = 9.31$  at 20 °C; Mudder, 1989). The equilibrium concentrations of the molecular and ionic species of cyanide are therefore determined by the pH of the solution and it follows that the molecular form, HCN, will be the predominant species at the pH values used most often in biological studies.

Cyanide can also form 72 possible complexes with metal ions (Ford-Smith, 1964, quoted in Chatwin and Smith, 1989). The particular metal ion involved determines the co-ordination type and stability of these cyano-metal complexes. Common complexes include those with cadmium, cobalt, copper, iron, mercury, nickel and zinc. The complexes of zinc and cadmium are relatively weak, whereas those of cobalt and iron are very strong (most stable).

The cyanide ion is classified as a pseudohalide (together with cyanate,  $\text{CNO}^-$ , and thiocyanate,  $\text{SCN}^-$ ) as its electronic structure imparts to it the properties of a halide (e.g.  $\text{F}^-$ ,  $\text{Cl}^-$ ,  $\text{I}^-$ ). Its complicated behaviour and high reactivity are explained in terms of its electronic structure, although its tendency to form complexes with metals cannot be explained in terms of its halogen-like behaviour (Chatwin and Smith, 1989).

### 1.1.2 CYANIDE IN NATURE

The chemical reactivity of cyanide accounts for the many chemical, biochemical and geochemical reactions in which it participates. This involvement of the molecular and ionic forms of cyanide in a diverse range of reactions have led to the proposal of a cyanide cycle in nature. Allen and Strobel (1966) proposed the idea of a biological cyanide microcycle in soil based on the potential interaction in the microatmospheric conditions of the soil between cyanide-producing organisms (such as certain plants and microorganisms) and cyanide-assimilating organisms (such as certain types of bacteria and fungi). Strobel (1967a) later showed that cyanide administered to non-sterile soils was converted to

carbonate and ammonia to a greater extent than sterilized soils. Furthermore, non-sterile soils taken from areas supporting the growth of cyanogenic plants had the greatest capacity to carry out this conversion, thereby lending some support to the theory of a cyanide microcycle in soil.

Thatcher and Weaver (1976) developed this hypothesis further by suggesting that a formamide-dependent carbon-nitrogen cycle may exist which involves cyanogenic plants, phytopathogenic fungi and formamide-metabolizing bacteria. In this theory the HCN, released from cyanogenic glycosides on infection of cyanogenic plants by phytopathogenic fungi, would be converted to formamide by the invading fungi. The formamide could then be converted to  $\text{CO}_2$  and  $\text{NH}_3$  by bacteria. The cycle would be completed by the direct fixation of the  $\text{CO}_2$  by cyanogenic plants and the reincorporation of the  $\text{NH}_3$  either directly, or indirectly as nitrate through the action of nitrifying microorganisms. A more general scheme, embracing both the biological and chemical reactions in the interconversion of cyanide in the environment was put forward by Mudder (1984, quoted in Chatwin and Smith, 1989). This scheme combines the various reactions of both HCN and  $\text{CN}^-$  in solution under aerobic and anaerobic conditions in a single diagram, and includes the effects of physical processes on the distribution and degradation of cyanide.

Natural cyanide removal or degradation mechanisms include complexing with metals (which reduces the toxicity of free cyanide and may reduce its mobility in the environment), precipitation of certain cyano-metal complexes, adsorption to mineral and organic components of soil, oxidation to cyanate, conversion to thiocyanate, loss to the atmosphere as volatile HCN, and biological degradation (Chatwin and Smith, 1989). Anaerobic biodegradation is considered to be far less effective than aerobic biodegradation.

### 1.1.3 CYANIDE TOXICITY

The poisonous nature of cyanide has long been the subject of scientific investigation. Sykes (1981) reviewed the early studies on the toxicity of cyanide, particularly the increase in physiological and biochemical investigation in experimental cyanide toxicology in animals. The toxicological and pharmacological effects of cyanide on metabolism have been reviewed by Solomonson (1981), Way (1981), Davis *et al.* (1988) and Way *et al.* (1988). The

antagonism of the toxic effects of cyanide in mammals has been specifically reviewed by Way (1981), Way *et al.* (1988) and Westley (1988).

Cyanide is probably best known as a respiratory inhibitor. Respiration is inhibited through the binding of cyanide to cytochrome oxidase which is the last enzyme in the electron transport chain in various eukaryotic and prokaryotic cells. However, cyanide also inhibits other metallo-enzymes (such as nitrogenase and nitrate reductase) and is thus not a specific poison of cytochrome oxidase. Enzymes containing a haeme group (iron), molybdenum, copper, zinc or selenium are all susceptible to inhibition by cyanide (Solomonson, 1981). Although there are a number of cyanide-sensitive metallo-enzymes, cyanide also carries out its role as a metabolic inhibitor by inhibiting certain enzymatic reactions involving the formation of a Schiff-base intermediate (Solomonson, 1981). Cyanide is also a potent nucleophile and can react with carbonyl groups to form cyanohydrins. It thus has the potential to react with certain enzyme substrate molecules to form inhibitors of enzymatic activity, or to react with an essential enzymatic cofactor to abolish activity (Solomonson, 1981).

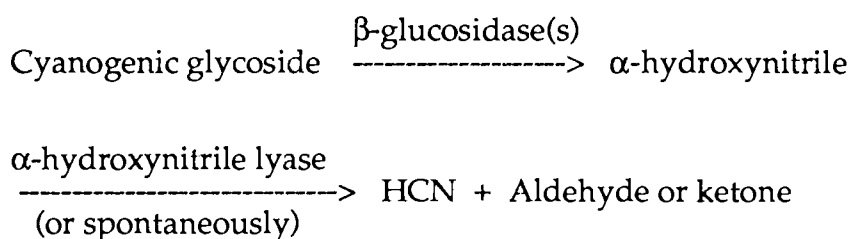
## 1.2 PLANT CYANOGENESIS

The synthesis of cyanogenic glycosides in plants and the release of HCN from these compounds has attracted considerable attention from biochemists for many decades. Robinson (1930) wrote an early review on the subject of cyanogenesis in plants. Cyanogenesis has been described in approximately 2000 species of higher plants and in all cases the HCN is derived from cyanogenic glycosides or cyanolipids (Conn, 1981).

The biosynthesis of cyanogenic glycosides has been reviewed by Conn (1981) and Halkier *et al.* (1988). The precursors of the cyanogenic glycosides are all amino acids: L-tyrosine, L-phenylalanine, L-valine, L-leucine, L-isoleucine, and the non-protein amino acid, L-2-cyclopentene-1-glycine (Seigler, 1981). The most detailed studies have dealt with the formation of the cyanogenic glycoside, dhurrin, in sorghum and a biosynthetic pathway has been proposed for its formation from L-tyrosine. However, some of the proposed intermediates have not been detected and although the enzymatic system for dhurrin biosynthesis has been partially purified it is still unclear whether it is a large, soluble enzyme

complex or a membrane-bound complex (Halkier *et al.*, 1988). The structures of the few known cyanolipids suggest that they are all derived from L-leucine (Seigler, 1975).

The isolation, purification and characterization of the naturally occurring cyanogenic compounds was reviewed by Seigler (1975) and Nahrstedt (1981). Classification of the structural types of the cyanogenic glycosides and lipids was discussed by Seigler (1975; 1981). Both cyanogenic glycosides and cyanolipids are derivatives of cyanohydrins ( $\alpha$ -hydroxynitriles). Although over 2000 species of higher plants belonging to 110 different families are known to contain cyanogenic glycosides (Seigler, 1981; Poulton, 1988), the structures of only about 75 of these compounds have been determined (Poulton, 1988). All are cyanohydrin derivatives in which the labile cyanohydrin is stabilized by O- $\beta$ -glycosidic linkage to a mono- or disaccharide (Poulton, 1988). The amino-acid precursor determines whether a particular cyanogenic glycoside is aliphatic, aromatic or cyclopentenoid in nature. Despite these structural differences all of these compounds are broken down by a similar mechanism involving one or two  $\beta$ -glucosidases and an  $\alpha$ -hydroxynitrile lyase:



(Equation 3)

These  $\beta$ -glucosidases exhibit a high degree of substrate specificity and have a number of properties in common (Hösel, 1981; Poulton, 1988). Although there is not much data on the specificity of plant  $\alpha$ -hydroxynitrile lyases there is preliminary evidence to suggest that they may be as substrate specific as the  $\beta$ -glucosidases involved in the catabolism of cyanogenic glycosides (Hösel, 1981).

Despite the presence of both cyanogenic glycosides and their catabolic enzymes in cyanogenic plants, the healthy tissues of these plants are free of detectable HCN (Poulton, 1988). However, damage to the plant results in the hydrolysis of the cyanogenic glycosides and the rapid production of HCN. This suggests that the cyanogenic glycosides are physically separated from their catabolic enzymes

within the plant by some form of compartmentation. In the case of the leaves of the cyanogenic plant, *Sorghum bicolor*, the cyanogenic glycoside, dhurrin, is located entirely in the epidermal tissue while the dhurrin  $\beta$ -glucosidase and hydroxynitrile lyase are located in the mesophyll tissue (Kojima *et al.*, 1979). Thayer and Conn (1981) showed further that there was subcellular compartmentation of these enzymes within the mesophyll cells. The  $\alpha$ -hydroxynitrile lyase was located in the cytoplasm, while the dhurrin  $\beta$ -glucosidase was associated with the chloroplasts. Compartmentation of the cyanogenic glycoside of cassava and its catabolic enzymes has also been demonstrated (Kojima *et al.*, 1983).

The role of cyanogenic glycosides in plants is not clear, although there is evidence that the products of their hydrolysis have a function in protecting cyanogenic plants from being eaten by herbivorous insects and vertebrate animals (Robinson, 1930; Jones, 1981; 1988). They may also be a defence against phytopathogenic bacteria and fungi (Fry and Munch, 1975). However, not all cyanogenic plants are toxic and in such plants the cyanogenic glycosides must serve some other function distinct from that of plant defence. One possibility is that they act as nitrogenous storage compounds (Poulton, 1988).

Cyanide is also known to be generated as a by-product during the formation of ethylene from 1-aminocyclopropane-1-carboxylic acid (Peiser *et al.*, 1984). Ethylene is a hormone which fulfils a number of functions in plants and its formation is thus widespread. This raises the interesting possibility that cyanogenesis by this route is widely distributed in the plant kingdom, although the amounts of cyanide involved are likely to be very small. The distribution of enzymes to detoxify cyanide should therefore also be widespread amongst plants.

### 1.3 MICROBIAL CYANOGENESIS

Many species of fungi and a few species of bacteria have been reported to be cyanogenic. However, both fungi and bacteria generate HCN directly from glycine by oxidative decarboxylation, rather than from a cyanogenic-glycoside intermediate (Knowles, 1988). The pathways of HCN production appear to be

similar in fungi and bacteria, although the biochemical details are not well established.

Fungal cyanogenesis occurs predominantly among the basidiomycetes and ascomycetes, with the most detailed studies having been performed on the snow mould basidiomycete and *Marasmius oreades* (Knowles, 1988). The subject of fungal cyanide production has been reviewed by Knowles (1976), Knowles and Bunch (1986), and discussed briefly by Vennesland *et al.* (1982) and Harris *et al.* (1987). Although there are many more known cyanogenic fungi than cyanogenic bacteria, much of the work on microbial cyanogenesis has been directed towards understanding bacterial cyanogenesis.

Cyanogenesis also occurs in photosynthetic microorganisms such as the microalga, *Chlorella vulgaris*, and the cyanobacterium, *Anacystis nidulans* (reviewed by Vennesland *et al.*, 1981; Solomonson and Spehar, 1981; Vennesland *et al.*, 1982; Knowles and Bunch, 1986; Harris *et al.*, 1987). Two pathways for cyanide formation have been described in these organisms. The D-amino-acid oxidase-peroxidase system requires oxygen,  $Mn^{2+}$  ions, peroxidase and a D-amino acid oxidase to form HCN from D-amino acids in *C. vulgaris* and *A. nidulans*. D-Histidine is the best precursor for cyanogenesis by this route in these organisms. A second HCN-forming pathway, involving the glyoxylic acid oxime system, has been described in *C. vulgaris*. Glyoxylic acid is converted, via glyoxylic acid oxime, to HCN in a reaction stimulated by  $Mn^{2+}$  and ADP. These pathways are distinct from those described for cyanogenesis in bacteria and fungi and produce much lower levels of cyanide.

Bacterial cyanogenesis was first reported in 1913 when Emerson *et al.* (1913) showed that bacteria could decompose egg protein to liberate HCN. One such bacterium was identified as a strain of *Bacillus pyocyaneus* (*Pseudomonas aeruginosa*) (Clawson and Young, 1913). Much effort has subsequently been made to understand bacterial cyanogenesis, which is a phenomenon confined to a very small number of bacterial species: *Chromobacterium violaceum*, many strains of *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*, and a few strains of *Pseudomonas chlororaphis* and *Pseudomonas aureofaciens* (Knowles and Bunch, 1986). The cyanogenic *Pseudomonas* species are all closely related and belong to the taxonomic group of fluorescent pseudomonads. Two excellent reviews on bacterial cyanogenesis are available (Castric, 1981; Knowles and Bunch, 1986) as

well as several less detailed discussions (Vennesland *et al.*, 1982; Harris *et al.*, 1987; Knowles, 1988).

The process of bacterial cyanide production exhibits features typical of secondary metabolism in that it occurs during a short period towards the end of the exponential phase of growth, requires *de novo* protein synthesis to form the cyanide synthase, is influenced by iron and phosphate in the growth medium within concentration ranges narrower than those required for growth, and is limited in its taxonomic distribution (Castric, 1975; Knowles, 1988). The regulation of the process is complex and a number of other factors such as the glycine concentration, the presence of methionine, the type of carbon source used for growth, and the degree of aeration of the culture at the time of cyanogenesis are also important (Castric, 1981).

The single, most-studied, cyanide-producing bacterial species is probably *C. violaceum*. Michaels and Corpe (1965) showed that glycine, in a chemically defined medium, induced and supported the production of micromolar concentrations of HCN by cells of *C. violaceum* strain 9. The amount of cyanide produced was greatly enhanced when glycine and DL-methionine were added together to the growth medium. However, these amino acids, either alone or together, did not support growth of the organism and additional sources of carbon and nitrogen were required. The role of methionine was not understood, but it was effective in enhancing cyanide production at lower concentrations than glycine. Michaels *et al.* (1965) used radiolabelling studies with  $^{14}\text{C}$ -glycine to show that HCN was derived from the methylene carbon atom (carbon-2) of glycine and not from the carboxyl carbon atom (carbon-1). The methyl group of methionine was also not a precursor of HCN. A hypothetical pathway for the conversion of glycine to cyanide was put forward. Further experiments using  $[2\text{-}^{14}\text{C}^{15}\text{N}]$ glycine showed that there was retention of the carbon-nitrogen bond in the conversion of glycine to cyanide (Brysk *et al.*, 1969b) i.e. the cyanide nitrogen atom was derived from the amino group of glycine.

Rodgers and Knowles (1978) showed that *de novo* protein synthesis was required in the late-exponential phase of growth of *C. violaceum* strain NCIB 9131 to furnish the enzyme(s) for cyanogenesis, with induction of the process being effected by glycine (2 mM) and methionine (0.5 mM). Addition to the growth medium of ferrous ions (in the micromolar range) or phosphate (in the millimolar range) led to greater maximal yields of cyanide. Collins *et al.* (1980)

showed that L-threonine was the only effective substitute for glycine in cyanogenesis by cells of *C. violaceum* strain NCIB 9131.

Bunch and Knowles (1982) investigated the ability of cell-free extracts of *C. violaceum* strain NCIB 9131 to form cyanide and showed that the cyanide synthase was sensitive to oxygen and required 1 mM dithiothreitol for stability. The presence of glycine provided partial protection against the effects of oxygen. The enzyme appeared to be located on the cell membrane and cell-free extracts required an electron acceptor such as phenazine methosulphate (PMS) for the production of cyanide from glycine. A specific activity for cyanogenesis of  $0.25 \pm 0.17$  nmol cyanide/min/mg protein was measured with 1 mM glycine and 100  $\mu$ M PMS. Attempts to show that compounds, proposed as intermediates in the biosynthesis of cyanide from glycine, could act as substrates for the cyanide synthase were unsuccessful. The ability of harvested whole cells to produce cyanide from glycine (the cyanogenic capacity) peaked in the late-exponential phase of growth and then decreased rapidly in the early stationary phase. This appearance and then disappearance of cyanogenic capacity was related to the changing levels of activity of the cyanide synthase in extracts and was suggested to be a pattern typical of enzyme formation in the production of other secondary metabolites.

Fluorescent cyanogenic pseudomonads appear to be common inhabitants of aquatic environments (Askeland and Morrison, 1983) and in soil they may be detrimental to plant growth through HCN-mediated inhibition of root-cell respiration (Bakker and Schippers, 1987). HCN production by certain cyanogenic pseudomonads may also help to suppress plant root pathogens (Voisard *et al.*, 1989). The characteristics of cyanogenesis in *Pseudomonas* species appear to be very similar to those in *C. violaceum*.

Wissing (1974) showed that the conversion of glycine to cyanide in whole cells of *Pseudomonas* sp. strain C was an oxidative process requiring oxygen, or an artificial electron acceptor, and at least one flavoprotein. A cell-free extract retained only weak HCN-forming activity in the presence of an oxidising agent (0.4 mM phenazine methosulphate) at pH 8.2 (Wissing, 1975). The presence of both 0.4 mM PMS and 0.3 mM FAD enhanced cyanogenic activity two-fold, but it was nevertheless still very weak (16 % of that of intact cells). The cyanide-producing activity appeared to be associated with the cytoplasmic membrane. Cell-free extracts prepared by sonication and detergent treatment contained

HCN-producing activity, which exhibited sensitivity to oxygen (Wissing and Andersen, 1981). Glycine provided a measure of protection against the effects of oxygen. The enzymatic activity could be partially purified by gel-filtration chromatography.

Castric (1975) carried out a survey of the occurrence of cyanogenesis in 110 strains of *P. aeruginosa*. Seventy four strains were reported to be cyanogenic and one (strain 9-D2) was studied in more detail. Cyanogenesis by this strain occurred in the late-exponential phase of growth and required *de novo* protein synthesis and oxygen. Ferric ions stimulated cyanide production (Castric, 1975). Phosphate at concentrations of 1 - 10 mM stimulated cyanogenesis, but concentrations of phosphate outside this range led to lower amounts of cyanide being produced (Meganathan and Castric, 1977). These results were taken to indicate that cyanogenesis was a secondary metabolic process in this organism, serving to regulate intracellular glycine levels which increased during the exponential phase of growth and became toxic to the cells as their metabolic rate slowed down at the end of this phase of growth (Castric, 1975; Meganathan and Castric, 1977).

It was subsequently reported that methionine enhanced cyanide production from glycine in *P. aeruginosa*, but was not itself a precursor of cyanide (Castric, 1977). Interestingly, the cyanide carbon atom could be derived from both carbon-1 and carbon-2 of glycine, although most of the cyanide (62.7 %) was derived from carbon-2. In *C. violaceum* the cyanide carbon is all derived from carbon-2 of glycine (Michaels *et al.*, 1965). Threonine and serine could be substituted for glycine as precursors of HCN, though they might have been indirect precursors through initial conversion to glycine. The termination of cyanogenesis in this organism occurs as the cells enter the stationary phase and appears to be the result of inactivation of the cyanide synthase by an oxygen build-up as cellular metabolic activity decreases (Castric *et al.*, 1981). Glycine provides some protection for the cyanide synthase from oxygen in cell-free extracts. Castric (1983) investigated the effect of dissolved-oxygen concentrations on cyanogenesis in *P. aeruginosa* and showed that this process was active at very low oxygen levels.

Freeman *et al.* (1975) reported cyanide production by two strains of *P. fluorescens*, but could not detect cyanide production by strains of *P. putida*. Askeland and Morrison (1983) investigated cyanide production from glycine by two strains of

*P. fluorescens*. Cyanogenesis in these strains was maximal during the transition from the exponential to the stationary phase of growth and was enhanced by iron and phosphate. The cyanide carbon atom was derived from carbon-2 of glycine (this was also shown to be the case with *P. aeruginosa* strain 1-73).

The reason for cyanogenesis in bacteria is unknown and there has been much speculation as to the possible functions it might have. Although HCN is converted into  $\beta$ -cyanoalanine in *C. violaceum* there is apparently little, if any, incorporation of cyanide or  $\beta$ -cyanoalanine into cellular components.  $\beta$ -Cyanoalanine is not converted further into asparagine and aspartate (Rodgers, 1981). Thus, although the formation of  $\beta$ -cyanoalanine may serve to detoxify cyanide, no pathway has been provided for the incorporation of HCN, via  $\beta$ -cyanoalanine, into cellular central metabolism and the question remains as to why HCN is produced at all. The characteristics of cyanogenesis which suggest that it is a process of secondary metabolism may provide the answer.

It has been proposed that the actual process of secondary metabolism is of greater significance than the end product because the process relieves some stress caused by a metabolic crisis within the cell (Castric, 1981; Knowles and Bunch, 1986). In terms of the immediate metabolic needs of the cell, the product is of minor importance and may have no value to the cell at all. Thus the process by which HCN is produced might have far more significance to the cyanogenic cell than HCN itself. The fact that HCN is so toxic may just confuse the issue by suggesting that it, as the product, has some particular function (for example, to increase the competitiveness or pathogenicity of the producing organism; Knowles, 1976; Castric, 1981). The induction of cyanogenesis in *C. violaceum* and *P. aeruginosa* as the cells switch from a state of high metabolic activity during vigorous growth to lower metabolic activity in stationary phase does suggest that cyanogenesis may be linked to changes in metabolic activity. The proposed secondary metabolic function of cyanogenesis in these bacterial species may be summarized as follows.

Cyanogenic bacteria require oxygen to maintain vigorous metabolic activity during the exponential phase of growth and cellular respiration during this period is expected to lead to a decline in the intracellular oxygen concentration. At the same time the intracellular concentration of glycine increases in response to an indirect need for this amino acid in such metabolic processes as the biosynthesis of purines, histidine, thymidine and methionine (Knowles and

Bunch, 1986). As cellular metabolic activity begins to slow down towards the end of the exponential phase of growth (in response to depletion of the carbon source), the high glycine concentrations are expected to become toxic to the cell. The decreased oxygen concentration in the cells at this time has allowed the induction of the membrane-bound, oxygen-sensitive cyanide synthase required to reduce the amount of glycine by converting it to HCN (Castric *et al.*, 1981). As the cellular metabolic rate declines further and the cells enter stationary phase, the intracellular oxygen concentration once again increases rapidly to its normal level of saturation. The increase in the amount of oxygen in the cell inhibits the cyanide synthase and cyanogenesis ceases in a process probably exacerbated by the decrease in the intracellular concentration of glycine (Castric *et al.*, 1981). The requirement for oxygen in cyanogenesis is probably indirect (Castric, 1981; Nazly *et al.*, 1981; Castric, 1983). It is worth noting in this regard that *C. violaceum* can grow anaerobically with fumarate as the electron acceptor and still produce HCN (Nazly *et al.*, 1981). The protein product of the *anr* gene of *P. aeruginosa* has been shown to be a positive control element which is required for both nitrate respiration and HCN biosynthesis in this organism under conditions of oxygen limitation (Zimmermann *et al.*, 1991).

This proposal that HCN is a secondary metabolite formed to reduce an excessive concentration of glycine within the cell is a somewhat vague explanation for cyanogenesis and, although it is well known that HCN is derived from glycine, speculation is still required to explain how a metabolic crisis involving glycine (and requiring some means of alleviating the stress) arises in the cell. Knowles and Bunch (1986) and Harris *et al.* (1987) have put forward a theory on how such a crisis might develop and how cyanogenesis might be involved in its alleviation. There is also plenty of room for conjecture when considering why glycine has to be converted to such a toxic secondary metabolite, and why so few bacterial species use cyanogenesis as a means of relieving this stress, since glycine metabolism must be widespread. Furthermore, the detoxification of HCN in *C. violaceum*, by reaction with cysteine to form  $\beta$ -cyanoalanine, could be considered a waste of cellular cysteine. Collins *et al.* (1980) suggested that cyanogenesis may be linked to the need in *C. violaceum* to remove excess nitrogenous compounds from the culture medium in some form other than ammonia.

However, it is still possible that cyanide plays a more direct role in the metabolism of cyanogenic microorganisms by acting as a metabolic regulator. The reversible inhibition of nitrate reductase by cyanide in the cyanogenic

unicellular green alga, *C. vulgaris*, has been suggested to be important in the regulation of nitrate assimilation in this organism (Solomonson, 1981; Vennesland *et al.*, 1981). This kind of explanation assumes a far more specific role for cyanogenesis and, with respect to bacteria, may be more compatible with cyanogenesis being restricted to so few species.

The mechanism by which methionine enhances cyanogenesis from glycine is unknown (Castric, 1981). Methionine is not a precursor of HCN in *C. violaceum* (Michaels *et al.*, 1965) and may be an activator, rather than an inducer, of the cyanide synthase (Collins *et al.*, 1980). The stimulatory effect of methionine on cyanogenesis may be the indirect result of its involvement in some other aspect of metabolism which creates conditions favourable for cyanogenesis (Collins *et al.*, 1980). While this may be true for *C. violaceum* and *P. aeruginosa*, it would not appear to hold for the snow mould fungus in which methionine does not enhance cyanide production from glycine (Bunch and Knowles, 1980).

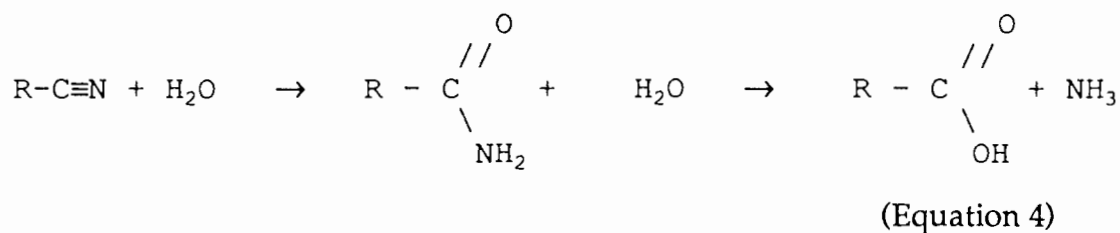
The production of a potent respiratory inhibitor by cyanogenic microorganisms, apparently without any adverse effects on the producing organism, indicates that they are insensitive to the inhibitory effects of cyanide. The study of cyanide-resistant respiration has enjoyed a great deal of attention particularly in eukaryotic organisms. The characteristics and regulation of the cyanide-resistant alternate terminal oxidase of plant mitochondria were reviewed recently (Moore and Siedow, 1991). There have also been many reports on cyanide-resistant respiration in the yeast, *Hansenula anomala* (for example, Sakajo *et al.*, 1990; 1991; Minagawa *et al.*, 1990; 1992). Bacterial cyanide-resistant respiration has been reviewed by Knowles (1976) and Henry (1981), however, this is a subject more related to respiration than to cyanide formation and degradation and is not relevant to this review.

## 1.4 THE ENZYMOLOGY OF CYANIDE DEGRADATION BY MICROORGANISMS

### 1.4.1 GENERAL

Nitriles can be considered to be cyanide-substituted carboxylic acids and the formal resemblance of  $C\equiv N$  to  $C=O$  suggests that the nitrile group might

undergo nucleophilic addition reactions analogous to those for the carbonyl group. The addition of  $\text{H}_2\text{O}$  (hydrolysis) may proceed by way of acid or base catalysis to yield the amide as the initial product. However, the amide may also undergo acid- or base-catalysed hydrolysis to yield the carboxylic acid as the final product:



Although this reaction was thought to belong predominantly to the realm of pure chemistry, there are now many reports providing evidence for its widespread distribution in biochemical systems too.

Investigations into the biochemical hydrolysis of nitriles have revealed two common pathways:



catalysed by a nitrile hydratase in which the nitrile is hydrated to the corresponding amide, and



catalysed by a nitrilase in which the nitrile is converted directly to the corresponding carboxylic acid, apparently without the formation of the amide as an intermediate. The nitrile hydratase pathway may be continued through the hydration of the amide by an amidase (acylamide amidohydrolase EC 3.5.1.4):



In each of these reactions the reactants and the products are at the same oxidation level and there is no overall oxidation or reduction of the reactants. Thus the conversion of a nitrile to the carboxylic acid, whether in one step or two, appears to be via a strictly hydrolytic mechanism (Chamberlain and Mackenzie, 1981).

Hydrocyanic acid (HCN) is the simplest nitrile, but there have been relatively few studies on the degradation of this compound. This may partly be due to the long-held belief that the high toxicity of cyanide precluded its metabolism in biological systems (Knowles, 1976). However, there have recently been a few reports on the enzymology of cyanide degradation in microorganisms, but as yet no descriptions of the mechanism by which this reaction might proceed. However, nitrile-converting enzymes such as nitrile hydratases and nitrilases, which have recently attracted a lot of attention as catalysts in organic chemical processing (Nagasawa and Yamada, 1989), have enjoyed very detailed study as a result of their potential commercial application in the chemical industry. The reaction mechanisms that have been proposed for these enzymes may, by analogy with cyanide-degrading enzymes, permit reaction mechanisms to be proposed for the latter.

#### 1.4.2.1 NITRILE HYDRATASES

Some confusion exists in the nomenclature of certain nitrile-degrading enzymes described in earlier studies since all such enzymes were described as nitrilases. This name was no doubt assigned because the overall reaction recorded for cells and cell-free extracts was the conversion of nitriles to their corresponding carboxylic acids and ammonia. However, this failed to make allowance for the possibility that there could be more than one way of converting a nitrile to its acid and ammonia.

The term, nitrile hydratase, was coined by Asano *et al.* (1980) who showed that the "nitrilase" activity of cell-free extracts of *Arthrobacter* sp. J-1 was in fact the result of the combined activities of two distinct enzymes: an amide-forming nitrile hydratase, and an amidase. A distinction clearly has to be made between those nitrile-degrading enzymes which catalyze the monohydration of the nitrile group to an amide, and those which catalyze the dihydration of this functional group directly to a carboxylic acid.

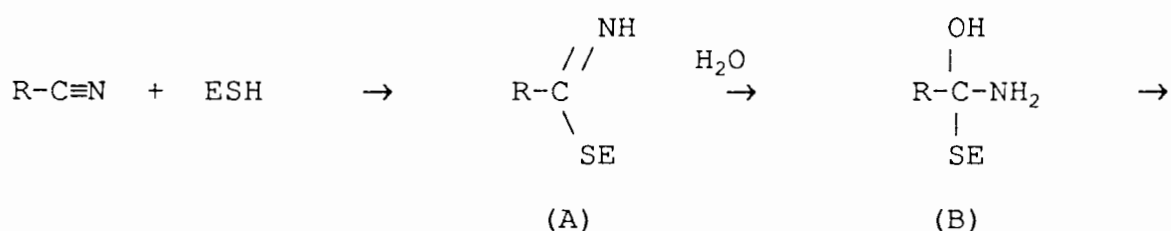
The metabolism of acetonitrile as the sole source of carbon and nitrogen by *Nocardia rhodochrous* LL100-21 was described by DiGeronimo and Antoine (1976). The enzyme system was shown to be intracellular and inducible and hydrolysed

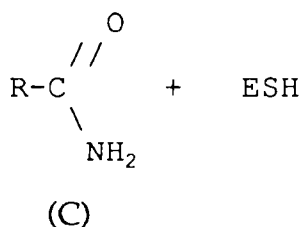
acetonitrile via acetamide to acetic acid and ammonia. Collins and Knowles (1983) showed that this organism could grow on a range of aliphatic nitriles and amides as sole sources of carbon and nitrogen, but could not grow on cyanide as either a carbon or a nitrogen source. The acetonitrile hydratase and the amidase activities were shown to be due to separate, inducible enzymes. The nitrile hydratase activity was lost on dialysis against EDTA, but was restored upon the addition of  $Mg^{2+}$  or  $Mn^{2+}$  to the dialysed enzyme.

It was suggested that the genes encoding the nitrile hydratase and the amidase might be regulated as a unit in the same operon or regulon with acetamide as the most likely inducer. Linton and Knowles (1986) extended the study of nitrile metabolism in *N. rhodochrous* LL100-21 and provided evidence which strongly supported the previous hypothesis that these enzymes are coordinately regulated in this organism.

Further evidence for the clustering of nitrile hydratase and amidase genes in operons was provided by sequence analysis of the DNA coding region of the enantiomer-selective-amidase gene in *Brevibacterium* sp. strain R312 (Mayaux *et al.*, 1990). This organism was suggested to be identical to, or very similar to, *Rhodococcus* sp. strain N-774. However, a different *Rhodococcus* species isolated from soil was also found to have an enantiomer-selective-amidase gene clustered with two downstream genes encoding two subunits of a nitrile hydratase (Mayaux *et al.*, 1991). The amino-acid sequence of the amidase had significant homology with that of the amidase from *Brevibacterium* sp. strain R312.

An inducible acetonitrile hydratase/acetamidase system has also been described in *Pseudomonas putida* (Nawaz *et al.*, 1989) and *Brevibacterium* sp. strain R312 (Bui *et al.*, 1984). The *Brevibacterium* nitrile hydratase also showed low activity towards cyanide. This enzyme had previously been shown to contain -SH, -NH<sub>2</sub> and -OH groups at the active site (Arnaud *et al.*, 1977). Bui *et al.*, (1984) proposed that their results for *Brevibacterium* sp. strain R312 were consistent with a nitrile hydratase reaction mechanism proposed by Asano *et al.* (1982):





(Equation 8)

An enzyme thiol group is assumed to initiate the reaction by nucleophilic attack at the nitrile carbon atom (with concomitant protonation of the nitrogen atom) to generate an enzyme-imino-thioether (A). Attack by H<sub>2</sub>O, accompanied by further protonation of the nitrogen atom, gives rise to the tetrahedral hydroxyamino-thioether intermediate (B). The collapse of the tetrahedral intermediate, with the enzyme as the leaving group, gives rise to the amide (C).

The *Rhodococcus* sp. N-774 acrylonitrile hydratase was purified and found to have two kinds of subunit ( $\alpha$  and  $\beta$ ) with different amino-terminal amino-acid sequences (Endo and Watanabe, 1989). The genes for these subunits were cloned into *E. coli* and found to be closely linked, suggesting that they were probably transcribed together as a single polycistronic mRNA species (Ikehata *et al.*, 1989). An open reading frame upstream of the gene encoding the  $\alpha$  subunit encoded an amidase (Hashimoto *et al.*, 1991) which was clustered with the nitrile hydratase subunit genes and transcribed in the same direction as these genes, lending further support to the hypothesis that there is co-ordinated regulation of the expression of genes for nitrile-degrading enzymes in this organism. Furthermore, the presence of at least part of the upstream amidase gene was required for the expression of *Rhodococcus* sp. N-774 nitrile hydratase (inactive) in *E. coli*. The native nitrile hydratase was similar to the nitrile hydratase from *Brevibacterium* sp. strain R312.

The purified nitrile hydratase from *Brevibacterium* sp. strain R312 contained two kinds of subunit ( $\alpha$  and  $\beta$ ) and tightly bound iron (Nagasawa *et al.*, 1986). Concentrated solutions of the purified enzyme had a pronounced greyish-green colour and an absorption maximum at 712 nm, but no absorption peaks corresponding to haeme or flavin groups. Iron was regarded as being important for enzyme function. The nitrile hydratase from *Rhodococcus* sp. N-774 was also shown to contain non-haeme iron (Ikehata *et al.*, 1989).

An unstable, aliphatic nitrile hydratase was purified from *P. chlororaphis* B23 by Nagasawa *et al.* (1987) and found to be stabilized by 22 mM n-butyric acid. The

100 kDa enzyme consisted of four subunits and contained one iron atom per subunit. Concentrated solutions of the purified enzyme had a pronounced greyish-green colour and exhibited a broad absorption peak in the visible range with a maximum at 720 nm. There were no absorption peaks corresponding to haeme or flavin groups. There were no acid-labile sulphur atoms, which information (when taken with that from the spectroscopy) suggested that this enzyme contained non-haeme iron differing from the [4Fe-4S] or [2Fe-2S] clusters known in other non-haeme iron proteins. The enzyme was very sensitive to certain thiol reagents and was also inhibited by carbonyl reagents such as hydroxylamine and phenylhydrazine. Slight inhibition of activity was recorded with 5 mM KCN. The enzyme displayed sensitivity to chelating agents and oxidizing agents such as  $\text{H}_2\text{O}_2$  and  $\text{FeCl}_3$ . These observations suggested that the catalytically active enzyme contained divalent iron ( $\text{Fe}^{2+}$ ), but there was no support for this (or for changes in the valency of the iron species during the course of the reaction), from subsequent electron spin resonance (ESR) spectroscopy (Sugiura *et al.*, 1987). The nitrile hydration reaction was proposed to be initiated by a nucleophilic thiol attack. The *P. chlororaphis* B23 enzyme was antigenically distinct from the nitrile hydratase of *Brevibacterium* sp. strain R312 (Nagasawa *et al.*, 1986), even though they had many properties in common.

ESR spectroscopy of the purified nitrile hydratases from *Brevibacterium* sp. strain R312 and *P. chlororaphis* B23 provided proof that these were the first non-haeme enzymes found to contain a typical low-spin Fe(III) co-ordination environment (Sugiura *et al.*, 1987). These enzymes were not ferredoxins. Interestingly, the *Brevibacterium* enzyme exhibited a spectral blue-shift from 712 to 690 nm on addition of its substrate, propionitrile, suggesting that binding of the substrate to the Fe(III) site of the enzyme had occurred. This was supported by the ESR spectra. ESR spectra showed that the addition of the enzyme inhibitor, isobutyronitrile, to the enzyme was accompanied by binding of isobutyronitrile to the  $\text{Fe}^{3+}$  site. However, this molecule was not hydrated and released from the active site as the spectrum did not revert to that of the native enzyme as it did for the preferred substrate, propionitrile. The axial positions in the co-ordination sphere were thought to be occupied by a thiolate group (probably a cysteine thiol group) and a water molecule. The direct binding of aliphatic nitrile substrates to the Fe(III) centre was proposed to proceed by displacement of the axial  $\text{H}_2\text{O}$  molecule.

Further work on the iron centre of this *Brevibacterium* enzyme showed that the low-spin ferric ion existed in a rhombically distorted octahedral complex with a ligand field of sulphur and nitrogen (or oxygen) donor atoms (Nelson *et al.*, 1991). This was regarded as being a biologically novel mononuclear, non-haeme iron complex with sulphur and nitrogen or oxygen atoms in the co-ordination sphere. A strong metal( $\text{Fe}^{3+}$ )-sulphur bond was indicated and the authors suggested that the metal binding site of the enzyme comprised three cysteine residues in the  $\alpha$  subunit.

It is interesting that the inducible aliphatic nitrile hydratase from *Arthrobacter* sp. J-1 was also a subunit enzyme inactivated by sulphydryl reagents, but was not affected by metal-chelating or carbonyl reagents (Asano *et al.*, 1982). Ferric ions had no influence on enzymatic activity. Potassium cyanide was shown to be a competitive inhibitor ( $K_i = 1.5 \mu\text{M}$ ) when acetonitrile was used as the substrate. Activity could be restored by overnight dialysis.

The nitrile hydratase of *Brevibacterium* sp. strain R312 was shown to contain a second prosthetic group which was identified as pyrroloquinoline quinone (PQQ) or a PQQ-like compound (Nagasawa and Yamada, 1987). The nitrile hydratases from *Rhodococcus* sp. N-774 and *P. chlororaphis* B23 have also been found to contain PQQ or a PQQ-like compound (Ikehata *et al.*, 1989). All other known quinoproteins are oxidoreductases and since nitrile hydratase is not an oxidoreductase, PQQ might be expected to fulfil a novel function in the nitrile-hydratase-catalysed hydration reaction. It is assumed that the hydrated form of PQQ ( $\text{PQQ-H}_2\text{O}$  or  $\text{PQQ-2H}_2\text{O}$ ) participates in the activation of a water molecule which is then added to the carbon atom of the nitrile group during the hydration reaction. The presence of PQQ would explain the sensitivity of the enzyme to carbonyl reagents.

Sugiura *et al.* (1988) showed that the addition of carbonyl reagents such as phenylhydrazine to the nitrile hydratases of *Brevibacterium* sp. strain R312 and *P. chlororaphis* B23 not only abolished enzymatic activity, but also induced significant changes in the ESR spectra of the native iron (III) enzymes. Treatment of isobutyronitrile-bound enzyme with phenylhydrazine also gave rise to altered ESR spectra which strongly indicated that phenylhydrazine reacted with an organic cofactor (thought to be PQQ) and not with the iron (III) site of the enzyme. The changes in the ESR spectra implied that there was weak, yet significant, interaction between the low-spin iron (III) centre and PQQ at the

active site of the native nitrile hydratase. This interaction is essential for the catalytic function of the enzyme. The nature of the interaction was suggested to be hydrogen-bonding between monohydrated PQQ and the axial ligand ( $\text{H}_2\text{O}$  or substrate) of the iron (III) complex. Reaction of phenylhydrazine with PQQ was thought to affect this hydrogen-bonding process.

As discussed earlier, the overall reaction in nitrile conversion to the amide or the acid is hydrolysis. The presence of  $\text{Fe}^{3+}$  and PQQ as cofactors in *Brevibacterium* sp. strain R312 and *P. chlororaphis* B23 nitrile hydratases suggests that an oxidation-reduction reaction may well be part of the enzymatic mechanism for the monohydration of nitriles to amides (Nagasawa and Yamada, 1989). The chemical conversion of nitriles to amides by the hydroperoxide anion is known to involve an oxidation-reduction mechanism.

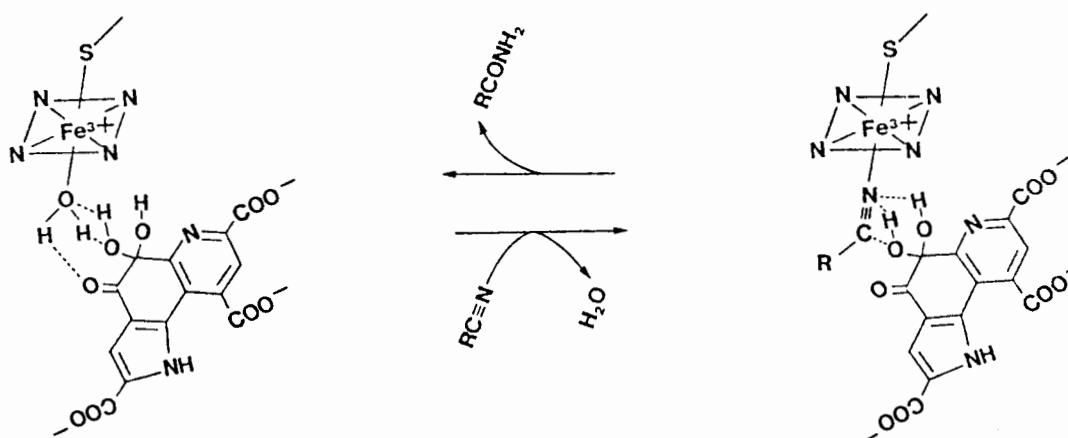
A 530 kDa benzonitrile hydratase comprising  $\alpha$  (29 kDa) and  $\beta$  (26 kDa) subunits was purified from *Rhodococcus rhodochrous* J1 and found to contain cobalt ions (Nagasawa *et al.*, 1988). No other transition-metal ions could replace the cobalt ions. The valency of the cobalt ions was unclear, but it was postulated that nitrile substrates might bind directly to the cobalt ion in a manner analogous to that proposed for the  $\text{Fe}^{3+}$  active site of *Brevibacterium* sp. strain R312 and *P. chlororaphis* B23 nitrile hydratases. The *R. rhodochrous* J1 nitrile hydratase was clearly different from the latter enzymes in its metal ion requirement and in its ability to act on an aromatic nitrile. It showed sensitivity to thiol reagents and had no ability to hydrate potassium cyanide. KCN was a competitive inhibitor of the enzyme ( $K_i = 0.75 \mu\text{M}$ ) when 3-cyanopyridine was used as the substrate (Nagasawa *et al.*, 1991). A pink, cobalt porphyrin-like pigment was isolated from the enzyme. PQQ or a PQQ derivative could not be isolated from the enzyme. It was suggested that the active nitrile hydratase contains  $\text{Co}^{3+}$ , but this seems unlikely as cobalt(III) is known to undergo very slow ligand replacement reactions (Cotton and Wilkinson, 1980) which would be incompatible with the type of reaction mechanism proposed for nitrile hydratases from *Brevibacterium* sp. strain R312 and *P. chlororaphis* B23 (Sugiura *et al.*, 1987).

During the course of the purification of the *R. rhodochrous* J1 nitrile hydratase it was discovered that this bacterium actually synthesized two distinct cobalt-containing nitrile hydratases differing in molecular mass (530 kDa and 130 kDa, respectively), but both comprising  $\alpha$  and  $\beta$  subunits. The substrate specificities of the enzymes were different and they were induced by different compounds

(Kobayashi *et al.*, 1991). The genes for the enzymes were cloned into *E. coli* and active enzymes were expressed at low levels only when cobalt ions were added to the culture medium. The amino acid sequences of the  $\alpha$  and  $\beta$  subunits from one enzyme exhibited similarities to the corresponding subunits of the other enzyme, and to the  $\alpha$  and  $\beta$  subunits of the *Rhodococcus* sp. N-774 nitrile hydratase. The findings suggested that the requirement for cobalt was manifested at the level of enzymatic activity (post-translational) rather than at the level of transcriptional regulation of the genes. It was proposed that amino acid differences between the metal binding sites in these enzymes and those in the nitrile hydratases from *Brevibacterium* sp. strain R312 and *P. chlororaphis* B23 could account for the binding of cobalt ions by the former and ferric ions by the latter enzymes. Although the subunit genes for each *R. rhodochrous* J1 nitrile hydratase were closely linked, the position of the gene-pairs relative to each other on the bacterial chromosome was unknown. No amidase-encoding sequences upstream or downstream of the pairs of nitrile-hydratase-subunit genes were reported.

Metal-ion-dependent nitrile hydratases have also been described in *Corynebacterium pseudodiphtheriticum* ZBB-41 (Li *et al.*, 1992), and in the fungus, *Myrothecium verrucaria* (Maier-Greiner *et al.*, 1991).

The mechanism for the nitrile-hydratase-catalysed hydration of nitriles shown in Equation 8 is now regarded as being incorrect and a more elaborate reaction mechanism has been proposed which takes into account the apparent requirement by these enzymes for metallic and carbonyl prosthetic groups:



(Equation 9)

There is increasing genetic evidence that organisms which utilize nitriles via conversion to the corresponding amide regulate the expression of the genes for the requisite enzymes in a nitrile-utilization operon. Comparison of amino-terminal amino-acid sequences from several nitrile hydratases from different organisms strongly suggests that there is significant similarity between them (Nagasawa *et al.*, 1991; Nishiyama *et al.*, 1991). Nitrile hydratases from different sources and with different substrate specificities thus seem to belong to a family. However, the ferric nitrile hydratases from *Brevibacterium* sp. strain R312 and *P. chlororaphis* B23 appear to be antigenically distinct from each other and from the cobalt-containing nitrile hydratase of *R. rhodochrous* J1 and the metal-free nitrile hydratase of *Arthrobacter* sp. J-1 (Nagasawa *et al.*, 1986; Nagasawa *et al.*, 1987; Nagasawa *et al.*, 1991). All the characterized nitrile hydratases are subunit enzymes comprising one or two kinds of subunit. The molecular weights of the active enzymes vary considerably with some being very large proteins. The pH optima vary in the range from 6.5 to 7.8 and the temperature optima are usually below 40 °C with enzyme activity greatly reduced, or lost altogether, at temperatures above 60 °C. Most of the enzymes appear to require iron or cobalt for activity and some also require the presence of a second cofactor (PQQ). All the enzymes are inhibited by thiol reagents and are therefore regarded as sulphhydryl enzymes; a mechanism for the hydration of nitriles has been proposed which takes this observation into account. Almost all these enzymes have no ability to hydrate cyanide and in some cases cyanide acts as a competitive or non-competitive inhibitor.

The conversion of nitriles to amides, and amides to acids, can also be effected chemically with hydrogen peroxide. The one-electron oxidation of cyanide to cyanyl radical by horseradish peroxidase, lactoperoxidase, chloroperoxidase, NADH peroxidase and methaemoglobin (ferric form of haemoglobin) in the presence of H<sub>2</sub>O<sub>2</sub> was reported recently (Moreno *et al.*, 1988). Lignin peroxidase H2 of the lignin-degrading fungus, *Phanerochaete chrysosporium*, has also been shown to oxidize cyanide to the cyanyl radical when incubated with H<sub>2</sub>O<sub>2</sub> and cyanide (Shah *et al.*, 1991). Lignolytic cultures of the fungus degraded cyanide to carbon dioxide.

#### 1.4.2.2 CYANIDE HYDRATASES

Very little detailed work has been carried out on cyanide hydratases and there is therefore not much information on the physicochemical and kinetic properties of these enzymes.

Cyanide hydratase (formamide hydrolyase, EC 4.2.1.66) was first described in the phytopathogenic fungus, *Stemphylium loti* (Fry and Millar, 1972), which causes disease in the cyanogenic plant, birdsfoot trefoil (*Lotus corniculatus* L.). Tolerance of the fungus to cyanide, which is released from birdsfoot trefoil upon injury, is thought to be one of the requirements for successful invasion of the host plant (Fry and Myers, 1981). This tolerance is an adaptive response to exposure to cyanide and requires protein synthesis (Fry and Millar, 1971). Cyanide hydratase was partially purified from spores of *S. loti* by anion-exchange and gel-filtration chromatography with an overall purification of 15-fold (Fry and Millar, 1972). The partially purified enzyme converted cyanide to formamide in a stoichiometric ratio of 1:1. The enzyme was stable at 4 °C, exhibited maximal activity over a broad pH range from pH 7.0 to pH 9.0, was not inhibited by 10 mM EDTA, and exhibited a Michaelis constant ( $K_m$ ) for cyanide of 15-20 mM at 25 °C. Sodium fluoride (NaF) at 10 mM caused a 50 % drop in activity, but had no effect on the enzyme when present at 1.0 mM. The molecular weight of the enzyme was not determined, but it appeared to be a very large protein as it eluted in the void volume of a Sephadex G-200 gel-filtration column (indicating a molecular mass of over 600 kDa).

Another cyanide hydratase was subsequently purified from the phytopathogenic fungus *Gloeocercospora sorghi* (Fry and Munch, 1975), which causes disease in the cyanogenic crop plant, sorghum (*Sorghum spp.*). The enzyme was partially purified from homogenized, cyanide-induced mycelia by anion-exchange and gel-filtration chromatography and shown to catalyze the stoichiometric conversion of cyanide to formamide. The partially purified enzyme was most stable at 4 °C and was insensitive to NaSCN, EDTA and  $\beta$ -mercaptoethanol (at 1 or 10 mM, pH 8.0). Sodium fluoride caused 50 % inhibition of enzymatic activity at 10 mM. Although the enzyme was inhibited by 10 mM  $\text{NaN}_3$ , exposure of fungal cells to 1 mM  $\text{NaN}_3$  for 18 h enhanced cyanide-hydratase activity (Fry and Myers, 1981).

Fry and Evans (1977) studied the distribution of cyanide hydratase amongst fungi by testing 31 species (from 24 different genera representing the Phycomycetes, Ascomycetes, Basidiomycetes and Deuteromycetes) for production of cyanide hydratase after exposure to a low concentration (1.0 mM) of cyanide. They showed that all 11 of the tested fungi that were pathogens of cyanogenic plants produced cyanide hydratase, while 9 out of 14 fungal pathogens of non-cyanogenic plants synthesized this enzyme. Only one of six of the tested fungi that were not pathogenic to plants could produce cyanide hydratase. This clearly indicated an association of cyanide hydratase activity with pathogens of cyanogenic plants. Furthermore, the average specific activities of cyanide hydratase in cell-free extracts of cyanide-induced pathogens of cyanogenic plants was 4-fold higher than the equivalent activities in cell-free extracts of cyanide-induced pathogens of non-cyanogenic plants. Preliminary data indicated that only cyanide served as a substrate for, or inducer of, cyanide-hydratase activity. Furthermore, acetonitrile, propionitrile and thiocyanate did not enhance cyanide hydratase activity in *S. loti*, *G. sorghi*, *Fusarium solani* f. sp. *pisi* or *Helminthosporium maydis* (Fry and Myers, 1981).

An interest in understanding the role of cyanide hydratase in the infection of cyanogenic plants by phytopathogenic fungi, and establishment of cyanide hydratase as a prerequisite for infection under these circumstances, has led to a reinvestigation of the cyanide hydratase of *G. sorghi* (Wang *et al.*, 1992). Other fungal isolates, including 5 species that had never before been tested for cyanide hydratase activity, were also investigated. All the phytopathogens of sorghum were shown to produce this activity after exposure to a low concentration of KCN (0.1 mM).

The cyanide hydratase from *G. sorghi* strain T-442 was purified 23.8-fold from homogenized, cyanide-induced mycelia (Wang *et al.*, 1992). The molecular mass of the enzyme was not determined, but appeared to be greater than 300 kDa. Denaturing polyacrylamide-gel electrophoresis, however, showed only a single polypeptide band at 45 kDa which, by isoelectric focusing, displayed three isozymes with pI values of 6.1, 6.3 and 6.5. This suggested that the large enzyme was a homomultimer of smaller protein subunits. This hypothesis was reported to be supported by (unpublished) electron micrographs of the purified enzyme. The enzyme displayed maximal activity in the pH range from pH 7 to pH 8 and had a  $K_m$  value for cyanide at 25 °C of 12 mM. The enzymatic activity was sensitive to inhibition by certain thiol-specific reagents, but was not affected by

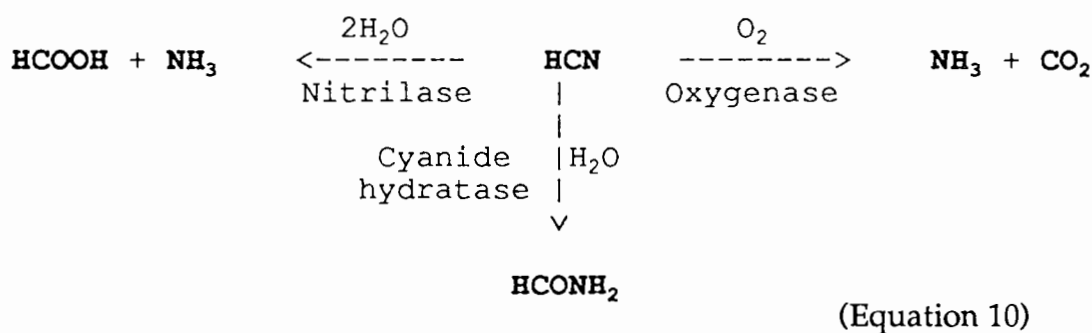
chelating agents such as EDTA. Polyclonal antibodies were raised against the active, purified enzyme.

Purification of the cyanide hydratase from the sorghum pathogen, *Colletotrichum graminicola* T-441 (by the same procedure as that used for the *G. sorghi* enzyme), also gave a single 45 kDa polypeptide on SDS-PAGE (Wang *et al.*, 1992). The antibodies raised against the *G. sorghi* cyanide-hydratase could completely inhibit cyanide-hydratase activity in both the *G. sorghi* and *C. graminicola* enzymes, suggesting antigenic similarity between the enzymes from these fungi.

A cDNA expression library was constructed for *G. sorghi* and screened (by Western-blotting procedures with the cyanide-hydratase-specific antibody) for clones expressing cyanide-hydratase protein (Wang and VanEtten, 1992). A positive clone was used as a Southern-hybridization probe against digested *G. sorghi* genomic DNA cloned into the pUC18 plasmid vector. One of the pUC18 clones, which hybridized with the probe, was cotransformed into the fungus *Aspergillus nidulans*. One transformant was selected that carried a *G. sorghi* DNA fragment containing a single open-reading frame of 1107 base pairs. The open-reading frame was predicted to encode a polypeptide of 40 904 Da. The polypeptide was enzymatically inactive on its own. These results confirmed the earlier hypothesis that the active enzyme is an aggregate of a single polypeptide species (Wang *et al.*, 1992). This was the first sequence to be published of a gene encoding a cyanide-degrading enzyme. Although the sequence of the cyanide-hydratase gene (*chy1*) from *Fusarium lateritium* is available in the EMBL Data Base (Accession number: M99046), a paper describing its cloning and sequencing has never been published. The *G. sorghi* cyanide-hydratase gene (*cht*) had no introns and the deduced amino-acid sequence showed 36.5 % identity with the bromoxynil nitrilase of *Klebsiella ozaenae* (Stalker *et al.*, 1988) which converted 3,5-dibromo-4-hydroxybenzonitrile (bromoxynil) to the corresponding acid and ammonia. The homology observed between a nitrile-hydratase-type enzyme and a nitrilase, which catalyze different reactions, may indicate that there is conservation in nitrile-converting enzymes of amino-acid sequences that interact with the nitrile group present in their different substrates (Wang and VanEtten, 1992). The cloning of the *cht* gene from *G. sorghi* will allow the role of cyanide hydratase (CHT) in pathogenesis to be tested directly through the creation of CHT- mutants of *G. sorghi* which can be tested for their ability to infect sorghum.

To date, 57 fungal isolates representing 37 species have been tested for cyanide hydratase. Fourteen of these species have been shown to lack this enzyme. This activity has never been demonstrated in plants or in phytopathogenic bacteria of both cyanogenic and non-cyanogenic plants (Wang *et al.*, 1992).

Despite the occurrence of cyanide hydratase in a number of fungi, and the apparent exclusive manifestation of this activity in some members of this group of organisms, recent work has shown that it also seems to occur in the mesophilic, non-phytopathogenic bacterium, *P. fluorescens* NCIMB 11764 (Kunz *et al.*, 1992). This organism was cultivated on cyanide as the sole source of nitrogen by a modified fed-batch method. Experimental results suggested that this organism may degrade cyanide by 3 distinct routes:



The selection of which pathways were used by the organism was shown to be dependent on the cyanide concentration and the availability of oxygen. The oxygen-dependent pathway leading to  $\text{CO}_2$  appeared to be the major degradative route under aerobic conditions at low concentrations of KCN (0.5 - 10 mM). However, under aerobic conditions at 20 and 50 mM KCN the route to  $\text{CO}_2$  became progressively less important as the KCN concentration increased. Under these conditions the pathway leading to formamide became more significant as the cyanide concentration increased (the production of formate increased with increasing cyanide concentration to 20 mM cyanide, but then remained at approximately the same level at 50 mM cyanide). Under anaerobic conditions (at KCN concentrations of 10 mM or less) formamide and formate were the major one-carbon products and there was very little production of  $\text{CO}_2$ . Cyanide degradation under anaerobic conditions was considerably slower than under aerobic conditions. The oxygen-independent pathways from cyanide to formamide and formate, respectively, appeared to be distinct as formamide was not degraded further. Unfortunately, the enzymes responsible for these activities

were not purified and it is not possible to assign specific enzymes to these apparently distinct activities.

It is interesting that both formamide and formate lie on the sequential pathways proposed by Knowles (1976) as one possible route from HCN to CO<sub>2</sub>, but in *P. fluorescens* NCIMB 11764 they are formed by distinct pathways with no apparent conversion of formamide to formate. This situation is different from that described in the previous section (1.4.2.1) for the sequential conversion of organic nitriles by a nitrile hydratase/amidase system in certain bacteria (see for example Collins and Knowles, 1983; Bui *et al.*, 1984; Linton and Knowles, 1986; Nawaz *et al.*, 1989; Mayaux *et al.*, 1990; Mayaux *et al.*, 1991; Nishiyama *et al.*, 1991). Whether this bacterial cyanide-hydratase-type enzyme bears any similarity to the fungal cyanide hydratases awaits further biochemical investigation.

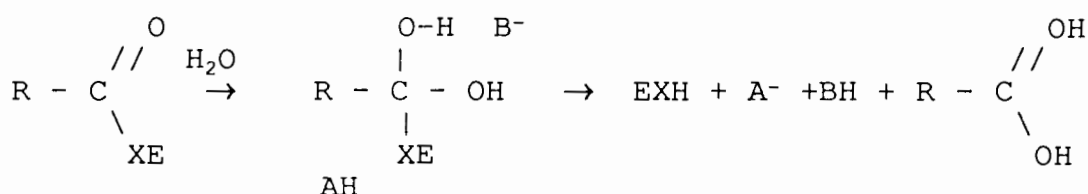
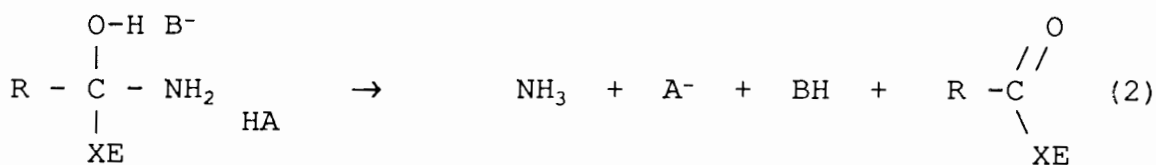
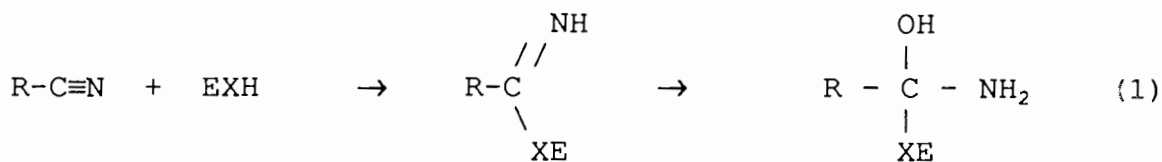
At present there is too little information on cyanide hydratases to make possible an extensive comparison of these enzymes with the organic nitrile hydratases discussed in the previous section (1.4.2.1). However, the cyanide hydratases from at least two of the fungi studied (namely, *G. sorghi* and *C. graminicola*) are homomultimeric enzymes (Wang *et al.*, 1992; Wang and VanEtten, 1992) and the cyanide hydratase from *G. sorghi* is a sulphhydryl enzyme (Wang *et al.*, 1992). These are properties exhibited by nitrile hydratases, although these enzymes often contain two different types of subunits ( $\alpha$  and  $\beta$ ). Further metabolism of formamide by fungi has not been observed (Fry and Myers, 1981) suggesting that there is no induction of a cyanide hydratase/formamidase system in fungi analogous to the nitrile hydratase/amidase systems of some bacteria that degrade organic nitriles. *P. fluorescens* NCIMB 11764 also does not appear to contain such a system. Importantly, the cyanide hydratases from *S. loti* (Fry and Millar, 1972) and *G. sorghi* (Wang *et al.*, 1992) do not appear to require divalent metal cations for activity as they are insensitive to EDTA. A number of organic nitrile hydratases have been shown to require transition-metal ions for activity (iron, cobalt or zinc, depending on the enzyme; see Nagasawa *et al.*, 1986; Sugiura *et al.*, 1987; Nagasawa *et al.*, 1988; Ikehata *et al.*, 1989; Maier-Greiner *et al.*, 1991). There is not enough data yet to propose a reaction mechanism for cyanide hydration by cyanide hydratases, but an initial nucleophilic attack on the cyanide carbon atom by an enzyme thiol group cannot be ruled out. If such a nucleophilic attack does occur the reaction mechanism may more likely resemble that of Equation 8 rather than the more detailed reaction mechanism proposed for iron- and pyrroloquinoline-quinone-containing nitrile hydratases (Equation 9).

### 1.4.3.1 NITRILASES

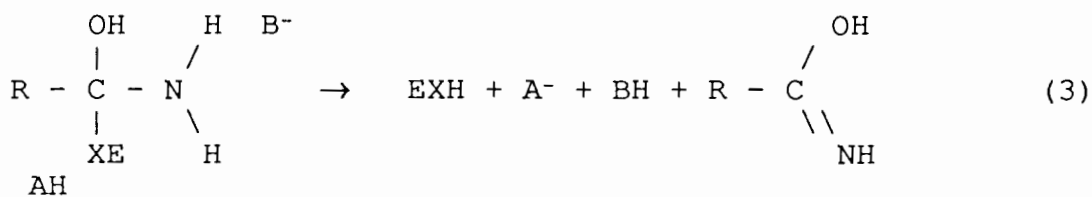
Nitrilases have been isolated from plants, fungi and bacteria and are characterized by their ability to hydrolyze nitriles directly to the corresponding carboxylic acid and ammonia. The observation that these enzymes are unable to use amides as substrates supports the view that nitrile hydrolysis by this pathway does not involve the amide as an intermediate. The nitrilases that will be described in this section are distinct from the oxynitrilases ( $\alpha$ -hydroxynitrile lyases) known to liberate HCN from plant cyanogenic glycosides.

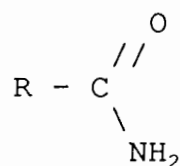
An indoleacetonitrilase which was partially purified from barley leaves by Thimann and Mahadevan (1964) was inhibited by thiol-specific reagents, but was insensitive to  $10^{-3}$  M KCN. It was shown that this enzyme was uncommon in the plant kingdom; of 21 plant families examined, only members of the *Gramineae* (grasses), *Cruciferae* (cabbage family) and *Musaceae* (banana family) exhibited this activity. The enzyme from barley leaves hydrolysed a number of aromatic nitriles as well as some aliphatic nitriles. The enzymatic reaction was considered to more nearly resemble alkaline chemical hydrolysis than chemical hydrolysis with acid (Mahadevan and Thimann, 1964). A mechanism was proposed in which the reaction was initiated by nucleophilic attack on the nitrile by an enzyme thiol or imidazole group.

A nitrilase which hydrolysed the nitrile group of ricinine (N-methyl-3-cyano-4-methoxy-2-pyridone) and a variety of structurally related aromatic compounds to the corresponding acids and ammonia was isolated from a species of *Pseudomonas* by Robinson and Hook (1964). No activity was exhibited towards the amide corresponding to ricinine. The enzyme was purified and found to be sensitive to inhibition by thiol-specific reagents and sodium cyanide (at  $2.9 \times 10^{-2}$  M), but was insensitive to a variety of metal ions and metal-chelating agents (Hook and Robinson, 1964). Although the amide of ricinine was not a substrate for the enzyme, a small but constant proportion (about 9%) of the amide was formed when ricinine was used as the substrate. A mechanism was proposed for the reaction catalysed by the nitrilase:



OR



$$\downarrow \uparrow$$


(Equation 11)

Nucleophilic attack by the enzyme (where X is an oxygen or sulphur atom) on the carbon atom of the nitrile gives rise to an enzyme iminoether which can be hydrated to the tetrahedral enzyme-hydroxyamino-ether intermediate (1). The presence of basic (B-) or acidic (HA) groups in the active site would facilitate the liberation of  $NH_3$  to form an acylenzyme, which could be hydrolysed to yield the acid and free enzyme (2).

To explain the formation of the small amount of the ricinine amide it was proposed that if the enzyme, and not  $\text{NH}_3$ , was the leaving group in step (2) the amide would be the final product. This clearly illustrates how closely related the nitrilase and nitrile hydratase mechanisms were believed to be. The two mechanisms proposed by Hook and Robinson (1964) are in fact identical up to the point where the tetrahedral intermediate collapses with either  $\text{NH}_3$  or the enzyme as the leaving group. It is only at this stage that the final product is determined. As has been discussed in the general section on nitrile hydratases, more recent evidence has suggested that the pathway for nitrile hydratases (Equation 9) is more complicated than originally proposed and bears no relation to that for nitrilases.

An unstable benzonitrilase from *Nocardia* sp. NCIB 11216, purified by Harper (1977), was found to be composed of 45 kDa subunits which required the presence of the substrate, benzonitrile, to associate into the active form of the enzyme (c. 560 kDa). Benzamide was not formed during the hydrolysis of benzonitrile to benzoic acid and ammonia, nor was it a substrate for the enzyme. Benzamide, benzoic acid and ammonia had no influence on the subunit association time. The enzyme was very sensitive to thiol-specific reagents, but it was not clear whether these reagents affected enzyme activity directly or indirectly through the subunit association process. Addition of azide and cyanide to 0.1 mM did not influence activity, nor did dialysis against EDTA.  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$  and  $\text{Fe}^{2+}$  (but not  $\text{Fe}^{3+}$ ) at 100  $\mu\text{M}$  inhibited activity. A reaction mechanism analogous to that proposed by Mahadevan and Thimann (1964), and Hook and Robinson (1964) for ricinine nitrilase, was suggested for the *Nocardia* nitrilase with an enzyme thiol group making the initial nucleophilic attack on the nitrile.

A nitrilase from *Fusarium oxysporum* f. sp. *melonis* was found to exhibit activity towards aliphatic, aromatic and heterocyclic nitriles over a very broad pH range from pH 6 to 11 (Goldlust and Bohak, 1989). The enzyme existed as a 550 kDa multimer of identical 37-kDa subunits in solution, but was also active as smaller aggregates with a tetrameric form being the smallest active species. The higher-molecular-weight active species appeared to be formed by the addition to the tetramer of two subunits at a time. Interestingly, the hydrolysis of benzonitrile, propionitrile and acrylonitrile was accompanied by the formation of the corresponding carboxylic acid as well as by the formation of a small (4 - 6 %), but constant, proportion of the corresponding amide irrespective of the reaction

conditions or the nature of the substrate. In this respect the fungal nitrilase was similar to ricinine nitrilase and indicated that the amide and the acid arose from a common intermediate in the reaction pathway as originally proposed by Hook and Robinson (1964), where the final product was determined by the leaving group released from a tetrahedral, enzyme-hydroxyaminothioether-intermediate (Equation 11). Divalent metal ions and metal-chelating reagents had no effect on the fungal nitrilase, although  $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Fe}^{2+}$  and  $\text{Mn}^{2+}$  seemed to stabilize the purified enzyme. Trivalent metal ions as well as certain thiol-specific reagents inhibited enzymatic activity.

*Arthrobacter* sp. strain J-1 was found to synthesize two distinct benzonitrilases (Bandyopadhyay *et al.*, 1986) in addition to the previously reported acetonitrile hydratase (Asano *et al.*, 1982). These nitrilases were small enzymes (30 kDa and 23 kDa, respectively) with no subunits and were only active towards aromatic nitriles. Both enzymes exhibited time-dependent substrate activation, but this must have been different from that described by Harper (1977) for *Nocardia* sp. NCIB 11216 as the *Arthrobacter* enzymes had no subunits. Azide, cyanide, EDTA and a range of transition metal ions had no effect on enzyme activity, but both enzymes were inhibited by thiol reagents. The similarity between the enzymes suggested that they might be isozymes.

It is apparent from this discussion that nitrilases generally act on aromatic nitriles as their preferred substrates, whereas nitrile hydratases generally hydrate aliphatic nitriles. The isolation of a nitrilase from *R. rhodochrous* K22 that preferentially hydrated aliphatic olefinic nitriles was regarded as a significant discovery (Kobayashi *et al.*, 1990a). This enzyme was composed of 15 or 16 identical 41-kDa subunits and was thus a very large protein, comparable in size to the benzonitrilase from *Nocardia* sp. NCIB 11216 (Harper, 1977). The aliphatic nitrilase had a very low pH optimum of 5.5 for the hydrolysis of crotonitrile. There was no requirement for metal ions and activity was not affected by the addition of carbonyl-modifying reagents. The enzyme exhibited a degree of sensitivity to thiol-specific reagents but was not as sensitive to these agents as previously-described nitrilases. Weak absorption between 320 and 380 nm was suggested to have arisen from some tightly bound cofactor, but this was not proved. The enzyme was also shown to carry out monohydrolysis of a variety of dinitrile compounds to the corresponding mononitrile-monocarboxylic acid compounds (Kobayashi *et al.*, 1990b).

Another unusual nitrilase which catalysed the direct hydrolysis of arylacetonitrile derivatives to the corresponding arylacetic acids and ammonia was isolated from *Alcaligenes faecalis* JM3 (Mauger *et al.*, 1990). This enzyme had no activity towards aliphatic nitriles, or aromatic nitriles where the nitrile group was attached directly to an aromatic ring. The purified enzyme was a homohexamer of 44 kDa subunits which was sensitive to thiol-specific reagents (Nagasawa *et al.*, 1990). There were no metals associated with the purified enzyme and EDTA, KCN and azide did not influence activity. No involvement of carbonyl groups could be deduced. Activity was enhanced by thiol compounds such as dithiothreitol and mercaptoethanol. The most likely mechanism for enzyme catalysis was believed to be analogous to that proposed by Harper (1977), which assumes an initial nucleophilic attack by a thiol group on the nitrile carbon atom.

A stable, two-subunit, 78-kDa nitrilase specific for nitrile groups attached directly to an aromatic or heterocyclic ring was purified from *R. rhodochrous* J1 (Kobayashi *et al.*, 1989). The enzyme displayed no activity towards benzamide or benzaldoxime and did not accumulate benzamide during the hydrolysis of benzonitrile. The enzyme contained no metals and was insensitive to chelating agents, cyanide and azide. Most carbonyl reagents did not affect enzymatic activity, although phenylhydrazine partially inhibited activity. Thiol-specific reagents inhibited enzymatic activity thereby implying that thiol groups played an important role at the active site. Reducing agents such as dithiothreitol, cysteine, 2-mercaptoethanol and reduced glutathione caused significant stimulation of enzymatic activity. This organism was previously shown to produce a cobalt-containing benzonitrile hydratase (Nagasawa *et al.*, 1988), but no cross-reaction could be demonstrated between antiserum to the benzonitrilase and the cobalt-containing benzonitrile hydratase.

Although a good deal of attention has been paid to the mechanism of nitrile-hydratase catalysis, there have been few reports in the literature concerning the mechanism of catalysis for nitrilases. The pathway that was proposed (Mahadevan and Thimann, 1964; Hook and Robinson, 1964) was speculative and was based on the observation that these enzymes (the nitrilase from barley, and ricinine nitrilase, respectively) were inhibited by thiol reagents. All the nitrilases that have subsequently been characterized also appear to contain a catalytically essential thiol group and are classified as sulphydryl enzymes (e.g. Harper, 1977;

Bandyopadhyay *et al.*, 1986; Kobayashi *et al.*, 1989; Kobayashi *et al.*, 1990a; Nagasawa *et al.*, 1990).

Stevenson *et al.*, (1990) analysed the interaction of the nitrilase of *Rhodococcus* ATCC 39484 with its aromatic-nitrile substrates (benzonitrile and 3-chlorobenzonitrile) using ion-spray mass spectroscopy and reported the first demonstration that a nitrilase formed a covalent intermediate with its substrate. It was not possible to determine whether this intermediate was the initial thioimide intermediate (Equation 11) or the subsequent acylenzyme after release of ammonia from the tetrahedral enzyme-hydroxyaminoether intermediate (Equation 11). The fact that a covalently-bound intermediate could be isolated indicated that the decomposition of this intermediate (enzyme thioimide or acylenzyme) was the rate-limiting step in the catalytic reaction. It appeared that nucleophilic attack on the nitrile carbon was not very specific as a covalent adduct was also formed between the enzyme and 2-methoxybenzonitrile (which was regarded as a very poor substrate). It was suggested that it was possible that the enzyme could bind to and carry out at least this initial step of the reaction with a variety of nitriles, both good substrates and poor substrates. Subsequent steps in the reaction would only occur at an appreciable rate with substrate molecules. This hypothesis seems to be reasonable as the substrate ranges of nitrilases are often quite broad and it would then be the geometry and charge environment of the active site which would determine substrate specificity rather than the initial nucleophilic attack by a thiol group.

The gene encoding the benzonitrilase from *R. rhodochrous* J1 was cloned into *E. coli* in which host the active enzyme was produced (Kobayashi *et al.*, 1992a). The amino-acid sequence predicted from the DNA sequence of the gene showed 42.7 % homology with the *K. ozaenae* bromoxynil nitrilase, but no homology with other protein sequences, including the cobalt-containing nitrile hydratase which is also produced by *R. rhodochrous* J1. The *R. rhodochrous* J1 nitrilase contained four free cysteines per enzyme subunit, of which only one could be shown to react with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). Modification of this single cysteinyl residue (Cys-165) to alanine or serine by site-directed mutagenesis abolished enzymatic activity, indicating that Cys-165 played an essential role at the active site. This was the first report identifying an active-site cysteinyl residue in a nitrilase and also showed that if an -SH group was important in the nucleophilic attack on the nitrile, the -OH group of serine could not function as a

nucleophilic substitute in the reaction. Interestingly, Cys-165 and a short piece of the adjacent sequence, were shown to be conserved at the corresponding positions in the bromoxynil nitrilase of *K. ozaenae*. Small differences in the amino-acid sequences of these enzymes, which might affect the active-site environment, appeared to account for their different substrate specificities.

Site-directed mutagenesis has also been used to identify an essential cysteinyl residue in the unusual aliphatic nitrilase from *R. rhodochrous* K22 (Kobayashi *et al.*, 1992b), and in indole-3-acetonitrilase (arylacetonitrilase) from *A. faecalis* JM3 (Kobayashi *et al.*, 1993).

It can be seen from the foregoing discussion that nitrilases are distinct from nitrile hydratases with respect to a variety of properties (for example, the requirement of the nitrile hydratases for transition-metal ions and organic cofactors). Nitrile hydratases generally contain two different types of subunit ( $\alpha$  and  $\beta$ ) and also exhibit much higher specific activities for their substrates than any known nitrilase for its substrates (Kobayashi *et al.*, 1990a; Kobayashi *et al.*, 1993). This information alone suggests that nitrile hydratases and nitrilases catalyze their reactions by different mechanisms, and distinct pathways have been proposed (Equations 9 and 11, respectively).

The substrate specificities are generally different with nitrile hydratases hydrating aliphatic nitriles and nitrilases almost exclusively hydrolysing aromatic nitriles. Indeed, it was believed that there might be a correlation between the nature of the nitrile and the kind of nitrile-degrading enzyme that acted on it. Thus saturated aliphatic nitriles were thought to be degraded in two stages by a nitrile hydratase/amidase system, whereas nitrile compounds in which the nitrile was conjugated with a double bond (i.e. aromatic, heterocyclic and unsaturated aliphatic nitriles) were thought to be degraded directly to the corresponding acid and ammonia by nitrilases (Bandyopadhyay *et al.*, 1986; Nagasawa and Yamada, 1989; Kobayashi *et al.*, 1990a). This hypothesis is clearly wrong because, for example, the arylacetonitrilase from *A. faecalis* JM3 (Nagasawa *et al.*, 1990) has low activity towards nitriles directly attached to an aromatic ring; the cobalt-containing nitrile hydratases of *R. rhodochrous* J1 (Nagasawa *et al.*, 1991) hydrate aromatic nitriles; and the nitrile hydratase from *C. pseudodiphtheriticum* ZBB-41 (Li *et al.*, 1992) hydrates acrylonitrile.

Amides are not substrates for nitrilases and are generally not formed during the hydrolytic reaction, except in two cases (Hook and Robinson, 1964; Goldlust and Bohak, 1989) where a small, but constant, proportion of the corresponding amide is formed from the nitrile substrates.

Nitrilases generally have pH optima in the range from pH 7 to 9 and temperature optima below 50 °C. Most nitrilases are sensitive to temperatures above 55 °C. They are all composed of identical subunits, except the benzonitrilases from *Arthrobacter* sp. strain J-1 (Bandyopadhyay *et al.*, 1986) which are small proteins with no apparent subunits. The molecular weights of active nitrilases vary up to very large aggregates of several hundred kilodaltons, but it has been suggested that the tendency of the enzyme subunits to associate into large aggregations may have led to very high active molecular weights being reported for some of these enzymes where it is possible that these large proteins are not the smallest active multimers (Goldlust and Bohak, 1989).

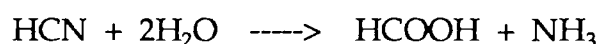
Transition-metal ions are not required for activity and generally do not affect enzymatic activity. Cyanide and azide also do not affect enzymatic activity. There is no evidence for the participation of organic cofactors in the catalytic reaction. All characterized nitrilases exhibit varying degrees of sensitivity to thiol-specific reagents and are therefore classified as sulphydryl enzymes.

Nitrile-degrading organisms usually only produce one kind of nitrile-degrading enzyme, although some, for example, *Arthrobacter* sp. strain J-1 (Bandyopadhyay *et al.*, 1986), *R. rhodochrous* J1 (Kobayashi *et al.*, 1989), and *N. rhodochrous* LL100-21 (Collins and Knowles, 1983) synthesize both nitrile hydratases and nitrilases. Which type of enzyme is produced depends on the inducer added to the growth medium.

#### 1.4.3.2 CYANIDE-DEGRADING NITRILASES

As is the case for cyanide hydratases, very little detailed information is available on the physicochemical and kinetic properties of cyanide-degrading nitrilases.

White *et al.* (1988) isolated a facultative methylotrophic *Pseudomonas* species from coke-plant activated sludge which displayed an inducible ability to degrade cyanide to formate and ammonia. The isolate could not grow on cyanide as the sole source of carbon, but grew when cyanide was supplied as the sole source of nitrogen in the presence of an added carbon source. The bacterium could also not grow on formamide as the sole source of carbon and nitrogen, and displayed no ability to further metabolize formate derived from cyanide. Cell-free extracts of induced cells stoichiometrically converted cyanide to formate and ammonia in a 1:1 ratio with specific activities of between 0.22 and 0.41  $\mu\text{g}$  cyanide (8.1 - 15.2 nmoles)/min/mg protein. The cyanide-degrading activity was unstable at room temperature and was lost upon dialysis at 4 °C for 5 h against dilute phosphate buffer, pH 7.2. Addition of 0.2 mM NAD(P)H or 0.3 mM ascorbate to the dialysed, inactive cell-free extract partially restored cyanide-degrading activity. Full restoration of activity was achieved on the addition of 1.5 mM NAD(P)H or 2.5 % yeast extract, but mineral salts, 1.5 mM NAD or 1 % yeast extract could not restore activity. The low molar ratios of the reducing agents to cyanide in the restoration of activity after dialysis indicated that these reducing agents were not directly involved as co-factors in the cyanide-degrading reaction. The cell-free extracts could not degrade formamide (2.2 mM) either in the presence or absence of cyanide. Although the enzyme responsible for the cyanide-degrading activity was not purified, the overall reaction appeared to be that catalyzed by a nitrilase:



(Equation 12)

The second of the two oxygen-independent cyanide-degradation pathways described by Kunz *et al.* (1992) appeared to involve the nitrilase-catalyzed hydrolysis of cyanide directly to formate and ammonia. The inability of cell-free extracts to degrade formamide ruled out the possibility that formate was generated from formamide by way of a formamidase. Under aerobic conditions and with low or high concentrations of KCN the pathway leading to formate and ammonia was less significant than that leading to formamide. However, under anaerobic conditions (when  $\text{CO}_2$  was produced in very small amounts) the pathway leading to formate and ammonia appeared to be far more important and formate and formamide were produced in almost equimolar amounts. Although it seems likely that the formate-producing activity in *P. fluorescens* NCIMB 11764 is that of a cyanide nitrilase, confirmation of this will require

detailed enzymological investigation of the three pathways proposed for cyanide degradation in this organism.

The first cyanide-degrading nitrilase to be purified was that from a soil isolate of the Gram-negative bacterium, *Alcaligenes xylosoxidans* subsp. *denitrificans* strain DF3 (Ingvorsen *et al.*, 1991). Production of this enzyme was induced when the bacterium was grown in nutrient broth with 1 % glycerol and 0.1 - 5 mM sodium cyanide. Induced cells converted cyanide to formate and ammonia in a stoichiometric ratio of 1:1 (as shown in Equation 12) in the presence or absence of oxygen. The presence of formate did not induce the cyanide nitrilase and nitrogen limitation of the cells in batch culture prevented induction of the enzyme. Induced cells could not degrade cyanate (CNO<sup>-</sup>) nor a range of aliphatic nitriles (both saturated and unsaturated) and aromatic nitriles. Cyanide hydrolysis by cells became progressively more inhibited as the cyanide concentration increased above 100 mM. Increasing concentrations of sodium formate, in the molar range, also caused increasing inhibition of cyanide degradation by induced cells, but this effect was less severe than that recorded with cyanide.

The *Alcaligenes* cyanide nitrilase was purified 162-fold (with a yield of 8 - 10 %) from cell-free extracts of induced cells. The optimum temperature for cyanide degradation was 26 °C and the optimum pH was  $7.8 \pm 0.2$ . The purified enzyme eluted in the void volume of a Sephacryl S-300 (suggesting a molecular mass of over 300 kDa). Denaturing polyacrylamide gel electrophoresis showed two protein bands of equal staining intensity at 39 and 40 kDa. Isoelectric focusing gave two protein bands with pI values of 4.55 and 4.65. However, when the enzyme was submitted to amino-terminal sequencing a single 25-amino-acid sequence was generated, suggesting that the two proteins seen on SDS-PAGE may have identical amino termini. The enzyme was named "cyanidase" to distinguish it from previously described nitrilases (EC 3.5.5.1) that act on organic nitriles and which are not known to degrade cyanide. It was suggested that the cyanidase should have a distinct Enzyme Commission number assigned to it.

The cyanidase enzyme was patented by Novo Nordisk A/S (Denmark) for use as a biocatalyst (Cyanidase®) in the treatment of cyanide-containing industrial wastewater. Although the cyanide-nitrilase-type enzyme from the pseudomonad was not purified (White *et al.*, 1988) it appears to be different from that of *A. xylosoxidans* subsp. *denitrificans* strain DF3 (Ingvorsen *et al.*, 1991).

The physicochemical and kinetic properties of Cyanidase® were investigated to develop an enzyme membrane reactor for the detoxification of cyanide-containing industrial wastewater without the need for pretreatment of these wastes (Basheer *et al.*, 1992). The biocatalyst, prepared in granular form by Novo Nordisk A/S, consisted of particles with sizes ranging from 50 - 2000  $\mu\text{m}$ . Tests on Cyanidase® were carried out in a fixed-bed reactor in phosphate buffer, pH 7.4, at 26 °C. Cyanide was supplied at 11.53 mM. When biocatalyst granules larger than 300  $\mu\text{m}$  were used significant internal diffusion effects were noted and the effectiveness of the biocatalyst decreased. As the maximum rate of cyanide degradation was achieved with smaller particles of the biocatalyst, kinetic measurements for cyanide degradation were performed on a finely powdered preparation of the biocatalyst (with particle sizes below 50  $\mu\text{m}$ ). The optimum pH range for the biocatalyst was shown to be pH 7.8 - 8.3 with activity dropping off sharply above pH 8.3. Activity was not affected by the ionic strength of phosphate buffer solutions up to an ionic strength (I) of 1.5 mole/l. Common ions present in cyanide-containing wastewaters such as chloride, sulphate, iodide,  $\text{Fe}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Ni}^{2+}$  (each at 70 ppm) had no effect on enzymatic activity. The organic compounds acetate, acetamide, acetonitrile and formamide (each at 100 ppm) also had no effect on enzymatic activity. Cyanide degradation by the biocatalyst obeyed simple Michaelis-Menten kinetics at pH 7.4 and 26 °C (I = 0.75 mole/l). Under these conditions the  $K_m$  was  $3.04 \pm 0.15$  mM for cyanide and the  $V_{\text{max}}$  was  $0.4 \pm 0.01$  mmole cyanide/l/min/g biocatalyst over the substrate range up to 11.53 mM cyanide. This range covers the cyanide concentrations found in most cyanide-containing industrial wastewaters. No substrate or product inhibition was recorded over this range of concentrations, but cyanide concentrations above 38.5 mM appeared to cause substrate inhibition. The activation energy ( $E_a$ ) over the temperature range from 20 - 30 °C was 52 kJ/mole cyanide. Strong thermal denaturation of the enzyme was observed at temperatures above 40 °C.

A strain of *Bacillus pumilus* isolated from Fargo clay, North Dakota (U.S.A.) was reported by Skowronski and Strobel (1969) to have an extreme tolerance to cyanide (2.5 M KCN). Radiolabelling experiments showed that in the presence of 0.1 M KCN the *B. pumilus* cells (grown in Trypticase-soy-yeast-extract broth without glucose, but containing 0.1 M KCN) degraded the cyanide to  $\text{CO}_2$  and  $\text{NH}_3$ . The cyanide-degrading activity was not purified from this organism and so it is impossible to determine whether these products were the result of the

activity of a single enzyme (i.e. an oxygenase), two enzymes (a nitrilase and formate dehydrogenase), or three enzymes (a cyanide hydratase, formamidase and formate dehydrogenase).

The investigation of the cyanide-degrading ability of another strain of *B. pumilus* is the subject of this thesis. The details of the studies on cyanide degradation by *B. pumilus* C1, isolated from a cyanide wastewater dam at a mine in the Transvaal province of South Africa, are described in the chapters that follow. Studies on the enzymology of cyanide degradation by this organism showed that a single enzyme, identified as a cyanide dihydratase, was responsible for this activity. The 417 kDa active enzyme displayed three distinct polypeptides (in the size range from 41 - 45 kDa) on SDS-PAGE and appears to be a sulphhydryl enzyme. The characterization of this enzyme is described in Chapter 3.

The reactions for the degradation of cyanide via the cyanide-hydratase and cyanide-nitrilase pathways have been presented as a single scheme by Knowles (1976).

Although there is yet too little information to propose a mechanism for the reaction catalyzed by cyanide nitrilases, it may be analogous to that proposed for ricinine nitrilase (Hook and Robinson, 1964) in Equation 11.

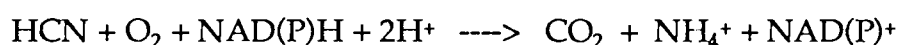
#### 1.4.4 CYANIDE OXYGENASES

*P. fluorescens* NCIMB 11764, isolated from river mud, was shown to produce an inducible cyanide-degrading system on exposure to cyanide in the growth medium (Harris and Knowles, 1983a). The rate of aeration of cyanide-grown cells controlled the rate of cyanide degradation to ammonia. Under anaerobic conditions there was no degradation of cyanide suggesting that there was an absolute requirement for oxygen in this reaction. Suspensions of harvested, cyanide-grown bacteria in 50 mM sodium phosphate buffer (at pH 7.0 or pH 7.6) exhibited cyanide-stimulated oxygen uptake. The addition of KCN to 80  $\mu$ M resulted in an approximately two-fold increase in the measured rate of O<sub>2</sub> consumption. The oxygen uptake in the presence of cyanide was 30 - 55 ng-atom oxygen/min/mg dry weight cells (corrected for the endogenous rate of respiration). The increased consumption of oxygen was proportional to the

amount of KCN added (although cyanide concentrations over 1 mM caused inhibition and the rate of respiration decreased). Suspensions of cells grown on ammonium salts did not exhibit this cyanide-stimulated increase in respiration. The requirement for O<sub>2</sub> in cyanide degradation by this organism suggested that the reaction, or part of it, was oxidative.

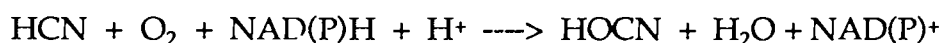
Cell-free extracts of cyanide-grown *P. fluorescens* NCIMB 11764 cells displayed a requirement for NADH or NADPH in order to degrade cyanide (Harris and Knowles, 1983b). The presence of NADH in cell-free extracts of ammonium-grown cells did not promote cyanide degradation. NADH was a better reducing agent for the reaction than NADPH. The cyanide-degrading activity remained in the supernatant after centrifugation of the cell-free extract for 90 min at 150 000 × g. The amounts of O<sub>2</sub> consumed and NADH oxidized were equimolar with the amount of KCN degraded. NADH was not oxidised in the presence of cyanide under anaerobic conditions. No detectable intermediates accumulated during the degradation of cyanide to ammonia and CO<sub>2</sub>. SDS-PAGE of the supernatant fractions of cyanide- and ammonium-grown cells after ultracentrifugation revealed an extra protein band in the cell-free extract of cyanide-grown cells. This protein was present mainly in the protein fraction precipitated by ammonium sulphate at 35 % saturation and had a molecular mass, by SDS-PAGE, of 15 - 17 kDa. It represented the major protein band in the 35 % ammonium sulphate fraction. However, preliminary data from the ammonium-sulphate fractionation studies suggested that at least two proteins were involved in the overall degradation of cyanide.

Although the overall stoichiometry of the reaction was that expected for a cyanide-dioxygenase-catalyzed reaction:



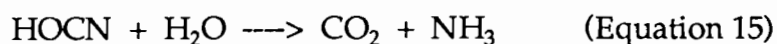
(Equation 13)

there was insufficient data to distinguish between a dioxygenase-catalysed reaction and a cyanide-monooxygenase-catalysed reaction:



(Equation 14)

If a cyanide monooxygenase was involved, the cyanate so formed could have been converted to CO<sub>2</sub> and ammonia by a cyanase (EC 3.5.5.3), or by chemical hydrolysis:



although the strictly chemical reaction may not proceed at a fast enough rate to account for the rapid generation of ammonia from cyanide under the reaction conditions used (30 °C, pH 7.0). The presence of cyanate-degrading activity was observed in cell-free extracts of both cyanide- and ammonium-grown bacteria (Harris and Knowles, 1983b).

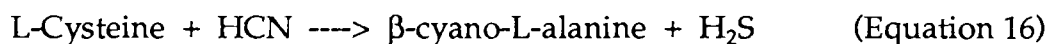
Growth of *P. fluorescens* NCIMB 11764 on 2 mM potassium cyanate (KOCN) as the nitrogen source led to an induction of cyanase activity to a maximum value of 2.34 μmol NH<sub>3</sub> formed/min/mg dry weight of cells, but no induction of cyanide oxygenase activity (Dorr and Knowles, 1989). Similarly, when grown on Ni(CN)<sub>4</sub><sup>2-</sup> as the nitrogen source, cyanide oxygenase activity was induced (to a maximum value of about 38 nmol O<sub>2</sub> consumed/min/mg dry weight of cells) without the induction of cyanate-degrading activity. Cyanide oxygenase and cyanase activities were never coinduced under a variety of culture conditions tested. Rapid degradation of 1 mM KCN by mid-exponential-phase cells in KCN-limited fed-batch cultures was accompanied by the formation of ammonia without the prior formation of cyanate. These results argue strongly in favour of a cyanide dioxygenase system being active in *P. fluorescens* NCIMB 11764. Induced cyanase activity (which was significantly higher than the induced cyanide-oxygenase activity) appeared to be independently regulated.

Kunz *et al.* (1992) have confirmed the presence of the cyanide oxygenase system in *P. fluorescens* NCIMB 11764, but have extended the understanding of cyanide degradation in this organism by showing that nitrilase-like and cyanide-hydratase-like pathways are also operative. Thus, CO<sub>2</sub> and NH<sub>3</sub> are not the only products of cyanide degradation in this organism. Furthermore, the bacterium was shown to degrade cyanide at much higher concentrations than previously described. The oxygenase-catalysed reaction to yield CO<sub>2</sub> and ammonia was the primary degradative pathway under aerobic conditions when the cyanide concentration was in the range 0.5 - 10 mM, but as the concentration increased beyond this range (up to 50 mM) the oxygenase pathway became progressively

less important and the other two pathways became more active. As expected very little  $\text{CO}_2$  was detected under anaerobic conditions. The control of the oxygenase pathway by the availability of  $\text{O}_2$  and the cyanide concentration was unexpected. This is, to date, the only microbial cyanide-oxygenase system which has been described. Unfortunately, no work has been published on the purification and properties of this inducible, intracellular, oxygen-dependent system. Detailed characterization of the enzyme involved may reveal similarities to NADH-requiring aromatic dioxygenases (Yeh *et al.*, 1977; Crutcher and Geary, 1979).

#### 1.4.5 $\beta$ -CYANOALANINE SYNTHASE

$\beta$ -Cyanoalanine synthase (L-cysteine hydrogen-sulphide-lyase (adding HCN), EC 4.4.1.9) catalyzes the reaction of L-cysteine with HCN to form  $\beta$ -cyanoalanine and  $\text{H}_2\text{S}$ :



(i.e. a  $\beta$ -replacement reaction in which the -SH of cysteine is replaced by -CN). This enzyme has been implicated in the assimilation of cyanide in higher plants. Blumenthal *et al.* (1968) showed that buffered extracts of sorghum (*Sorghum vulgare*), blue lupine (*Lupinus angustifolia* L.), bird's-foot trefoil (*Lotus tenuis*), and common vetch (*Vicia sativa* L.) catalyzed the formation of  $\beta$ -cyanoalanine and  $\text{H}_2\text{S}$  from L-cysteine and HCN. L-Serine could not be substituted for L-cysteine as the donor of the 3-carbon-atom unit for  $\beta$ -cyanoalanine synthesis. Partially purified  $\beta$ -cyanoalanine synthase from mitochondria of blue lupine seedlings required no added co-factors for catalytic activity (Hendrickson and Conn, 1969). Although the enzyme could not use L-serine as a substrate, it could use O-acetyl-L-serine at a very low rate for the formation of  $\beta$ -cyanoalanine. Akopyan *et al.* (1975) subsequently purified the  $\beta$ -cyanoalanine synthase from blue lupine seedlings and undertook a detailed investigation of the catalytic properties of the enzyme.

A survey of the occurrence of  $\beta$ -cyanoalanine synthase in cyanogenic and non-cyanogenic species of higher plants showed that this enzyme was present in all

15 of the plants tested and that there was a general correlation between cyanide-metabolizing activity of these plants and their potential to produce HCN i.e. cyanogenic plants exhibited higher  $\beta$ -cyanoalanine-forming activity than non-cyanogenic plants (Miller and Conn, 1980). This supported the hypothesis that  $\beta$ -cyanoalanine synthase may play a role in cyanide detoxification. Recent work showed that  $\beta$ -cyanoalanine synthase activity increased during the formation of ethylene (and therefore cyanide, Peiser *et al.*, 1984) in ripening apple, and in ethylene-treated pepper (Lurie and Klein, 1989).  $\beta$ -Cyanoalanine synthase activity was ample to detoxify the cyanide produced (Peiser *et al.*, 1984; Lurie and Klein, 1989). Fry and Myers (1981) reported that  $\beta$ -cyanoalanine synthase activity could be detected in both healthy and *G. sorghi*-infected tissues of sorghum, but that this activity decreased in infected leaves as the fungal disease developed. No  $\beta$ -cyanoalanine synthase activity was detected in *G. sorghi*.

Meyers and Ahmad (1991) reported that induction of  $\beta$ -cyanoalanine synthase in insects is used as a defense against cyanide toxicity. Furthermore, the level to which this enzyme was induced by cyanide determined the degree of cyanide tolerance exhibited by the insects.

Cell-free extracts of *E. coli* strain K12 grown on glucose-mineral salts medium produced an enzyme which catalyzed the formation of  $\beta$ -cyanoalanine from serine and cyanide (Dunnill and Fowden, 1965). However, a four-fold increase in the rate of  $\beta$ -cyanoalanine formation was recorded when ATP was present in the reaction mixture at concentrations above 0.01 M, indicating that the enzyme catalyzing this reaction may have been an ATP-dependent enzyme such as serine sulphydrase. This latter enzyme catalyzes the formation of cysteine from serine and  $H_2S$ . Interestingly, L-cysteine was also a substrate for the formation of  $\beta$ -cyanoalanine. Since the dependence of cysteine conversion to  $\beta$ -cyanoalanine on the ATP concentration was not determined, and the  $\beta$ -cyanoalanine-forming activity was not purified, it was impossible to determine whether the reaction was catalyzed by a serine sulphydrase with broad substrate specificity, or by the independent activities of such a serine sulphydrase and a separate  $\beta$ -cyanoalanine synthase.

Wissing and Andersen (1981) detected  $\beta$ -cyanoalanine-synthase activity in a *Pseudomonas* species during the investigation of cyanide production from glycine in this organism. The  $\beta$ -cyanoalanine-synthase activity could be separated from that of the cyanide-synthesizing enzyme by gel-filtration chromatography and

was shown to have a 10-fold higher specific activity than that of the cyanide-synthesizing enzyme. Further purification of the  $\beta$ -cyanoalanine synthase was not undertaken.

A strain of *Bacillus megaterium*, isolated from the same Fargo clay soil as the *B. pumilus* isolate of Skowronski and Strobel (1969), was shown to grow on 1 mM KCN and could incorporate the label of  $K^{14}CN$  into asparagine, aspartate and  $CO_2$  (Castric and Strobel, 1969). It was suggested that cyanide was being assimilated in this organism by conversion, via  $\beta$ -cyanoalanine, to asparagine and aspartate.

Castric and Conn (1971) subsequently showed that cyanide could not induce  $\beta$ -cyanoalanine-forming activity in this strain of *B. megaterium*. An *O*-acetylserine sulphydrylase (EC 4.2.99.8) was purified 40-fold from this organism and shown to have  $\beta$ -cyanoalanine-forming activity with cyanide and *O*-acetyl-L-serine, or L-cysteine, as the substrates for the reaction. Thus a broad-substrate-range enzyme involved in cysteine biosynthesis, and not a true  $\beta$ -cyanoalanine synthase, was shown to be responsible for the formation of  $\beta$ -cyanoalanine in this organism.

Resting cells of *C. violaceum* strain 9 were reported to produce  $\beta$ -cyanoalanine in the culture medium when incubated with glycine, methionine and succinate (Brysk *et al.*, 1969a). Cells incubated with serine and  $K^{14}CN$  accumulated  $\beta$ -cyanoalanine labelled in the cyano-carbon atom. Further radiolabelling experiments suggested that serine was synthesized from glycine *in vivo*. As carbon-2 of glycine is the precursor of cyanide in this organism (Michaels *et al.*, 1965), it appeared that glycine ultimately supplied all the carbon atoms of  $\beta$ -cyanoalanine. The  $\beta$ -cyanoalanine pathway appeared to be the only metabolic route by which cyanide was incorporated into cells of this organism. Brysk *et al.* (1969b) established that in the synthesis of HCN from carbon-2 and the amino group of glycine by this strain of *C. violaceum*, the carbon-nitrogen bond was not broken. The cyanide nitrile group was incorporated as a unit to become the cyano group of  $\beta$ -cyanoalanine (as suggested for *B. megaterium*).

Under culture conditions which promoted cyanogenesis in *C. violaceum*, induction of  $\beta$ -cyanoalanine synthase occurred only after cyanide production had occurred (Rodgers and Knowles, 1978). It was not known whether cyanide or glycine acted as the inducer. Interestingly,  $\beta$ -cyanoalanine synthase activity

was also induced in early stationary-phase cultures under conditions when relatively little cyanide was produced. Addition of chloramphenicol to cultures, after cyanogenesis had occurred, prevented the induction of  $\beta$ -cyanoalanine synthase and reduction of the cyanide concentration in the medium.

Further studies on cyanide metabolism and the factors which influence the formation of  $\beta$ -cyanoalanine in *C. violaceum* were undertaken by Rodgers (1981, 1982). Radiolabelling experiments with  $\beta$ -cyanoalanine indicated that this compound was not metabolized further to asparagine and aspartate, thus arguing for  $\beta$ -cyanoalanine formation being a means of cyanide detoxification rather than utilization in this organism. The amount of  $\beta$ -cyanoalanine produced was highest in cultures that were highly cyanogenic and the increase in the  $\beta$ -cyanoalanine concentration was accompanied by a decrease in the cyanide concentration of the medium (Rodgers, 1981). Both *O*-acetyl-L-serine and L-serine were shown to be good substrates for  $\beta$ -cyanoalanine synthesis, whereas L-cysteine inhibited  $\beta$ -cyanoalanine formation. L-Methionine was also shown to inhibit  $\beta$ -cyanoalanine formation by cells of *C. violaceum* (Rodgers, 1982).

Macadam and Knowles (1984) confirmed that  $\beta$ -cyanoalanine was the major product of cyanide degradation in *C. violaceum* and that its formation coincided with the disappearance of cyanide from the growth medium. The  $\beta$ -cyanoalanine-forming activity was purified to homogeneity and shown to be due to a true  $\beta$ -cyanoalanine synthase which converted cyanide and L-cysteine, or *O*-acetyl-L-serine, to  $\beta$ -cyanoalanine. Interestingly, the thiol compounds: ethanethiol,  $\beta$ -mercaptoethanol and dithiothreitol could replace cyanide as the co-substrate in the reaction. The  $K_m$  value for cyanide was 3.2 mM and those for  $\beta$ -mercaptoethanol and ethanethiol were 2.1 mM and 38 mM, respectively. The enzyme consisted of two identical 35-kDa subunits, exhibited optimal activity at pH 9.15, and contained approximately 2 moles of pyridoxal phosphate per mole of enzyme. The presence of this co-factor was manifested as an absorbance peak at 410 nm on spectrophotometric examination of purified  $\beta$ -cyanoalanine synthase. The identity of the cofactor was verified by its extraction from the enzyme followed by colourimetric analysis. Interestingly, the addition of exogenous pyridoxal phosphate did not enhance enzyme activity. Addition of the amino-acid substrates to the purified enzyme resulted in the disappearance of the pyridoxal phosphate absorbance peak, indicating an interaction between the enzyme-bound cofactor and the amino-acid substrate. The addition of cyanide on its own did not affect the absorbance spectrum. The enzyme

exhibited interesting inhibition behaviour with *O*-acetyl-L-serine (an alternative amino-acid substrate) exerting strong competitive inhibition when L-cysteine was the substrate. Both KCN and L-cysteine exerted substrate inhibition at high concentrations.  $\beta$ -Cyanoalanine exerted product inhibition. L-Methionine acted as a non-competitive inhibitor. Addition of  $Mg^{2+}$ ,  $Ca^{2+}$  and  $Zn^{2+}$  had no significant effect on activity, but low concentrations of EDTA (380  $\mu$ M) caused complete inhibition of activity which could be partially reversed on addition of 2 mM  $Mg^{2+}$ . These results indicated a requirement for divalent metal ions. The enzyme was inhibited by thiol-specific reagents. The purified  $\beta$ -cyanoalanine synthase exhibited no L-cysteine synthase (*O*-acetylserine sulphydrylase) activity.

This report is the only one, to date, on the purification and characterization of a true  $\beta$ -cyanoalanine synthase from a bacterium. It is at variance with the reports of Brysk *et al.* (1969a) and Rodgers (1981; 1982) which claimed that L-serine was a good substrate for  $\beta$ -cyanoalanine formation in *C. violaceum*, unless there is an alternative pathway for  $\beta$ -cyanoalanine formation in this organism (e.g. via an *O*-acetylserine sulphydrylase). The *C. violaceum*  $\beta$ -cyanoalanine synthase appeared to be a typical pyridoxal-phosphate-containing enzyme and is notably similar to bacterial L-cysteine synthases with respect to this, and other, physico-chemical properties (Macadam and Knowles, 1984). Although the true  $\beta$ -cyanoalanine synthases of higher plants are also pyridoxal-phosphate-containing enzymes they appear to be distinct from the *C. violaceum*  $\beta$ -cyanoalanine synthase.

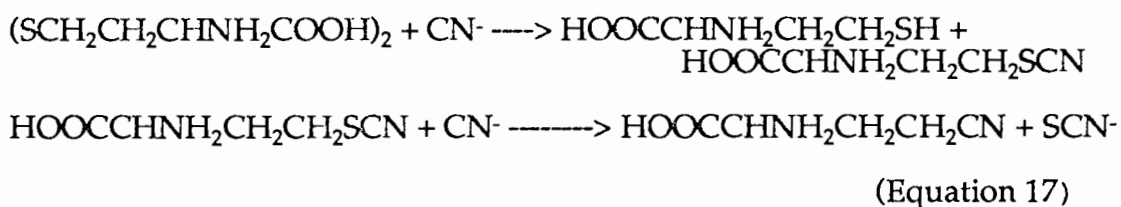
It is evident that three biochemical pathways have been described for the formation of  $\beta$ -cyanoalanine. The first was described in *E. coli* in which L-serine and cyanide serve as substrates (Dunnill and Fowden, 1965). The second involves  $\beta$ -cyanoalanine formation as described in blue lupine (Hendrickson and Conn, 1969); and the third involves the reaction of *O*-acetyl-L-serine with cyanide to form  $\beta$ -cyanoalanine as reported in *B. megaterium* (Castric and Conn, 1971). The  $\beta$ -cyanoalanine-forming enzyme in *B. megaterium* has been shown to be an L-cysteine synthase (*O*-acetyl-L-serine sulphydrylase) and not a true  $\beta$ -cyanoalanine synthase. It seems very likely that the  $\beta$ -cyanoalanine-forming reaction described in *E. coli* was also not catalyzed by a true  $\beta$ -cyanoalanine synthase.

True  $\beta$ -cyanoalanine synthases appear to utilize L-cysteine as a preferred amino-acid substrate with cyanide as the co-substrate. O-acetyl-L-serine may serve as a less favoured secondary amino-acid substrate.

#### 1.4.6 $\gamma$ -CYANO- $\alpha$ -AMINO BUTYRIC ACID SYNTHASE

Brysk and Ressler (1970) noted that young cultures of *C. violaceum* strain D341, which had been administered cyanide, accumulated  $\beta$ -cyanoalanine and a previously undetected compound. Thorough investigation led to the identification of this compound as  $\gamma$ -cyano- $\alpha$ -aminobutyric acid, which had never before been described as a biological molecule. Radiolabelling studies showed that the cyano group of this new compound was supplied by cyanide and that the rest of the carbon chain was derived from aspartic acid. Although 26 % of  $K^{14}CN$  was incorporated into  $\gamma$ -cyano- $\alpha$ -aminobutyric acid, this compound was not metabolized any further suggesting that it played a role in cyanide detoxification rather than cyanide utilization.

Ressler *et al.* (1973) subsequently purified an enzyme from *C. violaceum* strain D341 which catalyzed the conversion of homocystine and cyanide to  $\gamma$ -cyano- $\alpha$ -aminobutyric acid, thiocyanate and homocysteine:



It was proposed that the first step in the reaction involved the non-enzymatic reaction of homocysteine with cyanide to form cysteine and  $\gamma$ -thiocyano- $\alpha$ -aminobutyric acid. The purified enzyme was thought to catalyze the reaction of  $\gamma$ -thiocyano- $\alpha$ -aminobutyric acid with another cyanide ion to form  $\gamma$ -cyano- $\alpha$ -aminobutyric acid and thiocyanate. The enzyme was named  $\gamma$ -thiocyanoaminobutyric acid thiocyano-lyase (adding cyanide) ( $\gamma$ -cyano- $\alpha$ -aminobutyric acid synthase). The purified enzyme had a molecular mass of 130 kDa, exhibited optimal activity in the pH range from 8.3 - 9.6, and required pyridoxal 5'-phosphate for activity (as does  $\beta$ -cyanoalanine synthase).  $\gamma$ -Cyano- $\alpha$ -aminobutyric acid and cyanide were produced in nearly stoichiometric

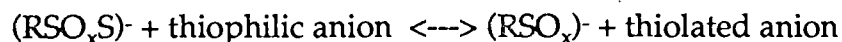
amounts. The  $K_m$  for cyanide was 30 mM and that for  $\gamma$ -thiocyano- $\alpha$ -aminobutyric acid was 29 mM. The enzyme was sensitive to product inhibition by thiocyanate, but not  $\gamma$ -cyano- $\alpha$ -aminobutyric acid. Although the action of the enzyme in converting cyanide to thiocyanate is superficially similar to the reaction catalyzed by rhodanese (thiosulphate: cyanide sulphurtransferase, EC 2.8.1.1) the enzymes are different: rhodanese generally has a much lower molecular weight than  $\gamma$ -cyano- $\alpha$ -aminobutyric acid synthase and also has no requirement for a pyridoxal-phosphate cofactor.

Despite the report by Rodgers and Knowles (1978) confirming the detection of  $\gamma$ -cyano- $\alpha$ -aminobutyric acid synthase in *C. violaceum* (strain NCIB 9131), and data showing that the activity of this enzyme was stimulated when cyanogenesis was maximal, subsequent attempts to detect the incorporation of the label of  $K^{14}CN$  into  $\gamma$ -cyano- $\alpha$ -aminobutyric acid and thiocyanate were unsuccessful (Rodgers, 1981; 1982). The reasons for these conflicting reports are not known, but may have been due to the use of cells harvested at different phases of growth (Rodgers, 1982).

The physiological significance of  $\gamma$ -cyano- $\alpha$ -aminobutyric acid synthase is unclear since it has only been described in one bacterial species and has a non-enzymatically produced substrate ( $\gamma$ -thiocyano- $\alpha$ -aminobutyric acid). The low affinity of the enzyme for its substrates (as indicated by the high  $K_m$  values) suggests that the reaction with cyanide may be a secondary catalytic function of the enzyme (Castric, 1981).

#### 1.4.7 RHODANESE

Biological sulphane-sulphur-containing molecules and the enzymes which act on these molecules have been reviewed by Westley (1981) and Volini and Alexander (1981). Rhodaneses are the best characterized of these enzymes and are very widely distributed in biology. Rhodanese (thiosulphate: cyanide sulphur transferase, EC 2.8.1.1) catalyzes the reaction between a sulphur-donating substrate containing sulphane sulphur and a thiophilic anion:

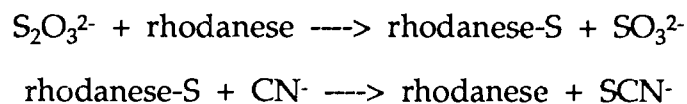


(Equation 18)

where R = aryl, alkyl, or  $\text{O}^-$  and  $x = 0, 1$ , or  $2$

Sulphane sulphur is sulphur in the 2-minus oxidation state which is covalently bonded only to another sulphur atom. Thiophiles are nucleophiles which can react with sulphane-sulphur-containing compounds to yield a thiol and a thiolated anion. Cyanide is a strong thiophile. The sulphur-donating substrate  $[(\text{RSO}_x\text{S})^-]$  can be almost any sulphane-sulphur-containing anion (e.g.  $\text{S}_2\text{O}_3^{2-}$ ) and the acceptor substrate can be any of a number of thiophilic anions (e.g.  $\text{CN}^-$ ).

The rhodanese-catalyzed reaction between thiosulphate and cyanide is unusual in that it is the only one of the rhodanese-catalyzed reactions which is irreversible (Knowles, 1976). The mechanism whereby rhodanese catalyzes the transfer of sulphane sulphur involves a double-displacement mechanism (i.e. an indirect transfer) in which the sulphane sulphur atom first becomes covalently bonded to the enzyme before being transferred to the acceptor substrate (Westley, 1981; 1988). Thus, in the reaction by which cyanide is detoxified:



(Equation 19)

An extensive collection of data has been compiled on the properties of rhodanese, including its catalytic mechanism, amino-acid sequence and 3-dimensional structure (Westley, 1981; 1988). Although rhodanese can catalyze the interconversion of many sulphane-containing compounds, it was first described in 1933 for its ability to transfer sulphane sulphur to cyanide and was thought to function specifically for the detoxification of cyanide. It is this reaction for which it is best known (Westley, 1981; Volini and Alexander, 1981). The enzyme occurs in some plants, fungi and bacteria as well as in all the phyla of the animal kingdom. Miller and Conn (1980) noted that rhodanese occurred less commonly in higher plants than did  $\beta$ -cyanoalanine.

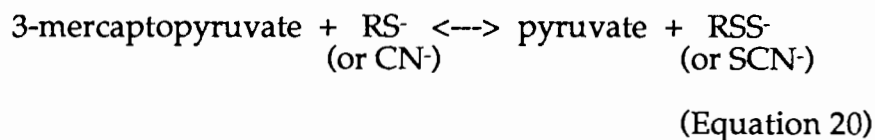
Rhodanese has been described in a number of heterotrophic microorganisms. A strain of *Bacillus stearothermophilus* was reported to contain a constitutively produced rhodanese (Atkinson *et al.*, 1975) which was of interest for the industrial removal of cyanide from wastewaters as thiocyanate (Atkinson, 1975). *P. aeruginosa* (Ryan *et al.*, 1979) and *E. coli* (Volini and Alexander, 1981) also contain rhodanese. The fungus *Rhizopus oryzae*, which causes spoilage of harvested cassava, was shown to induce an extracellular rhodanese on exposure to low concentrations of cyanide (1.0 mM) (Padmaja and Balagopal, 1985). This enzyme was also induced to low levels in the absence of cyanide suggesting a more general role for the enzyme in metabolism than just cyanide detoxification. Rhodanese activity was also reported in *C. violaceum* (Rodgers and Knowles, 1978), but this could not be verified in subsequent experiments (Rodgers, 1981; 1982).

Data supporting a wider role for rhodanese (than just cyanide detoxification) in bacterial metabolism were provided from studies with *E. coli* CSH61-1. The rhodanese activity in this organism was about 10-fold lower under anaerobic conditions than under aerobic conditions and provided evidence for the involvement of rhodanese in some way in aerobic energy metabolism in this bacterium. An indirect involvement of rhodanese was suggested, possibly by the involvement of the enzyme in the biosynthesis of the iron-sulphur group of proteins, some of which are important participants in aerobic respiration (Volini and Alexander, 1981).

Thus although the original role proposed for rhodanese was that of a sulphur donor in cyanide detoxification, this role can be seen as being complementary to its role as a sulphur donor to other ions or molecules (proteins in the case of Fe-S cluster synthesis). Cyanide detoxification by rhodanese could therefore be regarded as an ancillary function of an enzyme involved in sulphur metabolism (Knowles, 1976; Volini and Alexander, 1981; Castric, 1981; Harris *et al.*, 1987).

#### 1.4.8 MERCAPTOPYRUVATE SULPHURTRANSFERASE

Mercaptopyruvate sulphurtransferase (3-mercaptopyruvate: cyanide sulphurtransferase, EC 2.8.1.2) catalyzes the transfer of a sulphur atom from 3-mercaptopyruvate to a thiol-acceptor substrate (Westley, 1981):



The enzyme is specific for 3-mercaptopyruvate and is distinct from rhodanese in that it cleaves a carbon-sulphur bond as opposed to a sulphur-sulphur bond i.e. the source of the sulphur is not a sulphane sulphur atom. Furthermore, catalysis involves a single-displacement-type mechanism in which both the sulphur-donor and -acceptor species have to be bound to the enzyme simultaneously before catalysis can proceed (Westley, 1988). However, like rhodanese this enzyme can use a wide variety of thiophilic anions as sulphur-acceptor substrates (Westley, 1988). There is relatively little information on the properties of 3-mercaptopyruvate sulphurtransferase. It functions as a transsulphurase in animals and has been isolated from mammalian kidney tissue (Westley, 1988). It has also been described in *E. coli* (Vachek and Wood, 1972), but its function is uncertain. It is likely that it is involved primarily in the metabolism of mercaptopyruvate in this organism with cyanide detoxification being a minor secondary function.

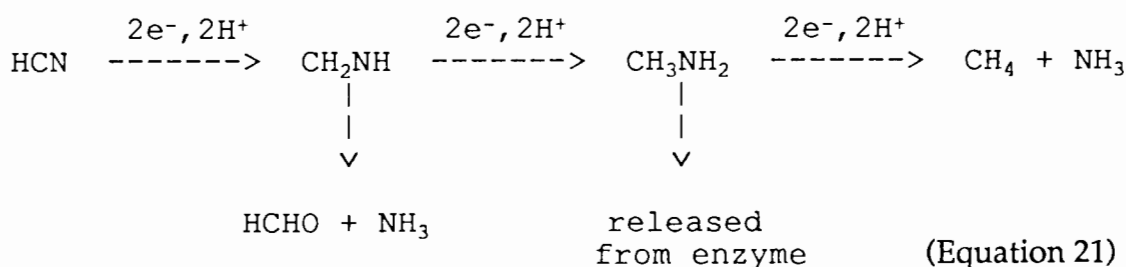
#### 1.4.9 NITROGENASE

Nitrogen fixation and the reduction of substrate analogues of N<sub>2</sub> by nitrogenase require two proteins: a molybdenum-iron (MoFe) protein and an iron (Fe) protein. The enzyme also requires a reducing agent, MgATP, and protons. The Fe protein is thought to be the specific one-electron donor to the MoFe protein in which the reduction of the substrate is thought to occur. Nitrogenase is known to catalyze the reduction of cyanide, alkyl cyanides, alkyl isocyanides and azide amongst others (Li *et al.*, 1982).

Cyanide reduction by nitrogenase was first reported by Hardy and Knight (1967) who used crude preparations of nitrogenase from *Azotobacter vinelandii* and *Clostridium pasteurianum* to show that cyanide underwent a six-electron reduction to form methane and ammonia plus small amounts of another basic product. This unidentified basic product was suggested to be methylamine, generated by the four-electron (incomplete) reduction of cyanide. The reduction of cyanide required the presence of both protein components, MgATP and a reducing agent.

Carbon monoxide (0.9 atm) completely inhibited the reaction. These results were subsequently confirmed by other investigators (for example Hwang and Burris, 1972; Rivera-Ortiz and Burris, 1975). However, the early work on cyanide reduction was confusing as there were conflicting reports on the effect of other nitrogenase substrates (such as  $\text{N}_2\text{O}$ ) on cyanide reduction, and there was no certainty regarding which cyanide species ( $\text{CN}^-$  or  $\text{HCN}$ ) actually served as the enzyme substrate (Li *et al.*, 1982). Furthermore, cyanide was observed to inhibit its own reduction at cyanide concentrations above 5 mM (Li *et al.*, 1982).

Li *et al.* (1982) clarified these issues by studying cyanide reduction using the purified MoFe and Fe protein components from *A. vinelandii*. This study showed that  $\text{CN}^-$  was a potent inhibitor of the total electron flow through nitrogenase by apparently uncoupling ATP hydrolysis and electron transfer to the substrate ( $K_i = 27.2 \pm 2.8 \mu\text{M CN}^-$ ). The inhibition by  $\text{CN}^-$  could be partially reversed by azide and fully reversed by carbon monoxide. This suggested a common binding site on nitrogenase for  $\text{CN}^-$  and CO. Interestingly, the  $\text{HCN}$  species of cyanide was shown to be a substrate for nitrogenase with a  $K_m$  of  $4.5 \pm 0.3 \text{ mM HCN}$ . The proposed overall reaction scheme for cyanide reduction by nitrogenase (including the products of partial cyanide reduction) was:



It was not known with which protein component the  $\text{CN}^-$  was interacting, but it appeared not to be binding to the site of  $\text{HCN}$  reduction.

It is important to bear in mind that nitrogenase is restricted to nitrogen-fixing microorganisms and has a primary role in nitrogen metabolism in these organisms. Since its substrate specificity happens to include cyanide, the detoxification of this compound can be regarded as a fortuitous minor function of the enzyme. For this reason, nitrogenase is unlikely to have any application in the industrial detoxification of cyanide. Furthermore, the  $\text{CN}^-$  form of cyanide inhibits the reaction and although the substrate ( $\text{HCN}$ ) form predominates at

physiological pHs, the small amounts of the ionic form that are present will influence the efficiency of cyanide removal. Application of the enzyme on its own would be difficult as it requires strictly anaerobic conditions for activity.

#### 1.4.10 OTHER CYANIDE-DEGRADING ENZYMES

There are a number of other cyanide-degrading activities which have been reported, but these are characterized by all having been described in the snow mould fungus or *Rhizoctonia solani* and are each represented by only a single report (Strobel 1964; 1966; 1967b; Mundy *et al.*, 1973). All these reactions were proposed to involve the formation of an aminonitrile from cyanide and an aldehyde. The importance of these pathways for the removal of cyanide is not known. There appear to have been no subsequent reports on cyanide incorporation into aldehydes.

The purification and characterization of more cyanide-degrading enzymes, particularly cyanide hydratases and cyanide nitrilases, will greatly advance this field of research and allow it to be determined whether such enzymes belong to the broader classes of nitrile hydratases and nitrilases, respectively, or whether such enzymes are a special cyanide-specific sub-group which deserve their own Enzyme-Commission nomenclature to distinguish them from the broader category of nitrile-degrading enzymes.

The microbial metabolism of the structurally related ions, thiocyanate ( $\text{SCN}^-$ ) and cyanate ( $\text{CNO}^-$ ), is a subject which is beyond the scope of this review. Thiocyanate degradation by microorganisms (for example, Stafford and Cally, 1969; Betts *et al.*, 1979) has not received much attention and until recently had not been investigated at the enzymological level. However, Katayama *et al.* (1992) have reported their findings on a thiocyanate hydrolase from *Thiobacillus thioparus* which catalyzes the conversion of thiocyanate to carbonyl sulphide ( $\text{S}=\text{C}=\text{O}$ ). The oxidation of thiocyanate by bovine lactoperoxidase and human myeloperoxidase has been investigated in some detail (Aune and Thomas, 1977; Pollock and Goff, 1992).

The conversion of cyanate to ammonia and bicarbonate ( $\text{HCO}_3^-$ ) by cyanase (EC 3.5.5.3) in *E. coli* has enjoyed considerable attention. The inducible, cyanate-

specific enzyme has been purified from *E. coli* and characterized (Anderson, 1980). It consists of four or five identical subunits for which the amino-acid sequence has been determined (Chin *et al.*, 1983). The kinetic properties of the enzyme have been examined (Anderson and Little, 1986; Anderson *et al.*, 1987) and the gene encoding it (*cynS*) has been cloned (Sung *et al.*, 1987a) and sequenced (Sung *et al.*, 1987b). The mechanism of cyanate degradation by cyanase involves bicarbonate as a co-factor and appears to be fundamentally different from that for cyanide degradation. Its detailed discussion here is thus not particularly relevant.

## 1.5 BIOLOGICAL DEGRADATION OF CYANIDE IN INDUSTRIAL WASTEWATERS

### 1.5.1 CYANIDE IN INDUSTRIAL PROCESSES AND DEVELOPMENT OF BIOLOGICAL DETOXIFICATION SYSTEMS

Sodium cyanide was first prepared in the laboratory in 1834, but remained a subject of predominantly academic interest until useful applications were discovered which were to lead to its widespread commercial application in the electroplating, gold-mining and other important industries. The discovery of the electroplating process for gold and silver in about 1840 made the alkali-metal cyanides chemicals of commercial significance. The MacArthur-Forrest cyanide process for gold extraction was patented in 1887 and described the employment of dilute cyanide solutions for the extraction of gold and silver from their ores. After being successfully tested on a commercial scale in the gold mines of New Zealand and South Africa in 1899, this process was applied in goldfields all over the world. This led to a rapid expansion of the cyanide-manufacturing industry with world production of alkali-metal cyanides increasing from 5900 metric tons per year in 1899 to 21 000 metric tons per year in 1915 (Jenks, 1979). The annual worldwide usage of sodium cyanide in 1977 was about 113 000 metric tons (Jenks, 1979). Apart from electroplating and mining, sodium cyanide also finds a major application in the synthetic-chemicals industries (Jenks, 1979).

Hydrogen cyanide (HCN) once found wide application as a fumigant, but its major use now is in the preparation of inorganic cyanides (sodium cyanide, potassium cyanide and iron cyanides), and in the synthesis of organic chemicals

such as methyl methacrylate, methionine, triazines, adiponitrile and chelates. The production of HCN in the United States of America in 1977 was estimated to be 259 000 metric tons (Jenks, 1979).

The worldwide use of cyanide in industry must clearly generate a large amount of cyanide-containing waste every year. The cyanide in this waste must either be recycled and re-used, or must be detoxified since its high toxicity to a wide variety of biota precludes its discharge directly into the environment. A study on the origins of cyanide contamination in small rivers in north-western Germany showed a correlation between the level of cyanide contamination and the population density, or the presence of steel and galvanic industries, in the catchment areas of the rivers (Krutz, 1981). Those rivers polluted by discharges from the galvanic industries had substantially higher cyanide concentrations than rural watersheds where the cyanide came from sources other than industry (i.e. biological sources).

High concentrations of free cyanide (as HCN) rapidly kill aerobic organisms by inhibiting cellular respiration and causing asphyxia. The toxicity of cyanide is however modified by several factors which include the nature of the cyanide compound (transition metal complexes of cyanide are less toxic than HCN or  $\text{CN}^-$ ) and the metabolic abilities of the organisms (some microorganisms can induce cyanide-insensitive respiratory pathways). Studies on the aquatic toxicology of cyanide have shown that fish are very sensitive to cyanide and that the lethal concentration for various species of freshwater fish is in the range 30 - 150  $\mu\text{g HCN/l}$  (Leduc, 1981). Although it is regarded as important to keep cyanide concentrations in natural waters below 5  $\mu\text{g HCN/l}$  to protect fish populations from the harmful sublethal effects of cyanide, this figure is based on laboratory studies in which the concentrations of cyanide were fixed. Little is known about the effects on fish in natural waters of varying levels of cyanide (and the factors which modify and reduce its toxicity), nor the adaptive resistance fish may develop to lethal concentrations of cyanide (Leduc, 1981). Furthermore, there is not much information on the long-term effects of discontinuous sublethal cyanide concentrations. There is also scant information on the effects of cyanide on marine organisms and on freshwater organisms other than fish (Leduc, 1981).

There is thus a need to reduce the concentration of cyanide in industrial wastewaters to a low, non-toxic level before release into the environment. The

maximum permissible level of free cyanide discharged into rivers in South Africa is 0.5 mg/l, although the maximum permissible level discharged into municipal sewers may be somewhat higher (20 mg/l in the Cape Town Municipality). Smith (1972) described a number of physical and chemical methods for the removal of cyanide from industrial wastewaters. These included air stripping of HCN from acidified waste solutions heated to 99 °C (with removal of the HCN by scrubbing with sodium hydroxide); electrolytic oxidation of cyanide; ozonation of metal-cyanide complexes with ozone; oxidation with chlorine ( $\text{Cl}_2$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), hypochlorite ( $\text{OCl}^-$ ), persulphates, or other oxidizing agents; and adsorption of cyanide onto charcoal. Chemical processes for the detoxification of cyanide are expensive, use chemicals which might be more profitably applied in other ways, may generate toxic by-products, and (in the case of chlorine-containing oxidizing agents) lead to an increase in the chloride content of the industrial effluent (Howe, 1965; Knowles, 1976).

These problems led to the investigation of alternative methods for destroying cyanide and resulted in the development of cheaper biological processes. The use of biological methods, however, was for a long time neglected as cyanide was considered to be far too potent a respiratory inhibitor to make biological cyanide detoxification possible (Howe, 1965; Knowles, 1976). Once the biological feasibility of cyanide detoxification had been realised in the 1940s, a variety of processes were investigated for the biological removal of cyanide from industrial wastewaters.

Howe (1965) reviewed some of the earlier outstanding achievements in this field which included (for the aerobic degradation of cyanide) the use of trickling filters; activated sludge processes; a two-stage, long-term sludge lagoon system; and a combination of treatments in sequence. Anaerobic biological detoxification processes employed single or two-stage digesters. A patent was taken out for the anaerobic, two-stage digestion of cyanide wastes (United States Patent 3,145,166), and another for the sequential treatment of cyanide wastes by sludge adsorption, sludge complexation, clarification and biological oxidation (United States Patent 3,165,166).

Raef *et al.* (1977a; 1977b) investigated the relative importance of four mechanisms of cyanide removal, namely, reaction of cyanide with glucose in solution; adsorption of cyanide onto biological solids (floc); loss of cyanide to the air (stripping); and biological metabolism of cyanide. The reaction of cyanide with

aldoses was regarded as a significant route for cyanide removal from solution only above pH 8.0; since the reaction is slow under the conditions used in biological studies it is probably of minor importance. The physical removal of cyanide by adsorption onto biological floc was regarded as a minor removal mechanism (Raef *et al.*, 1977b). Stripping of HCN from stirred, aerated systems (pH 7.0 and 30 °C), and biological removal, were regarded as the most significant removal mechanisms (Raef *et al.*, 1977b).

Imperial Chemical Industries (ICI, United Kingdom) filed an application for a patent describing the conditions for production of cyanide hydratase in *F. lateritium* Nees CMI 300533 for the treatment of cyanide-containing solutions (European Patent Application No. 87300840.3; Publication No. 0 233 719 A2, 1987). ICI Bio-Products markets a preparation of the cyanide-induced fungus as a granular biocatalyst (dry powder or in solution) called Cyclear which offers a biological alternative to the conventional chemical methods of cyanide detoxification. This product can degrade free cyanide in the concentration range 150 - 10000 ppm, but works optimally at 5000 ppm cyanide at 25 - 35 °C in the pH range 8.0 - 8.5. Under optimal conditions 5000 ppm cyanide can be reduced to less than 10 ppm in 6 h. An application of 1 kg of Cyclear is claimed to be capable of treating up to 50 kg of free cyanide. The preparation has very little activity toward metal-cyanide complexes (ICI Bio-Products, Cyclear product description).

The advantages of biological cyanide degradation include low running costs for established biological treatment facilities because there is no requirement for oxidizing chemicals, chemical storage, chemical handling equipment and expensive control instruments. Furthermore, existing biological-treatment facilities for non-cyanide wastes can be adapted, with some modification and adjustment, to treat cyanide wastes (Howe, 1965).

However, there are also several disadvantages to using biological processes for detoxifying cyanide: small volumes of cyanide wastes are better treated by chemical means if no biological treatment facility is already available; appreciable concentrations of heavy metals in many cyanide wastewaters may interfere with biological activity; biological treatment processes can be upset by a variety of factors (e.g. heavy metal ions) and therefore require the provision of emergency chemical or lagoon facilities to continue the treatment process during periods of low biological activity (so as to prevent interruption of the industrial

production process which generates the cyanide wastewater); and acclimatization of microorganisms to cyanide, or recovery after an interruption, require a week's or two weeks' time. Finally, the efficiency of chemical processes can be tightly controlled, whereas the efficiency of a biological treatment process depends on the characteristics of the waste received (for example, with respect to cyanide and heavy metals concentrations), the composition of the microbial population, and factors which influence microbial activity, including pH, temperature, nutrient supply, degree of aeration and the presence of toxic compounds (Howe, 1965).

Biological processes cannot compete with chemical processes for cyanide detoxification when the volume of cyanide to be treated is small, and are limited to the treatment of low concentrations of cyanide (Howe, 1965; 1969). Nevertheless, they are both practical and economical under certain conditions. For such biological processes to operate efficiently it is extremely important that the acclimatized microorganisms receive the required nutrients and that the composition of the cyanide waste is thoroughly understood (Howe, 1965). Chemical or physical methods of cyanide removal should not be overlooked where they are economically appropriate (Howe, 1969) and do not exacerbate the toxicity of the waste. However, under the current global circumstances of heightened environmental awareness it seems likely that biological detoxification methods will be favoured, which means that there is a need to develop more robust, more efficient biological processes to meet the anticipated need for them.

In view of the foregoing discussion it is worth looking at the achievements of the Homestake Mining Co. (U.S.A.) in developing a commercial-scale biological facility for the treatment of cyanide-containing mining wastewater at the Homestake gold mine in Lead, South Dakota (Mudder and Whitlock, 1984). Although several chemical and physical treatment processes were tested on a pilot-plant scale (either alone or in combination), none of these methods was found to be suitable because their treatment performance was inadequate, the toxicity of the treated effluent was too high, or the cost of establishing and operating them would have been prohibitive. As a result, attention was focussed on evaluating the possibility of developing a simple, inexpensive, biological-treatment facility which would overcome the limitations of the other treatment processes while producing a high-quality, low-toxicity effluent.

The effectiveness of biological detoxification of cyanide was tested by assessing both suspended-growth (activated sludge) and attached-growth treatment processes (trickling filters, biological towers, aerated biological filters, and rotating biological contactors) with and without raw sewage as a supplemental carbon source. The addition of supplemental carbon was found to be unnecessary for biological degradation of cyanide and thiocyanate as these compounds served as the sole carbon source for the bacteria. The bacterial inoculum was obtained on the Homestake property and was indigenous to the Homestake wastewaters.

The only chemical requirements for the biological treatment processes were an inorganic carbon source (soda ash) to aid nitrification and phosphorus as a trace nutrient. The biological treatment processes removed thiocyanate, free and metal cyanides (including iron cyanides), metals (mainly copper), and ammonia (a by-product of cyanide and thiocyanate degradation). The detoxification products included sulphate, nitrate and carbonate.

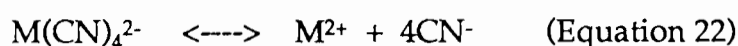
Although activated sludge removed cyanide most efficiently, microbial growth was slow and accumulated metals became toxic during the course of operation. All the attached-growth processes worked well and gave an effluent of the required quality, but the rotating-biological-contactor process proved to be the best and least expensive for this application. A large biological treatment facility was constructed to treat the cyanide-containing wastewater from the Homestake gold mine (containing up to 10 mg/l total cyanide). The facility employed 48 rotating biological contactors followed downstream by a clarifier and pressure sand filters to improve the quality of the final effluent which was discharged into Whitewood Creek. The plant was designed to treat a maximum of 21000 m<sup>3</sup> of wastewater per day with a total hydraulic residence time through the plant of approximately 5 h. Toxicological testing of the final effluent was undertaken (using young rainbow and brown trout, and several macroinvertebrates) to ensure that the treated water was suitable for release into the environment.

The success of the Homestake operation represents one of the remarkable achievements of biological detoxification of industrial cyanide-containing wastewaters and is apparently the only commercial-scale facility for the treatment of cyanidation wastewaters from the gold-mining industry.

### 1.5.2 BIOLOGICAL REMOVAL OF CYANO-METALS

The degradation of cyano-metal complexes by microbial cyanide-degrading systems has received surprisingly little attention despite the presence of these complexes in many industrial cyanide wastewaters. Pettet and Mills (1954) showed that small sewage-treatment percolating filters which had been acclimatized to cyanide could completely degrade the tetracyano complexes of  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$ , but could only degrade about 75 % of the tetracyano complexes of  $\text{Cu}^+$  and  $\text{Ni}^{2+}$ . Ferrocyanide,  $\text{Fe}(\text{CN})_6^{4-}$ , was oxidized to ferricyanide but was not degraded to any great extent. Smith (1972) reported that ferricyanides were also stable to ozonation.

The tetracyano complexes of the divalent transition-metal ions in solution are expected to exist in equilibrium with the free metal and free cyanide ions:



although the relationship may be complicated by the potential to form complexes with fewer cyanide ions. The tendency of the complex to dissociate into its constituent ions is indicated by the stability constant:

$$\text{stability constant (K)} = \frac{[\text{M}^{2+}][\text{CN}^-]^4}{[\text{M}(\text{CN})_4^{2-}]}$$

(Equation 23)

The complexes tested by Pettet and Mills (1954) had stability constants which increased in the order:  $\text{Cd}^{2+} < \text{Zn}^{2+} < \text{Cu}^+ < \text{Ni}^{2+} \ll \text{Fe}^{2+}$ . Since the weaker complexes were degraded best it appeared that these must have existed in solution in the predominantly dissociated form. This also suggests that the microbial flora in the filters were degrading free cyanide rather than the metal-bound cyanide. The resistance of the very stable ferrocyanide complex to degradation lends support to this hypothesis.

Rollinson *et al.* (1987) showed that *P. fluorescens* NCIMB 11764 (originally isolated on HCN; Harris and Knowles, 1983a) could be grown on tetracyanonickelate(II),  $\text{Ni}(\text{CN})_4^{2-}$ , as the source of nitrogen at a concentration (0.25 mM) below the MIC

of free  $\text{Ni}^{2+}$ . Tetracyanocuprate(II),  $\text{Cu}(\text{CN})_4^{2-}$ , was also able to serve as a nitrogen source, but with a lower maximum cell yield. Ferrocyanide and tetracyanozincate(II) supported very little growth. The use of  $\text{Ni}(\text{CN})_4^{2-}$  overcame the toxic effects observed when KCN was used as the nitrogen source in cultivation of the organism for the induction of cyanide-oxygenase activity. The high stability constant for  $\text{Ni}(\text{CN})_4^{2-}$  ( $K = 10^{22}$ ) indicated that the concentrations of free cyanide in the medium would have been very low. It was not possible to determine whether the cyano-metal complex or the free cyanide anion (or HCN) induced the cyanide-oxygenase activity, nor whether the induced enzyme acted on the complexed cyanide or only on the free cyanide.

Silva-Avalos *et al.* (1990) subsequently isolated several species of *Pseudomonas* and *Klebsiella* that were able to grow on  $\text{Ni}(\text{CN})_4^{2-}$  as the sole source of nitrogen. More detailed investigation of one of the isolates, *P. putida* strain BCN3, showed that it grew concomitantly with  $\text{Ni}(\text{CN})_4^{2-}$  utilization. Comparison with *P. fluorescens* NCIMB 11764 showed that strain BCN3 could grow faster on 0.25 mM  $\text{Ni}(\text{CN})_4^{2-}$ . The presence of ammonia completely suppressed the utilization of the cyano-metal complex. Growth of the organism on 8 - 16 mM  $\text{Ni}(\text{CN})_4^{2-}$  was accompanied by the formation of a green, flocculent precipitate of  $\text{Ni}(\text{CN})_2$  in the medium. This compound appeared to arise from the metabolism of  $\text{Ni}(\text{CN})_4^{2-}$ , but neither the mechanism of its formation, nor whether it was metabolized further, were investigated. It was not possible to determine whether  $\text{Ni}(\text{CN})_4^{2-}$  itself was attacked or whether dissociated cyanide (as  $\text{CN}^-$  or HCN) was the substrate. Preliminary evidence suggested that the organism was utilizing the free cyanide thereby shifting the equilibrium away from the complex in favour of the formation of the cyanide anion.

*Acinetobacter* sp. strain RFB1 was shown to assimilate a wide variety of compounds including simple cyanides, cyano-metal complexes, potassium thiocyanate, sodium cyanate, and a number of aliphatic and aromatic nitriles (Finnegan *et al.*, 1991a, 1991b). The organism grew on  $\text{KAu}(\text{CN})_2$  as the sole source of carbon and nitrogen, and could precipitate gold(I) cyanide from solution. An extracellular complex of mixed fatty acids and proteins was shown to be responsible for the degradation of cyanide-containing substrates. This complex was partially purified by gel-filtration chromatography but could not be purified further. The complex showed optimal cyanide-degrading activity at 20 °C and pH 6.5. Its wide range of substrates suggested that it was a suitable

candidate for use in the degradation of industrial cyanide-containing wastes, as well as in the recovery of gold from solution (Finnegan *et al.* 1991a; 1991b).

Novo Nordisk A/S (Denmark) filed a patent describing the specific decomposition of metal-cyanide complexes using whole cells or a purified enzyme from *Alcaligenes* sp. strains DSM 4010 and DSM 4009 (Danish Patent Application No. 1283/87 of 1987; South African Patent Application No. 894989). The patent describes optimum degradation of metal cyanides at 37 °C in the pH range 6.0 - 8.5 with cyanide-complex concentrations from 1 mM to more than 50 mM depending on the type of metal-cyanide complex. The cyanide complexes of Ni, Cu, Co, Zn, Cd, Au, Ag, Fe and Pd are all claimed to be degraded by cells or enzyme preparations of these organisms. Thus 20 mM  $\text{Ni}(\text{CN})_4^{2-}$  can be degraded to less than 60  $\mu\text{M}$  after 72 h, and 26 mM  $\text{Cu}(\text{CN})_4^{3-}$  can be degraded to less than 5  $\mu\text{M}$  after 96 h.

It would appear from the foregoing discussion that, at least in the cases mentioned, degradation of cyano-metal complexes was an indirect consequence of microbial utilization of the small amount of free cyanide in equilibrium with its metal complex, thereby favouring the formation of more of the free cyanide anion. However, this has not been proven, and in the case of *Acinetobacter* sp. strain RFB1 there may well be a direct attack on cyanide complexes by the extracellular enzyme complex. This work with *Acinetobacter* is still at an early stage, but shows that a single organism has the potential to degrade both simple cyanides and cyanide complexes. Such organisms are of great value in the development of efficient biological systems for the treatment of the heterogenous cyanide wastes generated by industry.

### 1.5.3 IMMOBILIZED CELLS AND ENZYMES FOR TREATMENT OF CYANIDE WASTES

Many of the studies on biological degradation of cyanide-containing industrial waste have not involved an examination of the types of microorganisms that were assumed to be responsible for the removal of the cyanide. Such investigations were aimed at developing sustainable biological processes for the continuous treatment of particular industrial wastewaters. As the study of cyanide degradation by microorganisms progressed to the detailed investigation of

distinct strains of bacteria and fungi, and more recently to the enzymology of cyanide degradation (Ingvorsen *et al.*, 1991; Wang *et al.*, 1992), so the potential for applying this knowledge in the commercial sector has developed.

The advantage of using well-studied cyanide-degrading microorganisms and their enzymes for addressing a distinct need in industry is that the conditions for optimum removal of cyanide are known, and importantly, the conditions which reduce or inhibit this activity (such as pH ranges, temperature ranges, substrate or product inhibition, and the effect of metal ions) are also known. Perhaps the best way of applying both the cyanide-degrading microorganisms and their enzymes to alleviate an industrial problem is to immobilize them in some kind of support which still allows cyanide access to the enzymes, but prevents the cells or enzymes from washing out of the reactor.

Development of immobilized, biological cyanide-degrading systems for use in industry requires a considerable amount of effort in applying the results of laboratory investigations, with cells or enzymes free in solution, to a process on a far larger scale with immobilized material and operated under potentially different conditions.

However, despite the problems that may be associated with applying a biological system to a potentially harsh industrial environment, the use of immobilization methods for cells and enzymes appears to be the best way to apply the knowledge gained in research to a commercial venture. This approach also offers the sponsors of this research (who may be interested in its commercial applications) the option of exercising considerable influence in the market through the protection by patent of processes using defined bacterial or fungal strains, or their purified enzymes.

Nazly and Knowles (1981), and Nazly *et al.* (1983) investigated the use of polyelectrolyte flocculating agents to immobilize cyanide-induced mycelia of the phytopathogenic fungi, *S. loti.*, *G. sorghi* and *Fusarium moniliforme*. Although immobilization reduced the cyanide-degrading activity of the mycelia, the immobilized forms displayed increased stability at room temperature over mycelia which had not been immobilized. The results of these studies provided preliminary evidence that such immobilized fungal systems could be useful for the treatment of industrial cyanide wastes.

The immobilization of Cyanidase<sup>®</sup> in a diffusional-type flat-membrane reactor (FMR) was investigated by Basheer *et al.* (1993). The immobilized Cyanidase<sup>®</sup> preparation was applied to the treatment of food-industry wastewaters generated by hot-water extraction of apricot seeds. These extracts contained the cyanogenic glycoside, amygdalin, which yields cyanide on hydrolysis. The results of the investigation indicated that the flat-membrane reactor had promising applications for treatment of industrial wastes on a large scale.

The application of immobilization methods to cyanide-degrading microbial cells and enzymes is arguably an under-represented area in the literature on microbial cyanide degradation, in view of the potential for certain industries to adopt these methods for the large-scale detoxification of cyanide-containing wastes. This particular area of research is relatively new and an effort has only been made very recently to assess the efficiency of these immobilized biological systems to treat industrial wastes directly. However, given the progressively stricter environmental standards being applied to cyanide (and other) effluents from industry, this area of research is bound to enjoy increased attention.

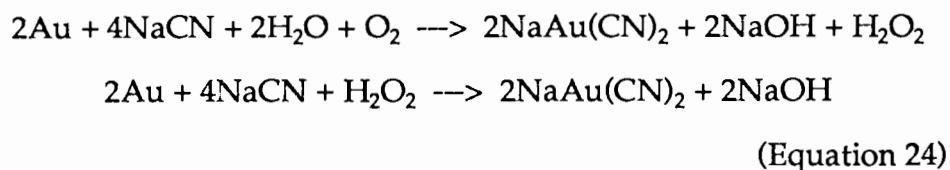
#### 1.5.4 CYANIDE IN THE GOLD MINING INDUSTRY

The gold mining industry can be taken as an example of an industry which uses cyanide extensively and therefore generates large volumes of cyanide-containing wastewaters. It is also of particular relevance to this study.

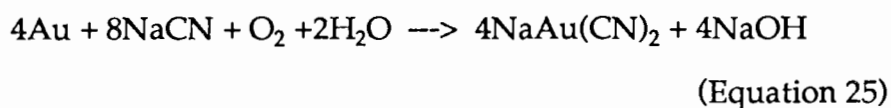
Gold-bearing ore mined underground is transported to a milling plant on the surface where the ore is broken down to a fine pulp in water. The pulp is pumped to aerated leaching tanks where cyanide (as calcium or sodium cyanide) is added to dissolve the gold in the ore pulp. Gold is recovered from the leachate by zinc cementation which precipitates elemental gold, or by adsorption of the dicyanoaurate complex onto activated carbon from which it can be stripped and recovered as elemental gold by an electrolytic process called electrowinning.

The availability of cyanide, the strength of the gold-cyanide complex and its solubility in water, and the efficiency with which gold can be extracted from ore and then recovered from solution, are the main reasons for the importance of the cyanidation process in the treatment of gold-bearing ores (Mudder, 1989).

The dissolution of gold by cyanide is believed to be a two-step reaction in which hydrogen peroxide is generated as an intermediate:

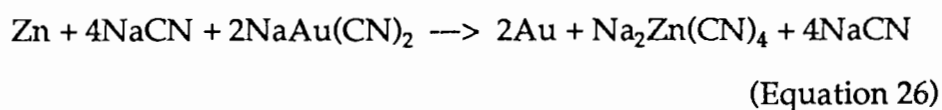


The overall reaction (Elsner's equation) is:



Cyanide also forms complexes with other metals such as silver, copper, iron, nickel, mercury, zinc and lead and these, together with inorganic components of the ore, contribute to the consumption of cyanide. However, the strength of the dicyanoaurate complex makes its formation a relatively specific reaction and allows quite dilute cyanide solutions to be used (0.1 g NaCN/l) (Mudder, 1989).

The Zinc Cementation Process (or Merrill-Crowe Process) for the recovery of gold from solution requires the further use of cyanide in an electrochemical reaction (in the absence of oxygen) in which metallic zinc powder, oxidized by reaction with free cyanide ions, reduces monovalent gold ions in the dicyanoaurate complex to the elemental metal (Mudder, 1989):



The Activated-Carbon Adsorption Method for gold recovery from solution has advantages over the Zinc Cementation Process in that there is no requirement for the addition of zinc or other reagents for dicyanoaurate adsorption to the carbon, but a hot solution of 0.1 % NaCN 1 % NaOH is required for stripping the

adsorbed gold complex from the carbon. The recovery of gold from this strip-solution eluate by electrowinning allows the recycling of the cyanide. The use of the activated-carbon process improves the efficiency of wastewater treatment by reducing the concentration of undesirable metals in solution (Mudder, 1989).

It can thus be seen that cyanide is used for both the dissolution of gold and its subsequent recovery in the cyanidation process, and although dilute cyanide solutions are used, the sheer scale of global gold mining activities results in the generation of vast quantities of cyanide wastes. This is particularly true of a country such as South Africa in which gold mining is one of the primary industries on which the economy is based, and is necessarily conducted on a very large scale.

## 1.6 MOTIVATION FOR THE STUDY OF CYANIDE DEGRADATION IN

### *B. pumilus*

The removal of cyanide from gold-extraction effluents has long been a problem in the gold mining industry. In most cases the cyanide-containing wastewaters discharged from gold recovery plants are stored in slimes dams where the cyanide is gradually removed by both biotic and abiotic processes. However, as legislation becomes progressively stricter and tightens the control on industrial pollution, industrial companies are becoming entirely responsible for the pollutants they release into the environment and also for combatting any toxic effects these pollutants may have on the environmental biota.

In a country such as South Africa with limited water reserves and intermittent droughts there is a need for industries to conserve water and recycle used water to some extent. The combined needs of reducing pollution as cheaply and efficiently as possible and conserving water resources has led to the anticipation of a major market for biological detoxification processes in this country.

To this end National Chemical Products (Germiston, Transvaal province, South Africa) in 1988 embarked on a project to isolate and test effective cyanide-degrading microorganisms with a view to supplying a biological means of treating cyanide wastes which would be both cheaper and less hazardous to the environment than conventional chemical means. It must be stressed, however,

that this project was intended to pre-empt an anticipated increase in the requirement for treatment of cyanide effluents following a governmental announcement of stricter pollution legislation. Although these laws have been imminent for several years they have not yet been promulgated and there is thus no strong incentive for mining companies to change their existing operating procedures.

The National Chemical Products effort is thus a project waiting for an application. However, there is good reason to believe that their approach is a valid one and that the future potential of the system is sufficiently promising to warrant intensive investigation. Changing public attitudes towards an increased awareness of the need for environmental protection will continue to encourage changes in governmental and industrial attitudes towards environmental protection. As the shift in environmental policy continues, and the knowledge of the impact of cyanidation wastewaters on the environment increases, the development of improved wastewater treatment technologies will be favoured. The development of an economically acceptable biological alternative to existing chemical industrial treatment technologies was the primary motivation for this project and for the detailed investigation of a single bacterial isolate at the cellular and molecular level.

The University of Cape Town was approached to collaborate on this project and assumed responsibility for the study of an efficient cyanide-degrading bacterial strain while National Chemical Products continued research on fungal cyanide degradation. This study led to the identification of a strain of *B. pumilus* which was shown to produce a soluble, intracellular, cyanide-degrading nitrilase enzyme which degraded cyanide to formate and ammonia. Production of the enzyme required the presence of a basal level of  $Mn^{2+}$  ions in the growth medium and was constitutive from the mid-exponential phase of growth into the stationary phase.

Although the scope of this work was to characterize a cyanide-degrading biological system of interest to industry, it also has significant academic interest in terms of the enzymology of cyanide degradation and has made a further contribution to the understanding of cyanide degradation by microorganisms.

## CHAPTER 2

### ISOLATION AND CHARACTERIZATION OF *B. pumilus* C1

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## CHAPTER 2

### ISOLATION AND CHARACTERIZATION OF *B. pumilus* C1

#### 2.1 SUMMARY

A cyanide-degrading, Gram-positive, aerobic, endospore-forming bacterium was isolated from a cyanide wastewater dam by an enrichment technique and was identified as a strain of *B. pumilus*. The bacterium was routinely cultured in Oxoid nutrient broth and rapidly degraded 100 mg/l of free cyanide in the absence of added inorganic and organic substances. The ability to degrade cyanide was linked to the growth phase and was not exhibited before late-exponential/early-stationary phase. Cyanide-degrading activity could not be induced in early exponential phase by the addition of cyanide or acetonitrile to 20 mg/l. Production of the cyanide-degrading activity required at least 0.01 mg  $Mn^{2+}$ /l in the growth medium (lower concentrations prevented the development of strong cyanide-degrading activity and also resulted in poor growth of the organism). No induction of cyanide-degrading activity occurred when  $Mn^{2+}$  ions were added to late-exponential-phase cells (or a cell-free extract from these cells) which had been grown with a low endogenous concentration of  $Mn^{2+}$ . However,  $Mn^{2+}$  ions could be added to cultures growing in low- $Mn^{2+}$  broth as late as the mid-exponential phase of growth with no apparent reduction of the cyanide-degrading activity exhibited in stationary phase. Culturing the organism in Difco nutrient broth resulted in poor growth and very low levels of cyanide-degrading activity; addition of  $Mn^{2+}$  to this medium did not significantly increase the levels of activity. Production of the cyanide-degrading activity required *de novo* transcription and translation. Cyanide-degrading activity was located intracellularly and cell-free extracts rapidly degraded cyanide ( $0.27 \pm 0.08$   $\mu$ mole cyanide/min/mg protein at 30 °C in pH 7.4 phosphate buffer). Eight strains of cyanide-utilizing fluorescent pseudomonads were also isolated from activated sewage sludge by an enrichment technique and tentatively identified as strains of *P. fluorescens* and *P. putida*. These isolates could not degrade cyanide rapidly.

## 2.2 INTRODUCTION

There have been several reports on the isolation of cyanide-degrading bacteria from a variety of sources (for example, Ware and Painter, 1955; Skowronski and Strobel, 1969; Harris and Knowles, 1983a; White *et al.*, 1988; Silva-Avalos *et al.*, 1990; Ingvorsen *et al.*, 1991). In 1988 National Chemical Products (Germiston, South Africa) embarked on a project to develop a biological cyanide-degrading system which could be patented and marketed to the gold-mining industry. The company therefore elected to isolate their own cyanide-degrading bacterial strains. A partnership with a South African university was sought to carry out detailed research into bacterial cyanide degradation.

The collaboration between the University of Cape Town and National Chemical Products began with the investigation of several cyanide-degrading bacterial strains which had been isolated at National Chemical Products. Work on these strains at the University of Cape Town was undertaken with a view to selecting a single strain for detailed investigation which would eventually be assessed for its industrial potential. An effort was also made to determine whether cyanide-degrading bacteria could be isolated independently at the University of Cape Town from activated sewage sludge (i.e. a source not expected to be exposed to significant concentrations of cyanide).

The initial work on the selected cyanide-degrading bacterial isolate was intended to provide general information about cyanide degradation at the cellular level and prepare the way for subsequent detailed investigations at the enzymological level. This chapter describes the initial investigation into cyanide degradation by a strain of *B. pumilus*.

## 2.3 MATERIALS AND METHODS

### 2.3.1 MATERIALS

Nutrient broth and nutrient agar were supplied by Oxoid Ltd. (England) and Difco Laboratories (U.S.A.). Brain-heart broth was supplied by E. Merck (Germany). Tryptone and yeast extract for Luria broth were supplied by Biolab Diagnostics (Pty) Ltd. (South Africa). Oxoid yeast extract (Code L21), bacteriological peptone (Code L37) and 'Lab-Lemco' powder (Code L29) were used where indicated. Potassium cyanide and 1.2 % picric acid solution GR (Art. 604) were from E. Merck. Folin & Ciocalteu's phenol reagent for protein determinations was supplied by BDH Chemicals Ltd. (U.K.). Bovine serum albumin (fraction 5) and chloramphenicol were from Boehringer Mannheim GmbH (Germany). Rifampicin was from the Sigma Chemical Company (U.S.A.). Acetonitrile was from Rathburn Chemicals Ltd. (Scotland). Chemicals used for reagents, buffers, taxonomic biochemical tests, and metal solutions were of high purity. Water, filtered through a Millipore Milli-RO apparatus, was used throughout unless otherwise indicated.

### 2.3.2 SELECTION AND ISOLATION OF CYANIDE-DEGRADING BACTERIA

The selection of cyanide-degrading bacteria was performed by Mr Pravin Gokool at the Biotechnology Division, Research and Development Department, National Chemical Products (Germiston, South Africa).

Two continuous selection systems were set up in the laboratory, each consisting of a stoppered round-bottomed flask supplied with a feed solution of 10 mg CN<sup>-</sup>/l (as NaCN) in tap water. The cyanide solution was fed into the flasks at a dilution rate of approximately 0.05 h<sup>-1</sup>. The pH of the cyanide solution was not controlled. One flask was inoculated with slurry from a cyanide wastewater dam and the other with a mixture of this slurry and garden soil. The systems were operated at 25 °C and the cyanide concentration in the feed solution was gradually increased to 100 mg CN<sup>-</sup>/l over the first 2 months of operation. The flasks were aerated by bubbling filtered air through the resident solutions. Overflow from each flask was diverted through a column of activated carbon (to trap any bacteria washed out of the flasks) before draining into a waste drum.

The activated carbon served as an additional source from which cyanide-degrading bacteria could be isolated.

At monthly intervals a 10-ml sample of the aqueous suspension in each flask was removed, serially diluted in sterile distilled water, and plated on Oxoid nutrient agar plates supplemented with 1 % glucose. A circular piece of Whatman's no. 1 filter paper (8 cm in diameter) was placed in the lid of each petri dish and moistened with 1.5 ml of a sodium cyanide solution (100 mg CN<sup>-</sup>/l, pH 10.5). The plates were sealed with Parafilm and incubated in plastic packets at room temperature in a fume cupboard. After 2-5 days bacterial colonies were subcultured onto Oxoid nutrient agar plates supplied with similar moist, cyanide-filter-paper discs. Bacterial isolates (70-90) were assayed for the ability to degrade cyanide.

An attempt was also made to isolate cyanide-degrading bacteria independently at the University of Cape Town using another continuous selection system. A sample of activated sewage sludge was used as the source of bacteria. Activated carbon was washed thoroughly with Millipore water and used to pack a 0.9 × 60 cm chromatography column. The outflow from the bottom of the column was fed via a tube through a rubber stopper into a 1-l conical flask containing 800 ml of a reservoir solution. Another tube led from the reservoir solution back out through the rubber stopper to a peristaltic pump and on to the inflow of the activated carbon column (thereby forming a closed system). None of the parts of the apparatus were sterilized.

The initial reservoir solution consisted of  $1/_{10}$  strength Luria broth containing 10 mg CN<sup>-</sup>/l. A 10-ml sample of activated sewage sludge (obtained from the Department of Chemical Engineering at the University of Cape Town) was used as the initial bacterial inoculum. The peristaltic pump was set to a low pumping rate and the system was run continuously at room temperature. After 2 days the turbid reservoir solution was replaced with 800 ml of 20 mM potassium phosphate buffer (pH 7.2) containing 0.5 % yeast extract, 8 ml of a trace-metals solution A (Appendix A), and 10 mg CN<sup>-</sup>/l. After 7 days of operation the turbid reservoir solution was replaced with 800 ml of 20 mM potassium phosphate buffer (pH 7.2) containing 8 ml of the same trace-metals solution and 20 mg CN<sup>-</sup>/l. The system was allowed to run continuously with occasional re-addition of cyanide. The cyanide concentration was gradually increased to 50 mg/l (day 144 of operation). During the first 50 days of operation the pH of the reservoir

solution remained in the range from pH 7.0 to 7.5. The number of viable cells in the reservoir solution was determined occasionally by dilution in sterile Millipore water and plating on Difco nutrient agar. The plates were incubated at 30 °C. Plate counts showed that there were approximately  $10^7$  colony-forming units/ml initially (first 30 days), but this number dropped to about  $10^5$  colony-forming units/ml after 85 days of operation.

Cyanide-degrading organisms were selected by plating 100- $\mu$ l samples of reservoir solution on glucose minimal agar plates (Appendix A). Cyanide (as HCN) was the only nitrogen source and was supplied from a sterile 25  $\times$  25 mm piece of Whatman 3MM filter paper moistened with 200  $\mu$ l of filter-sterilized 20 mM KCN on the lid of the petri dishes (as described by Harris and Knowles, 1983a). The plates were sealed with Parafilm and incubated at room temperature in a fume cupboard. After several days tiny white and off-white colonies appeared on the plates. The largest of these were streaked onto nutrient agar and eight isolates were kept for cyanide-degradation assays and taxonomic classification.

### **2.3.3 ASSAY FOR BACTERIAL CYANIDE DEGRADATION**

Subcultured bacterial colonies growing on the sealed nutrient agar/glucose plates with cyanide were resuspended in 3 ml of sterile distilled water and inoculated into 200-ml screwcap glass bottles containing 100 ml sterile distilled water and 50 mg CN<sup>-</sup>/l (pH 10.4). The bottles were tightly sealed with screw caps containing rubber seals and incubated at 30 °C. Samples were removed at 24-h intervals and assayed for remaining cyanide. In subsequent experiments with selected strains, 100-ml cultures in Oxoid nutrient broth (pH 7.4) were grown in 1-l conical flasks at 30 °C with shaking for 14 h. The cultures were harvested by centrifugation ( $10000 \times g$ , 10 min), the cell pellets were resuspended in 50 ml of sterile distilled water, and the suspensions were transferred to screwcap bottles. Cyanide (as KCN) was added to a final concentration of 50 or 100 mg CN<sup>-</sup>/l (final pH 10.5). The sealed bottles were incubated at 30 °C without shaking and 600- $\mu$ l samples were removed at half-hourly or hourly intervals. These samples were microfuged at room temperature for 3 min to remove the bacterial cells and the supernatant was used to determine the residual cyanide concentration at each time point.

#### 2.3.4 COLOURIMETRIC ASSAY FOR CYANIDE

Cyanide was assayed by a modification of the picric acid method of Fisher and Brown (1952). Cyanide solutions (50  $\mu$ l) were added to 100  $\mu$ l of a solution containing 0.5 % (w/v) picric acid and 0.25 M  $\text{Na}_2\text{CO}_3$  (Appendix A). The solutions (in glass test tubes with loose-fitting plastic caps) were placed in boiling water for 5 min and were then diluted to 1 ml with 850  $\mu$ l of distilled water. Solutions were allowed to cool in water for 15-30 min before the absorbance at 520 nm was measured (against a blank containing distilled water mixed with the picric acid/ $\text{Na}_2\text{CO}_3$  reagent). A linear calibration curve was obtained with standard cyanide solutions of up to 100 mg CN<sup>-</sup>/l. Cyanide concentrations determined by the picric acid test correlated well with measurements performed on the same solutions by gas chromatography (Pravin Gokool, personal communication). The simplicity and reliability of the picric acid test made it suitable for the requirements of this work. The absorbance of the picric acid-cyanide product was stable for many hours.

#### 2.3.5 CLASSIFICATION OF CYANIDE-DEGRADING BACTERIA

Forty eight bacterial isolates from National Chemical Products were tested for the ability to degrade free cyanide. The 12 best strains were chosen for further study and were characterized with respect to cell morphology, Gram stain, catalase and oxidase reactions, and ability to grow anaerobically. Further characterization was carried out using biochemical tests for bacterial classification described by Gordon (1973), Cruickshank *et al.* (1975), Norris *et al.* (1981) and Sneath (1986). *Bacillus cereus* var. *mycoides* ATCC 7068 and *B. megaterium* NCIB 2602 were used as positive controls for the Gram reaction, catalase test, test for anaerobic growth, Voges-Proskauer test, citrate-utilization test, and test for reduction of nitrate. *B. cereus* var. *mycoides* ATCC 7068 was also used as a positive control for the formation of gas from D(+)-glucose and the hydrolysis of casein and starch. *B. megaterium* NCIB 2602 was used as a positive control for the hydrolysis of urea, for growth in 7 % NaCl, and for growth at 50 °C. A strain of *B. coagulans* was used as a positive control for the hydrolysis of gelatin.

The bacterial isolates from activated sewage sludge were classified taxonomically using biochemical tests described by Cruickshank *et al.* (1975) and Palleroni (1984). *E. coli* strain CSH62, *B. pumilus* strain C1, and strains of *P. aeruginosa* and *P. fluorescens* were used as controls.

### 2.3.6 PREPARATION OF CELL-FREE EXTRACTS

Oxid nutrient broth cultures (200 ml in 1-l conical flasks) were grown at 30 °C with shaking for 14-15 h. Cells were harvested by centrifugation at  $10\,000 \times g$  for 10 min at 4 °C and then washed twice with 20 ml ice-cold 50 mM potassium phosphate 50 mM NaCl buffer, pH 7.4 (in later experiments this buffer was changed to a 10 mM Tris-HCl 50 mM NaCl, pH 8.0 buffer). The cells were finally resuspended in 10 ml of the same buffer and disrupted by two passages through an Aminco French pressure cell at  $1.1 \times 10^5$  kPa. The homogenate was centrifuged at  $27\,000 \times g$  for 30 min at 4 °C and the supernatant was retained. A sample of this low-speed supernatant was subsequently centrifuged at  $145\,000 \times g$  for 60 min at 4 °C to determine whether the cyanide-degrading activity could be removed from solution. To assay cyanide-degrading activity, a sample of the cell-free extract (low-speed fraction) was diluted 1-in-20 in the phosphate/NaCl buffer to give a final volume of 2 ml. The diluted sample was prewarmed in air at 30 °C for 1 h and KCN was added to give a final concentration of 102 mg CN<sup>-</sup>/l. The incubation was continued at 30 °C and triplicate 50- $\mu$ l samples were removed after 0, 15, 30, 60, 120 and 240 min for the cyanide assay. In subsequent assays only the low-speed supernatant fraction was used. Assay conditions were similar, but the duration of the assay was limited to 40 min.

### 2.3.7 PROTEIN DETERMINATION IN CELL-FREE EXTRACTS

Protein concentrations were determined by the method of Lowry *et al.* (1951). Protein samples (200  $\mu$ l, containing 5-100 mg of protein) were mixed with 1 ml of reagent C (Appendix A) and allowed to stand at room temperature for 10 min (or longer). The solutions were then mixed with 100  $\mu$ l of reagent E (Appendix A) - rapid mixing was required as reagent E is unstable at alkaline pH. Samples were allowed to stand at room temperature for a further 30 min before the absorbance at 750 nm of the blue protein-Cu<sup>2+</sup> complex was determined against a

blank prepared with distilled water. Bovine serum albumin (BSA) was used as the standard.

### **2.3.8 TESTING OF CYANIDE AND ACETONITRILE AS INDUCERS OF CYANIDE DEGRADATION**

Glucose (0.2 %) minimal medium (Appendix A) was prepared and dispensed into 100-ml conical flasks in 20-ml volumes. The sterile medium was supplemented with sterile biotin (to 0.0005 %), prewarmed at 30 °C and inoculated with 87 µl of an overnight culture of *B. pumilus* C1 grown at 30 °C in Oxoid nutrient broth ( $OD_{600} = 1.836$ ). The minimal medium cultures were grown at 30 °C with shaking for 7 h and the optical density of each culture was measured. Two of the cultures received cyanide and acetonitrile, respectively, to a final concentration of 20 mg/l. A third (unsupplemented) culture served as a control. Incubation at 30 °C with shaking was continued for a further 90 min and the optical density of each culture was measured again. The cells were harvested by centrifugation ( $10\,000 \times g$ , 10 min) and resuspended in 10 ml of sterile Millipore water in standard containers. Cyanide was added to a final concentration of 100 mg/l and the containers were incubated at 30 °C. Samples were removed at 30-min intervals and assayed for remaining cyanide after the cells had been removed by centrifugation (3 min) in a microfuge. A 20-ml Oxoid nutrient broth culture (in a 100-ml conical flask) which was inoculated at the same time as the minimal media (with the same seed culture) was allowed to grow without interruption for the full 8.5 h of the experiment. This culture served as a positive control to show that the cells used in the experiment had the ability to produce cyanide-degrading activity.

### **2.3.9 ASSAY FOR EXTRACELLULAR CYANIDE-DEGRADING ACTIVITY**

A 20-ml Oxoid nutrient broth culture of *B. pumilus* C1 (in a 100-ml conical flask) was grown overnight (14 h) at 30 °C with shaking. The cells were harvested by centrifugation ( $10\,000 \times g$ , 10 min), resuspended in 10-ml of sterile distilled water and assayed for their ability to degrade 100 mg CN<sup>-</sup>/l (section 2.3.8). The culture supernatant was divided into two equal parts. One part was heated in boiling water for 10 min and allowed to cool to room temperature. Cyanide was added

to both supernatant samples (final concentration 100 mg CN<sup>-</sup>/l) and cyanide-degrading activity was assayed at 30 °C.

### **2.3.10 THE EFFECT OF METAL IONS ON THE PRODUCTION OF CYANIDE-DEGRADING ACTIVITY**

Oxoid nutrient broth was prepared in Millipore water or tap water and dispensed into 100-ml conical flasks in 20-ml volumes. The media were sterilized by autoclaving. Broths prepared in Millipore water were supplemented with sterile trace-metals solutions or single-metal solutions to the concentrations indicated in the text. Broths used in the same experiment were inoculated (20 µl each) from the same late-exponential-phase seed culture of *B. pumilus* C1 grown in Oxoid nutrient broth. Cultures were grown overnight at 30 °C with shaking. Cells were harvested by centrifugation and assayed for the ability to degrade cyanide as described previously (section 2.3.8).

### **2.3.11 THE EFFECT OF TEMPORAL ADDITION OF Mn<sup>2+</sup> TO A GROWING CULTURE**

Oxoid nutrient broth was prepared in Millipore water, dispensed into thirteen 100-ml conical flasks in 20-ml volumes, and sterilized by autoclaving. The broths were prewarmed at 30 °C, inoculated with 20 µl of a late-exponential-phase seed culture of *B. pumilus* C1 (OD<sub>600</sub> = 1.256) and incubated at 30 °C with shaking for 15 h. During the course of incubation the cultures received Mn<sup>2+</sup> at different stages in the growth curve (final concentration 0.206 mg Mn<sup>2+</sup>/l). The optical density of one culture (containing no added manganese) was monitored hourly to follow the growth curve of the cultures (it was assumed that all thirteen cultures behaved similarly). Pairs of cultures received manganese at the following stages in the growth curve: the time of inoculation; at the end of the lag phase (3 h of growth); in mid-exponential phase (7 h of growth); in late-exponential phase (10 h of growth); and at the start of the stationary phase (13 h of growth), respectively. A sixth pair of cultures received no manganese and served as a negative control. All six pairs of cultures were harvested by centrifugation after 15 h of growth and the cells were resuspended in 10 ml of sterile distilled water in standard containers. Samples were prewarmed at 37 °C for 5 min in a waterbath and cyanide was added to a final concentration of 100

mg/l. Samples (120  $\mu$ l) were removed at 30-min intervals and microfuged for 3 min at room temperature. Duplicate 50- $\mu$ l samples of the resulting supernatant were pipetted into picric acid reagent in test tubes on ice and used to determine remaining cyanide (as described in section 2.3.4).

### **2.3.12 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATIONS OF CHLORAMPHENICOL AND RIFAMPICIN**

A 350-ml Oxoid nutrient broth in Millipore water in a 2-l conical flask was supplemented with 13.1  $\mu$ l of a sterile solution of 5 mM  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  (final concentration 0.01 mg  $\text{Mn}^{2+}$ /l). The medium was inoculated with 350  $\mu$ l of a stationary-phase seed culture of *B. pumilus* C1 (grown in Oxoid nutrient broth) and grown at 30 °C with shaking. The optical density of the culture was monitored hourly from mid-exponential phase to late-exponential phase. When the culture reached late-exponential phase ( $\text{OD}_{600} = 1.8\text{-}2.2$ ) it was dispensed in 15-ml volumes into sterile, prewarmed 200-ml conical flasks containing increasing amounts of chloramphenicol or rifampicin (the solvent alcohols evaporated almost immediately on addition of the small volumes of antibiotic to the prewarmed flasks). The 15-ml cultures were incubated with the antibiotics at 30 °C with shaking for a further 3 h after which the optical density of each culture was measured. The minimum inhibitory concentration of each antibiotic was defined as the minimum concentration which prevented further growth of a late-exponential phase culture under the conditions of the experiment.

### **2.3.13 THE EFFECTS OF CHLORAMPHENICOL AND RIFAMPICIN ON PRODUCTION OF CYANIDE-DEGRADING ACTIVITY**

A sterile 400-ml Oxoid nutrient broth (prepared in Millipore water in a 2-l conical flask) was supplemented with 15  $\mu$ l of a sterile solution of 5 mM  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  (final concentration 0.01 mg  $\text{Mn}^{2+}$ /l). The broth was inoculated with 250  $\mu$ l of an overnight culture of *B. pumilus* C1 (grown in Oxoid nutrient broth) and grown at 30 °C with shaking. The optical density of the culture was monitored regularly from 4 h after inoculation onwards. After 6.2 h the culture ( $\text{OD}_{600} = 0.795$ ) was divided into three parts. Two 120-ml samples were transferred to sterile, prewarmed 1-l conical flasks and the remainder of the culture (160 ml) was dispensed into a third prewarmed flask. Incubation at 30

°C with shaking was continued for 30 min to allow the cells to recover from the interruption. Chloramphenicol was added to one 120-ml culture (final concentration 40 µg/ml) and rifampicin was added to the other 120-ml culture (final concentration 5 µg/ml). The 160-ml culture served as a control and its optical density was measured hourly after the addition of antibiotics to the 120-ml cultures. A 40-ml sample was removed from the control culture at the time of addition of the antibiotics and was used for the preparation of a cell-free extract. Samples (40 ml) were also removed from all three cultures at 1 h and 5.8 h after the addition of the antibiotics for the preparation of cell-free extracts. The last set of samples for cell-free extracts (5.8 h) was removed as the control culture was entering stationary phase.

Cell-free extracts were prepared as described in section 2.3.6 with the exception that the cells were washed twice in 8 ml of buffer (10 mM Tris-HCl 50 mM NaCl, pH 8.0) and were finally resuspended in 4 ml of this buffer. The cell-free extracts were stored overnight at 4 °C before being assayed for cyanide-degrading activity.

Samples (250 µl) of the cell-free extracts were prewarmed at 37 °C for 5 min and the reaction was initiated by adding 50 µl of a 610 mg CN<sup>-</sup>/l solution (final concentration 101.7 mg CN<sup>-</sup>/l). Duplicate 50-µl samples were removed at 0 and 60 min and assayed for residual cyanide. Protein concentrations in the cell-free extracts were measured as described in section 2.3.7 after appropriate dilution in buffer (10 mM Tris-HCl 50 mM NaCl, pH 8.0). Cyanide-degrading activity in the cell-free extracts was expressed as the change in absorbance at 520 nm divided by the amount of protein in the assay:

$$\text{activity} = (A_{520\text{ nm } t=0} - A_{520\text{ nm } t=60\text{ min}}) \div (\text{mg protein in assay})$$

## 2.4 RESULTS

### 2.4.1 SELECTION OF THE BEST CYANIDE-DEGRADING BACTERIA

Subculturing of the cyanide-degrading bacterial isolates supplied by National Chemical Products revealed that some of the nutrient agar plates contained mixed cultures (as judged by colony colour and morphology). Each colony type was streaked to purity on Oxoid nutrient agar plates (without glucose and cyanide) and assayed for cyanide-degrading activity in the batch-bottle test. Forty eight strains were assayed by this method and the best strains in each test were noted. The cyanide-degrading activity of the 12 best strains was tested in a single experiment (Table 2.1). Although the initial pH in this assay was about 10.5, it dropped as the cyanide was degraded (pH 8.5 - 9.0 after 7 h at 30 °C). The bacteria from all 12 strains were aerobic and the cells were slender, endospore-forming, Gram-positive rods. The spores were located centrally and caused a slight swelling of the cells (making them slightly cigar-shaped). No vacuolation was visible in safranin-stained cells grown on glucose agar. These characteristics indicated that all 12 cyanide-degrading bacterial isolates were strains of *Bacillus*. The strains were subjected to biochemical tests for the identification of *Bacillus* species (Table 2.2).

All 12 strains were unable to hydrolyze starch or reduce nitrate. Furthermore, they could only grow on Spizizen minimal medium (Appendix A) if it was supplemented with biotin (0.0005 %). These are characteristics of *B. pumilus* (Knight and Proom, 1950) and strongly suggested that all 12 of the cyanide-degrading isolates were strains of this organism. None of the strains could grow aerobically on glucose-biotin minimal agar plates with KCN as the only source of nitrogen (supplied as HCN gas by the moist, cyanide-filter-paper method of Harris and Knowles, 1983a). The strains were stored as spore stocks in sterile water at 4 °C. Based on four comparative cyanide-degradation assays, strain C3b(G)a was chosen for further study and was renamed *B. pumilus* C1.

The eight bacterial isolates from activated sewage sludge consisted of short, motile, Gram-negative rods. They were all oxidase positive, unable to grow anaerobically and were able to oxidize, but not ferment, glucose in the oxidation-fermentation test. Acid, but not gas, was produced from glucose by all strains. The other biochemical characteristics are presented in Table 2.3. All strains

produced a pigment which fluoresced blue-green on exposure to ultraviolet radiation after growth on King's medium B, and also exhibited arginine dihydrolase activity on Thornley's medium (Thornley, 1960) (Appendix A). These results suggested that all the strains were species of the fluorescent pseudomonads (belonging to rRNA Group I of *Pseudomonas*).

Strains 4, 20, 22 and 26 were nitrate-reductase positive. All of these strains could hydrolyse gelatin and utilize D-xylose, thereby ruling them out as strains of *P. aeruginosa*, *P. putida*, *P. chlororaphis* and *P. aureofaciens*, and suggesting that they were strains of *P. fluorescens*. Since these strains were unable to utilize sucrose they could not have been strains of *P. fluorescens* biovars I, II or IV, suggesting that they were strains of *P. fluorescens* biovar III or V.

Strains 23a, 24, 27a and 28a were nitrate-reductase negative and could not hydrolyse gelatin indicating that they were strains of *P. putida* biovars A and B. The inability of strains 23a and 27a to utilize L-arabinose suggested that they might belong to *P. putida* biovar A. Strains 24 and 28a were able to utilize L-arabinose and D-galactose suggesting that they might belong to *P. putida* biovar B.

The *P. fluorescens* and *P. putida* strains were tested for their ability to degrade cyanide (Table 2.4). The results showed that none of these strains could degrade cyanide as rapidly as *B. pumilus* C1 and they were not investigated further.

Table 2.1 Comparison of the cyanide-degrading activities (towards 100 mg CN-/l) of the twelve best cyanide-degrading bacterial isolates from the cyanide-wastewater dam. The control consisted of Millipore water and cyanide.

| STRAIN     | CYANIDE REMAINING (mg/l) |       |      |       |                  |       |      |       |      |  |  |
|------------|--------------------------|-------|------|-------|------------------|-------|------|-------|------|--|--|
|            | 0 h                      | 0.5 h | 1 h  | 1.5 h | <sup>a</sup> 2 h | 2.5 h | 3 h  | 3.5 h | 4 h  |  |  |
| CHEMAP (A) | 75                       | 42.5  | 20.5 | 9     | 85.5             | 56.5  | 40   | 25.5  | 17.5 |  |  |
| D2 (B) b   | 68.5                     | 25    | 11.5 | 3.5   | 78.5             | 43.5  | 29.5 | 17    | 11.5 |  |  |
| C3b (D) b  | 73.5                     | 28.5  | 11   | 4     | 81               | 49    | 32   | 18.5  | 12.5 |  |  |
| D5 (F) b   | 77                       | 33    | 17   | 6     | 84.5             | 56    | 38.5 | 25    | 17   |  |  |
| C3b (G) a  | 75                       | 33.5  | 14.5 | 5     | 79.5             | 51.5  | 33.5 | 22    | 15   |  |  |
| D2 (H) a   | 70                       | 24    | 8.5  | 2.5   | 79               | 46.5  | 31.5 | 19.5  | 14   |  |  |
| D2 (L) W   | 72.5                     | 30    | 12.5 | 3.5   | 75.5             | 47.5  | 32.5 | 25.5  | 18   |  |  |
| D2         | 75                       | 33    | 10   | 3     | 78.5             | 46    | 29.5 | 19.5  | 16.5 |  |  |
| D2*W       | 77                       | 31.5  | 11.5 | 3.5   | 80               | 50.5  | 34.5 | 21.5  | 15   |  |  |
| D5 (J) W   | 71.5                     | 31.5  | 13.5 | 6.5   | 79               | 52.5  | 33.5 | 20.5  | 13.5 |  |  |
| D5*        | 72.5                     | 39.5  | 17.5 | 7.5   | 83.5             | 52.5  | 33.5 | 19    | 13   |  |  |
| D2Bb       | 72                       | 35    | 15.5 | 5     | 84               | 56    | 42.5 | 27.5  | 20.5 |  |  |
| CONTROL    | 86.5                     | 88    | 87.5 | 85.5  | 93               | 80    | 93   | 88    | 87.5 |  |  |

<sup>a</sup> Cyanide was re-added to approximately 100 mg CN-/l at 2 h. Experimental details are described in section 2.3.3.

**Table 2.2 Results of the taxonomic tests performed on the cyanide-degrading bacterial strains isolated from a cyanide-wastewater dam.**

| TEST                         | ALL<br>CYANIDE-<br>DEGRADING<br>ISOLATES | <i>B.cereus</i><br>var.<br><i>mycoides</i><br>ATCC<br>7068 | <i>B.megaterium</i><br>NCIB 2602 |
|------------------------------|------------------------------------------|------------------------------------------------------------|----------------------------------|
| GRAM<br>REACTION             | +                                        | +                                                          | +                                |
| CATALASE                     | +                                        | +                                                          | +                                |
| OXIDASE                      | +                                        | N.D.                                                       | N.D.                             |
| ANAEROBIC<br>GROWTH          | -                                        | +                                                          | +                                |
| VOGES-<br>PROSKAUER<br>TEST  | +                                        | +                                                          | +                                |
| pH IN<br>V-P BROTH           | 5.0-5.5                                  | 5.0-5.5                                                    | 5.0-5.5                          |
| ACID FROM:<br>D (+) -GLUCOSE | +                                        | +                                                          | N.D.                             |
| L (+) -<br>ARABINOSE         | + <sup>a</sup>                           | N.D.                                                       | N.D.                             |
| D (+) -XYLOSE                | + <sup>b</sup>                           | N.D.                                                       | N.D.                             |
| D-MANNITOL                   | +                                        | N.D.                                                       | N.D.                             |
| GAS FROM<br>GLUCOSE          | -                                        | -                                                          | N.D.                             |

<sup>a</sup> Strains C3b(D)b, D5(F)b and D5\* were not tested.

<sup>b</sup> Only strains C3b(D)b, D5(F)b and D2 were tested.

N.D. = not determined.

Table 2.2 continued

| TEST                                                                                | ALL<br>CYANIDE-<br>DEGRADING<br>ISOLATES | <i>B.cereus</i><br>var.<br><i>mycoides</i><br>ATCC<br>7068 | <i>B.mega-</i><br><i>terium</i><br>NCIB 2602 | <i>B.licheni</i><br><i>formis</i> | <i>B.coagul</i><br><i>ans</i> |
|-------------------------------------------------------------------------------------|------------------------------------------|------------------------------------------------------------|----------------------------------------------|-----------------------------------|-------------------------------|
| HYDRO-<br>LYSIS OF:<br>CASEIN                                                       | +                                        | +                                                          | N.D.                                         | N.D.                              | N.D.                          |
| GELATIN                                                                             | +                                        | N.D.                                                       | N.D.                                         | N.D.                              | +                             |
| STARCH                                                                              | -                                        | +                                                          | N.D.                                         | N.D.                              | N.D.                          |
| UREA                                                                                | -                                        | +                                                          | N.D.                                         | N.D.                              | N.D.                          |
| UTILIZ-<br>ATION OF<br>CITRATE                                                      | +                                        | +                                                          | +                                            | +                                 | +                             |
| REDUCTION<br>OF<br>NITRATE                                                          | - <sup>c</sup>                           | +                                                          | +                                            | N.D.                              | N.D.                          |
| GROWTH IN<br>7 % NaCl                                                               | +                                        | N.D.                                                       | N.D.                                         | +                                 | +                             |
| GROWTH<br>AT: 30 °C                                                                 | +                                        | N.D.                                                       | N.D.                                         | N.D.                              | N.D.                          |
| 37 °C                                                                               | +                                        | N.D.                                                       | N.D.                                         | N.D.                              | N.D.                          |
| 50 °C                                                                               | +                                        | -                                                          | N.D.                                         | +                                 | N.D.                          |
| GROWTH ON<br>SPIZIZEN<br><i>Bacillus</i><br>MINIMAL<br>MEDIUM:<br>WITHOUT<br>BIOTIN | -                                        | -                                                          | N.D.                                         | +                                 | N.D.                          |
| WITH<br>BIOTIN                                                                      | +                                        | N.D.                                                       | N.D.                                         | +                                 | N.D.                          |

<sup>c</sup> The results for strains D2(B)b and D2\*W were inconclusive.  
N.D. = not determined.

**Table 2.3 Results of the taxonomic tests performed on the cyanide-utilizing bacterial strains isolated from activated sewage sludge.**

| STRAIN                | 4 | 20 | 22 | 23a | 24 | 26 | 27a | 28a | <i>E.coli</i><br>CSH62 | <i>B.</i><br><i>pumilus</i><br>C1 |
|-----------------------|---|----|----|-----|----|----|-----|-----|------------------------|-----------------------------------|
| GRAM STAIN            | - | -  | -  | -   | -  | -  | -   | -   | -                      | +                                 |
| CATA-LASE             | - | +  | -  | +   | +  | -  | +   | +   | N.D.                   | +                                 |
| OXIDASE               | + | +  | +  | +   | +  | +  | +   | +   | -                      | +                                 |
| ANAEROBIC GROWTH      | - | -  | -  | -   | -  | -  | -   | -   | +                      | -                                 |
| <sup>a</sup> O-F TEST | O | O  | O  | O   | O  | O  | O   | O   | F                      | N.D.                              |
| ACID FROM: GLUCOSE    | + | +  | +  | +   | +  | +  | +   | +   | +                      | +                                 |
| MALTOSE               | - | -  | -  | -   | -  | -  | -   | -   | +                      | -                                 |
| SUCROSE               | - | -  | -  | -   | -  | -  | -   | -   | -                      | +                                 |
| MANN-ITOL             | - | -  | -  | -   | -  | -  | -   | -   | +                      | +                                 |
| XYLOSE                | + | +  | +  | +   | +  | +  | +   | +   | +                      | -                                 |
| L-ARABIN-<br>OSE      | + | -  | +  | -   | +  | -  | -   | +   | +                      | -                                 |

<sup>a</sup> O-F TEST = oxidation-fermentation test; O = glucose only oxidized; F = glucose oxidized and fermented.  
N.D. = not determined.

Table 2.3 continued

| STRAIN                              | 4              | 20  | 22             | 23a | 24  | 26 | 27a | 28a | <i>P. fluorescens</i> | <i>P. aeruginosa</i> |
|-------------------------------------|----------------|-----|----------------|-----|-----|----|-----|-----|-----------------------|----------------------|
| GROWTH ON:<br>CITRATE               | +              | +   | +              | +   | +   | +  | +   | +   | +                     | +                    |
| <sup>b</sup> CETRI-<br>MIDE<br>AGAR | +              | +   | +              | +/- | +   | +  | +/- | +/- | N.D.                  | N.D.                 |
| HYDRO-<br>LYSIS<br>OF:<br>UREA      | -              | -   | -              | -   | -   | -  | -   | -   | -                     | +                    |
| STARCH                              | +              | +/- | +              | +/- | +/- | +  | +/- | +   | N.D.                  | N.D.                 |
| GELATIN                             | +              | +   | +              | -   | -   | +  | -   | -   | N.D.                  | N.D.                 |
| CASEIN                              | +              | -   | +              | -   | -   | +  | -   | -   | -                     | +                    |
| NITRATE<br>REDUCT-<br>ION           | + <sup>d</sup> | +   | + <sup>d</sup> | -   | -   | +  | -   | -   | -                     | +/-                  |
| INDOLE<br>TEST                      | -              | -   | -              | -   | -   | -  | -   | -   | -                     | -                    |
| <sup>c</sup> FLUOR-<br>ESCENCE      | +              | +   | +              | +   | +   | +  | +   | +   | + <sup>e</sup>        | + <sup>e</sup>       |
| ARG-<br>ININE<br>DIHYDRO-<br>-LASE  | +              | +   | +              | +   | +   | +  | +   | +   | +                     | +                    |

<sup>b</sup> Cetrimide-agar test determines ability to grow in the presence of cetyltrimethylammonium bromide.

<sup>c</sup> Fluorescence on King's medium B. Strains 20 and 27a fluoresced blue under ultraviolet light. The other six strains isolated from activated sewage sludge fluoresced blue-green.

<sup>d</sup> Strains 4 and 22 were weakly positive for nitrate reductase after 3 d.

<sup>e</sup> *P. fluorescens* and *P. aeruginosa* fluoresced green under ultraviolet light.

+/- = variable result.

N.D. = not determined.

Table 2.3 continued

| STRAIN                                         | 4 | 20 | 22 | 23a | 24 | 26 | 27a | 28a | <i>P. fluorescens</i> | <i>P. aeruginosa</i> |
|------------------------------------------------|---|----|----|-----|----|----|-----|-----|-----------------------|----------------------|
| GROWTH ON CH <sub>3</sub> CN <sup>f</sup>      | + | +  | +  | +   | +  | +  | +   | +   | +                     | +                    |
| LIPASE <sup>g</sup>                            | + | +  | +  | -   | -  | +  | -   | -   | +                     | +                    |
| <sup>h</sup> UTILIZATION OF: D (+) - GALACTOSE | + | +  | +  | +   | +  | +  | +   | +   | +                     | +                    |
| L-RHAMNOSE                                     | - | -  | -  | +   | +  | -  | +   | +   | -                     | -                    |
| ADONITOL                                       | - | -  | -  | -   | -  | -  | -   | -   | -                     | -                    |
| ERYTHRITOL                                     | - | -  | -  | -   | -  | -  | -   | -   | -                     | -                    |
| meso-INOSITOL                                  | + | -  | +  | -   | -  | +  | -   | -   | -                     | -                    |
| SORBITOL                                       | - | -  | -  | -   | -  | -  | -   | -   | -                     | -                    |
| GLYCINE <sup>i</sup>                           | + | +  | +  | +   | +  | +  | +   | +   | +                     | +                    |

<sup>f</sup> Growth on agar plates with acetonitrile as the sole source of nitrogen.

<sup>g</sup> Tested for hydrolysis of Tween 80 using Sierra's medium (Palleroni, 1984).

<sup>h</sup> A colour change in the liquid medium from green to yellow (with bromothymol blue as indicator) was recorded as a positive result.

<sup>i</sup> Test for deamination of glycine. A colour change from green to blue (with bromothymol blue as indicator) was recorded as a positive result.

<sup>j</sup> Strain 20 exhibited weak lipase activity.

**Table 2.4 Comparison of the cyanide-degrading activities (towards 100 mg CN-/l) of the cyanide-utilizing bacterial isolates from activated sewage sludge. The control consisted of Millipore water and cyanide. Experimental details are described in section 2.3.3.**

| STRAIN                                     | CYANIDE REMAINING (mg/l) |       |       |       |        |        |
|--------------------------------------------|--------------------------|-------|-------|-------|--------|--------|
|                                            | 0.5 h                    | 1 h   | 1.5 h | 3.5 h | 20.5 h | 44.5 h |
| 4                                          | 90                       | 93    | 94    | 93.5  | 73     | 65.5   |
| 20                                         | 91.5                     | 91.5  | 92.5  | 90    | 77.5   | 68.5   |
| 22                                         | 94                       | 94.5  | 94.5  | 96.5  | 82.5   | 71     |
| 23a                                        | 100.5                    | 102.5 | 99.5  | 99    | 90.5   | 81     |
| 24                                         | 91.5                     | 92    | 92    | 87.5  | 82     | 72     |
| 26                                         | 95                       | 95    | 95.5  | 93    | 80.5   | 71.5   |
| 27a                                        | 99.5                     | 99    | 99    | 99    | 85.5   | 75.5   |
| 28a                                        | 101                      | 99    | 99.5  | 98    | 87.5   | 76.5   |
| CONTROL<br>(H <sub>2</sub> O +<br>CYANIDE) | 103.5                    | 105   | 107   | 106.5 | 103    | 101.5  |

#### **2.4.2 EFFECT OF GROWTH MEDIUM ON CYANIDE-DEGRADING ACTIVITY**

During the course of assaying bacterial isolates for cyanide-degrading activity, it was noticed that the bacteria grew poorly in Difco nutrient broth and showed very little ability to degrade cyanide after growth in this medium. Fig. 2.1 shows a comparison of the cyanide-degrading activity of strain D2\*W grown in 200 ml of Oxoid nutrient broth, Difco nutrient broth, and brain-heart broth, respectively. It is clear that the cells grown in Difco nutrient broth displayed very little cyanide-degrading activity, whereas those grown in Oxoid nutrient broth degraded cyanide relatively rapidly. These results indicated that a component of the Difco medium was inhibiting the production of the cyanide-degrading activity, or that a component common to Oxoid nutrient broth and brain-heart broth promoted the ability to degrade cyanide and was lacking in the Difco medium.

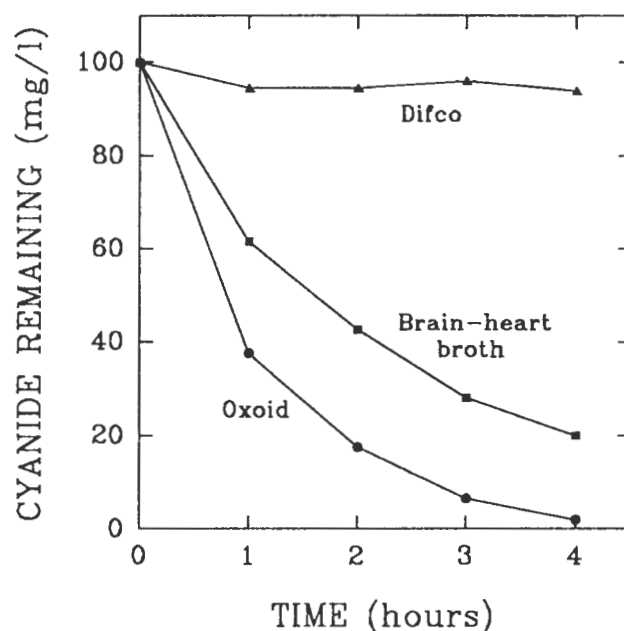


Fig. 2.1 The effect of growth medium on the cyanide-degrading activity of *B. pumilus* isolate D2\*W. The organism was grown overnight at 30 °C with shaking in 200 ml of the indicated broths. Cells were harvested by centrifugation, resuspended in 100 ml of sterile Millipore water, and assayed for their ability to degrade 100 mg CN-/l (as described in Materials and Methods sections 2.3.3 and 2.3.4). No cyanide-control sample was used. Oxoid nutrient broth, (●); Difco nutrient broth, (▲); and brain-heart broth, (■).

Further experiments (with *B. pumilus* C1) showed that the organism did not display cyanide-degrading activity when grown in Difco nutrient broth supplemented with the various components of Oxoid nutrient broth (at the same concentrations used in Oxoid nutrient broth), either singly or in combination (Tables 2.5 and 2.6). It is worth noting that the supplemented Difco broths supported better growth of the organism even though it could not degrade cyanide when grown in these media. Table 2.6 also shows that when the organism was grown in a mixed broth (half-strength with respect to each of Oxoid and Difco nutrient broths) it could not degrade cyanide even though the medium supported good growth. This result argued very strongly for the inhibition of cyanide-degrading activity by a component of Difco nutrient broth. However, the identity of the inhibitory component was not determined.

Tables 2.5 and 2.6 Growth and cyanide-degrading activity of *B. pumilus* C1 grown in Difco nutrient broth supplemented with the components of Oxoid nutrient broth. Cyanide assays were performed on harvested cells as described in sections 2.3.3 and 2.3.4.

**Table 2.5**

| COMPOSITION OF MEDIA                                                          | OVERNIGHT GROWTH | CYANIDE REMAINING (mg/l) |      |       |
|-------------------------------------------------------------------------------|------------------|--------------------------|------|-------|
|                                                                               |                  | 0.5 h                    | 1 h  | 1.5 h |
| OXOID NUTRIENT BROTH                                                          | +++              | 21.5                     | 5    | 1.5   |
| DIFCO NUTRIENT BROTH                                                          | +                | 96.5                     | 88   | 100   |
| DIFCO BROTH + 1 g/l OXOID LAB-LEMCO POWDER                                    | ++               | 98                       | 90.5 | 100   |
| DIFCO BROTH + 1 g/l OXOID LAB-LEMCO POWDER + 2 g/l YEAST EXTRACT              | ++/+++           | 95.5                     | 98   | 100   |
| DIFCO BROTH + 1 g/l OXOID LAB-LEMCO POWDER + 2 g/l YEAST EXTRACT + 5 g/l NaCl | ++/+++           | 98                       | 93.5 | 96    |

**Table 2.6**

| COMPOSITION OF MEDIA                                             | OVERNIGHT GROWTH | CYANIDE REMAINING (mg/l) |      |       |
|------------------------------------------------------------------|------------------|--------------------------|------|-------|
|                                                                  |                  | 0.5 h                    | 1 h  | 1.5 h |
| OXOID NUTRIENT BROTH                                             | +++              | 40                       | 16.5 | 6     |
| DIFCO NUTRIENT BROTH                                             | +                | 98                       | 96.5 | 94.5  |
| MIXED BROTH (HALF OXOID, HALF DIFCO)                             | ++/+++           | 94.5                     | 92   | 93    |
| DIFCO NUTRIENT BROTH + 2 g/l OXOID YEAST EXTRACT + 5 g/l PEPTONE | ++++             | 90                       | 89   | 87.5  |
| OXOID YEAST EXTRACT (2 g/l) + 5 g/l PEPTONE                      | +++ / +++++      | 58.5                     | 29.5 | 16    |

### 2.4.3 CYANIDE DEGRADATION BY *B. pumilus* C1

Overnight Oxoid nutrient broth cultures of *B. pumilus* C1 rapidly degraded 100 mg CN-/l (supplied as KCN) and could remove all the cyanide within 2 h (Fig. 2.2). When cyanide was re-added to 100 mg/l the cells continued to degrade it, albeit at a slower rate. When the organism was cultivated in Oxoid nutrient broth and harvested in the lag- or exponential-phase of growth it displayed no cyanide-degrading activity (Fig. 2.3). However, cells harvested in the stationary phase of growth degraded cyanide rapidly.

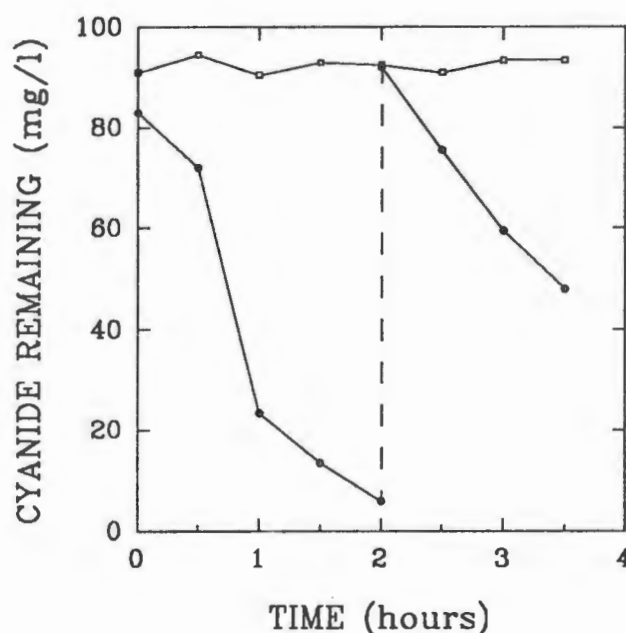


Fig. 2.2 Degradation of free cyanide by *B. pumilus* C1. An overnight Oxoid nutrient broth culture (200 ml) was harvested and the cells were resuspended in 100 ml of sterile Millipore water. Cyanide was added to the indicated concentration and the cell suspension was incubated at 30 °C without shaking. Remaining cyanide, (●), was assayed at 30-min intervals as described in Materials and Methods (sections 2.3.3 and 2.3.4). Cyanide loss from a control containing sterile water and cyanide was also measured, (□). Cyanide was re-added to the bacterial suspension to 100 mg CN-/l after 2 h of incubation.

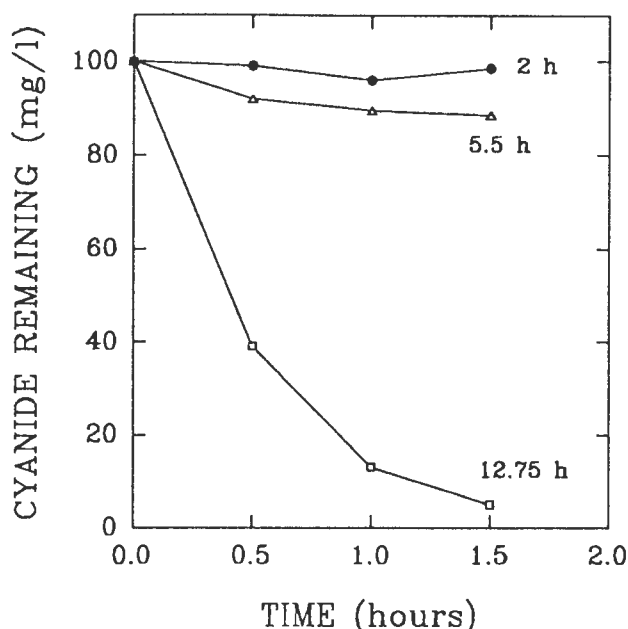


Fig. 2.3 The influence of growth phase on the ability of *B. pumilus* C1 to degrade cyanide. An overnight culture of the organism in Oxoid nutrient broth was used as the inoculum for three 200-ml Oxoid nutrient broths. Cultures were grown at 30 °C with shaking for 2, 5.5, and 12.75 h respectively. At the appropriate time the optical density of the culture was measured and the cells were harvested by centrifugation. Cells were resuspended in 50 ml of sterile Millipore water and assayed for their ability to degrade 100 mg CN/l (as described in Materials and Methods sections 2.3.3 and 2.3.4).

Cyanide and its structural analogue, acetonitrile ( $\text{CH}_3\text{CN}$ ), were tested as inducers of cyanide-degrading activity in pre-stationary-phase cells. Cells were cultivated in separate flasks, containing 0.2 % glucose minimal medium until they were just entering the exponential phase of growth (Fig. 2.4). At this time the optical density of each culture was measured and then either cyanide or acetonitrile was added to the cultures to a final concentration of 20 mg/l. A third culture was left untreated as a control. Incubation was continued for a further 90 min after which the optical density of the cultures was measured again, the cells were harvested, and assayed for cyanide-degrading activity (Table 2.7). None of the minimal medium cultures displayed any ability to degrade cyanide, indicating that neither cyanide nor acetonitrile could induce cyanide-degrading activity at this early stage in the growth curve.

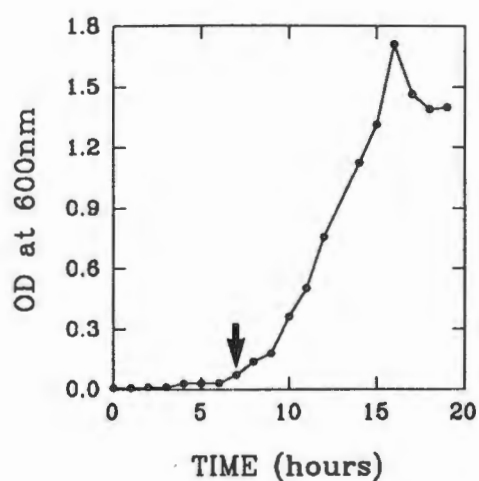


Fig. 2.4 Growth of *B. pumilus* C1 in 0.2 % glucose minimal medium. The approximate place in the growth curve where cyanide and acetonitrile were added is indicated by an arrow. Experimental details are provided in the Materials and Methods (section 2.3.8).

Table 2.7 Induction of cyanide-degrading activity by cyanide and acetonitrile in *B. pumilus* C1 grown in glucose minimal medium. OD at 600 nm = optical density of cultures at 600 nm. N.D. = not determined. Experimental details are provided in section 2.3.8.

| COMPOSITION OF MEDIA                    | O.D. AT 600 nm |       | CYANIDE REMAINING (mg/l) |     |       |
|-----------------------------------------|----------------|-------|--------------------------|-----|-------|
|                                         | 7 h            | 8.5 h | 0.5 h                    | 1 h | 1.5 h |
| MINIMAL MEDIUM CONTROL                  | 0.116          | 0.252 | 95                       | 99  | 93    |
| MINIMAL MEDIUM + ACETONITRILE FOR 1.5 h | 0.088          | 0.188 | 98                       | 96  | 95    |
| MINIMAL MEDIUM + CYANIDE FOR 1.5 h      | 0.088          | 0.084 | N.D.                     | 102 | 97    |
| COMPLETE MEDIUM (POSITIVE CONTROL)      | N.D.           | 1.672 | 27                       | 7   | 2     |
| CYANIDE CONTROL (CYANIDE + WATER)       | ---            | ---   | 104                      | 105 | 108   |

#### 2.4.4 THE REQUIREMENT FOR $Mn^{2+}$

It was subsequently discovered that cells grown in Oxoid nutrient broth prepared in Millipore water, which had been filtered through a new set of filters, exhibited very low cyanide-degrading activity. However, cells grown in the same medium prepared in tap water, and cells grown in Luria broth, or brain-heart broth prepared in the Millipore water, exhibited high cyanide-degrading activity (Fig.2.5). This indicated that the tap water (as well as the Luria and brain-heart broths) were providing some ionic species (probably a metal ion) required for the production of cyanide-degrading activity by the bacteria. Moreover, cyanide degradation by bacteria grown in Oxoid nutrient broth prepared in tap water was as fast, or faster, than the rate of cyanide degradation recorded in previous experiments.

To determine the identity of the ionic species required for cyanide degradation, Oxoid nutrient broths were prepared in Millipore water and supplemented with two different trace-metals solutions (Appendix A). The composition of these trace-metals solutions was generally similar with respect to ionic components, but trace-metals solution A contained  $Mn^{2+}$ ,  $Cu^{2+}$  and borate which were not components of trace-metals solution B. Cells grown overnight in the broth supplemented with trace-metals solution A exhibited rapid cyanide degradation, whereas those grown overnight in the broth supplemented with trace-metals solution B exhibited very low cyanide-degrading activity (Table 2.8). This indicated that  $Mn^{2+}$ ,  $Cu^{2+}$  or borate (or a combination of these) was required for the production of cyanide-degrading activity.

As it seemed most likely that a metal ion was required for the production of this activity, cells were inoculated into Oxoid nutrient broth prepared in Millipore water and supplemented with  $Mn^{2+}$  or  $Cu^{2+}$  (or both) at the same final concentrations as used in the experiment with the trace-metals solutions (i.e. final concentrations of 0.064 mg  $Cu^{2+}$ /l and 0.208 mg  $Mn^{2+}$ /l). Both metals were supplied as their sulphate salts. After overnight growth at 30 °C only the cultures supplemented with  $Mn^{2+}$  showed an enhanced ability to degrade cyanide over the unsupplemented control (Table 2.9). Cells grown in broth supplemented with both  $Mn^{2+}$  and  $Cu^{2+}$  showed enhanced cyanide-degrading activity, but this was lower than that of the culture containing just  $Mn^{2+}$ . This suggested possible antagonism by  $Cu^{2+}$  of the  $Mn^{2+}$ -mediated enhancement of cyanide-degrading activity.

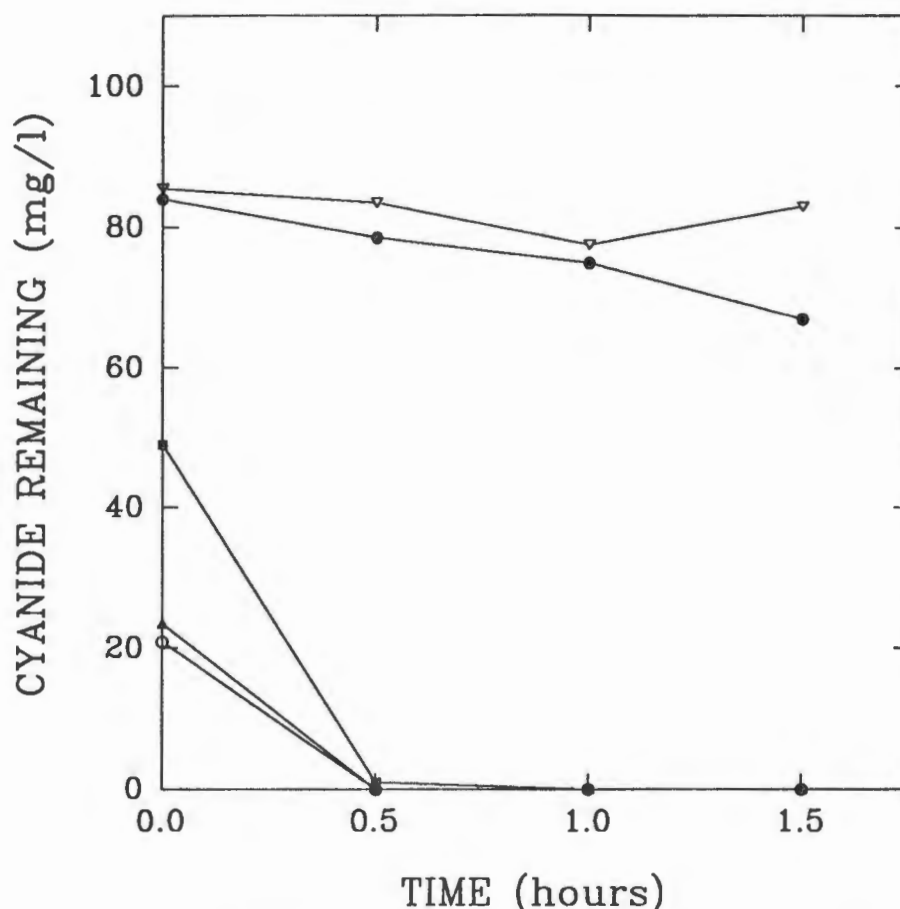


**Fig. 2.5** The effect of the growth medium on cyanide-degrading activity of *B. pumilus* C1. The organism was grown in Oxoid nutrient broth, Luria broth and brain-heart broth prepared in Millipore water or tap water (as described in Materials and Methods section 2.3.10). Oxoid broth in Millipore water, (●); and tap water, (○); Luria broth, (▲), and brain-heart broth, (■), in Millipore water; cyanide control, (▽) containing Millipore water and cyanide.

The effect of varying the amount of supplementary  $Mn^{2+}$  was tested by adding  $Mn^{2+}$  to Oxoid nutrient broth (prepared in Millipore water) to a final concentration of 0.002-0.2 mg/l. Cells grown overnight at 30 °C with shaking in these broths exhibited increasing cyanide-degrading activity with increasing  $Mn^{2+}$  concentration (Fig. 2.6). These results indicated that as little as 0.01 mg  $Mn^{2+}$ /l was sufficient for the appearance of high cyanide-degrading activity after overnight growth of the organism in  $Mn^{2+}$ -supplemented broth. Interestingly, cells grown in Difco nutrient broth (prepared in Millipore water and supplemented with 0.2 mg  $Mn^{2+}$ /l) displayed only a slightly enhanced ability to

degrade cyanide over cells grown in the unsupplemented medium or in Difco broth prepared in tap water (Table 2.10).

**Table 2.8 Cyanide-degrading activity of *B. pumilus* C1 grown in Oxoid nutrient broth prepared in Millipore water and supplemented with trace metals from trace-metals solution A or B. Bacteria grown in unsupplemented broth served as a negative control for bacterial cyanide degradation. The broth prepared in tap water was not supplemented with trace metals. The cyanide + water control served to determine the loss of volatile HCN during the experiment. Experimental details are described in section 2.3.10.**

| COMPOSITION OF MEDIA                    | CYANIDE REMAINING (mg/l) |       |      |       |
|-----------------------------------------|--------------------------|-------|------|-------|
|                                         | 0 h                      | 0.5 h | 1 h  | 1.5 h |
| OXOID BROTH IN MILLIPORE WATER          | 85.5                     | 83    | 78   | 73    |
| OXOID BROTH IN TAP WATER                | 24                       | 1     | 0    | 0     |
| OXOID BROTH + TRACE-METALS SOLUTION (A) | 54                       | 5.5   | 1    | 0     |
| OXOID BROTH + TRACE-METALS SOLUTION (B) | 83                       | 77.5  | 68.5 | 64    |
| CONTROL (CYANIDE + WATER)               | 84.5                     | 85.5  | 82.5 | 85.5  |

Since the L-threonine dehydrogenase of *E. coli* is activated by  $Mn^{2+}$  and  $Cd^{2+}$  (Craig and Dekker, 1986; 1988; 1990), cadmium was also tested for its ability to promote cyanide-degrading activity in *B. pumilus* C1. The effect of  $Cr^{3+}$  was tested as a higher-oxidation-state substitute for  $Mn^{2+}$ . Cells were grown in broths supplemented with  $Mn^{2+}$ ,  $Cd^{2+}$  or  $Cr^{3+}$  at a final concentration of 0.2 mg/l. Cells grown in the presence of  $Cr^{3+}$  exhibited similar low-level cyanide-degrading activity to cells grown in the unsupplemented medium (Fig. 2.7) indicating that chromium ions had no effect on the production of this activity. However, cells grown in the presence of  $Cd^{2+}$  appeared to have no ability to degrade cyanide

suggesting that cadmium ions inhibited the production of even low levels of cyanide-degrading activity.

**Table 2.9 Cyanide-degrading activity of *B. pumilus* C1 grown in Oxoid nutrient broth prepared in Millipore water and supplemented with  $Mn^{2+}$  or  $Cu^{2+}$ . Bacteria grown in unsupplemented broth served as a negative control for bacterial cyanide degradation. The broth prepared in tap water was not supplemented with metal ions. The cyanide + water control served to determine the loss of volatile HCN during the experiment. Experimental details are described in section 2.3.10.**

| COMPOSITION OF MEDIA                | CYANIDE REMAINING (mg/l) |       |      |       |
|-------------------------------------|--------------------------|-------|------|-------|
|                                     | 0 h                      | 0.5 h | 1 h  | 1.5 h |
| OXOID BROTH IN MILLIPORE WATER      | 86                       | 83.5  | 84   | 82    |
| OXOID BROTH IN TAP WATER            | 54                       | 5     | 0    | 0     |
| OXOID BROTH + 0.064 mg $Cu^{2+}$ /l | 86.5                     | 84.5  | 85   | 80    |
| OXOID BROTH + 0.208 mg $Mn^{2+}$ /l | 79                       | 44.5  | 23.5 | 11    |
| OXOID BROTH + $Cu^{2+}$ + $Mn^{2+}$ | 83                       | 60    | 41   | 26    |
| CONTROL (WATER + CYANIDE)           | 86                       | 86.5  | 86.5 | 83.5  |

Further experiments were undertaken to understand the role of manganous ions in promoting cyanide-degrading activity in *B. pumilus* C1. An experiment was conducted to test the effect on cellular cyanide-degrading activity of adding manganous ions to a late-exponential-phase culture grown in Oxoid nutrient broth prepared in Millipore water (i.e. a broth that does not support high cyanide-degrading activity). The late-exponential-phase culture was monitored for an increase in cyanide-degrading activity with time after the addition of  $Mn^{2+}$  (final concentration 0.22 mg  $Mn^{2+}$ /l). Such an increase would signal the induction of cyanide-degrading activity by these ions. The results showed that under the conditions of the experiment there was no such induction of activity

by  $\text{Mn}^{2+}$  ions *in vivo* (Fig. 2.8 and Table 2.11). The sharp decline in the optical density of the culture on addition of the manganese (Fig. 2.8) may have been the result of  $\text{Mn}^{2+}$  toxicity at this stage of growth, although the concentration of the metal was extremely low.

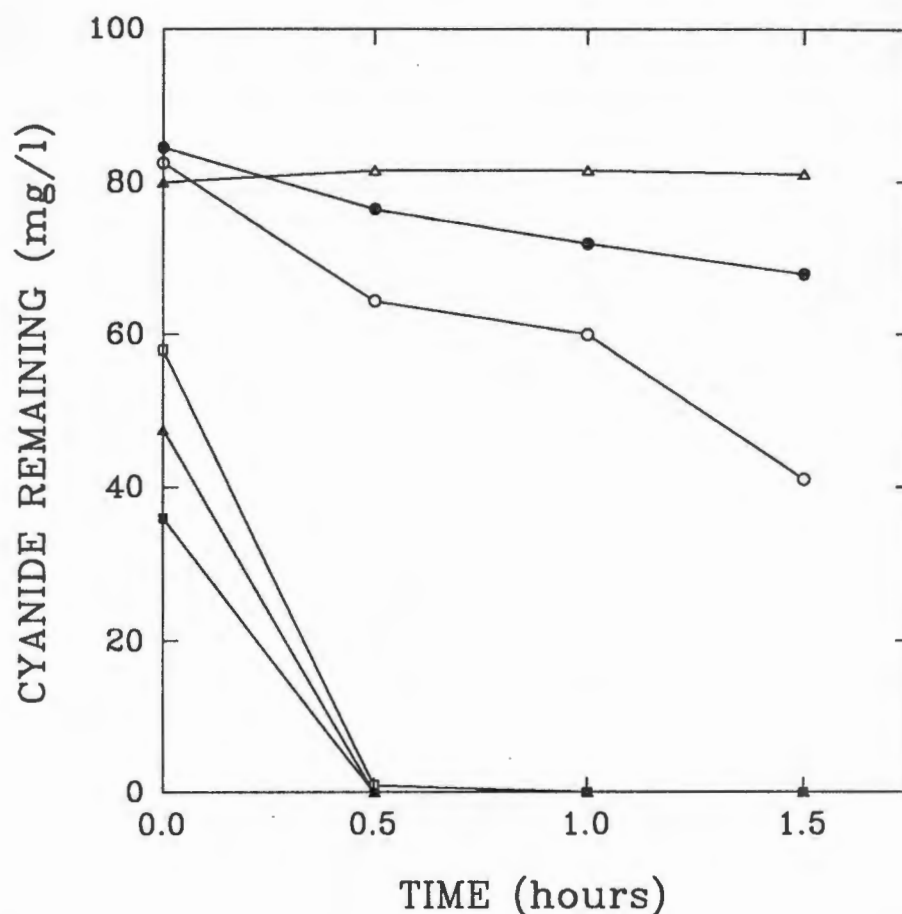


Fig. 2.6 The effect of varying concentrations of  $\text{Mn}^{2+}$  on cyanide-degrading activity of *B. pumilus* C1. Experimental details are provided in the Materials and Methods (section 2.3.10). Oxoid broth in Millipore water supplemented with 0, (●); 0.002, (○); 0.01, (□); 0.05, (▲); and 0.2, (■) mg  $\text{Mn}^{2+}/\text{l}$ ; cyanide control, (△) containing Millipore water and cyanide.

**Table 2.10 Cyanide-degrading activity of *B. pumilus* C1 grown in Difco nutrient broth prepared in Millipore water and supplemented with  $Mn^{2+}$ . Bacteria grown in unsupplemented broth served as a negative control for bacterial cyanide degradation. The broth prepared in tap water was not supplemented with  $Mn^{2+}$ . Bacteria grown in Oxoid broth supplemented with  $Mn^{2+}$  served as a positive control for bacterial cyanide degradation. The cyanide + water control served to determine the loss of volatile HCN during the experiment. Experimental details are described in section 2.3.10.**

| COMPOSITION OF MEDIA              | CYANIDE REMAINING (mg/l) |       |      |       |
|-----------------------------------|--------------------------|-------|------|-------|
|                                   | 0 h                      | 0.5 h | 1 h  | 1.5 h |
| DIFCO BROTH IN MILLIPORE WATER    | 85                       | 84.5  | 83.5 | 83    |
| DIFCO BROTH IN TAP WATER          | 83.5                     | 78    | 82   | 78    |
| OXOID BROTH + 0.2 mg $Mn^{2+}$ /l | 43                       | 1     | 0    | 0     |
| DIFCO BROTH + 0.2 mg $Mn^{2+}$ /l | 80                       | 77.5  | 71.5 | 70.5  |
| CONTROL (WATER + CYANIDE)         | 84.5                     | 85.5  | 87.5 | 88    |

A related experiment was undertaken to establish the effect of manganous ions on the activation of cyanide-degrading activity *in vitro* in a cell-free extract. A cell-free extract was prepared (in 10 mM Tris-HCl 50 mM NaCl, pH 8.0 buffer) from a stationary phase culture of *B. pumilus* C1 and divided into two parts which were incubated at 30 °C. Manganous ions were added to one sample to a final concentration of 0.2 mg  $Mn^{2+}$ /l. Samples were removed at intervals after the addition of the metal ions and assayed for their ability to degrade cyanide (Table 2.12). The results clearly indicate that manganous ions have no ability to activate cyanide-degrading activity *in vitro* in a cell-free extract of stationary-phase *B. pumilus* C1 cells grown in a medium low in  $Mn^{2+}$ .

These results suggested that the activation of cyanide-degrading activity by  $Mn^{2+}$  ions could not be effected in the late-exponential or stationary phase of growth in cultures grown with low endogenous levels of the metal. There was an indication that the process whereby this activity is formed could involve a multi-step cascade-type process in which  $Mn^{2+}$  ions are required by an early step for the production of cyanide-degrading activity at the end of the process.

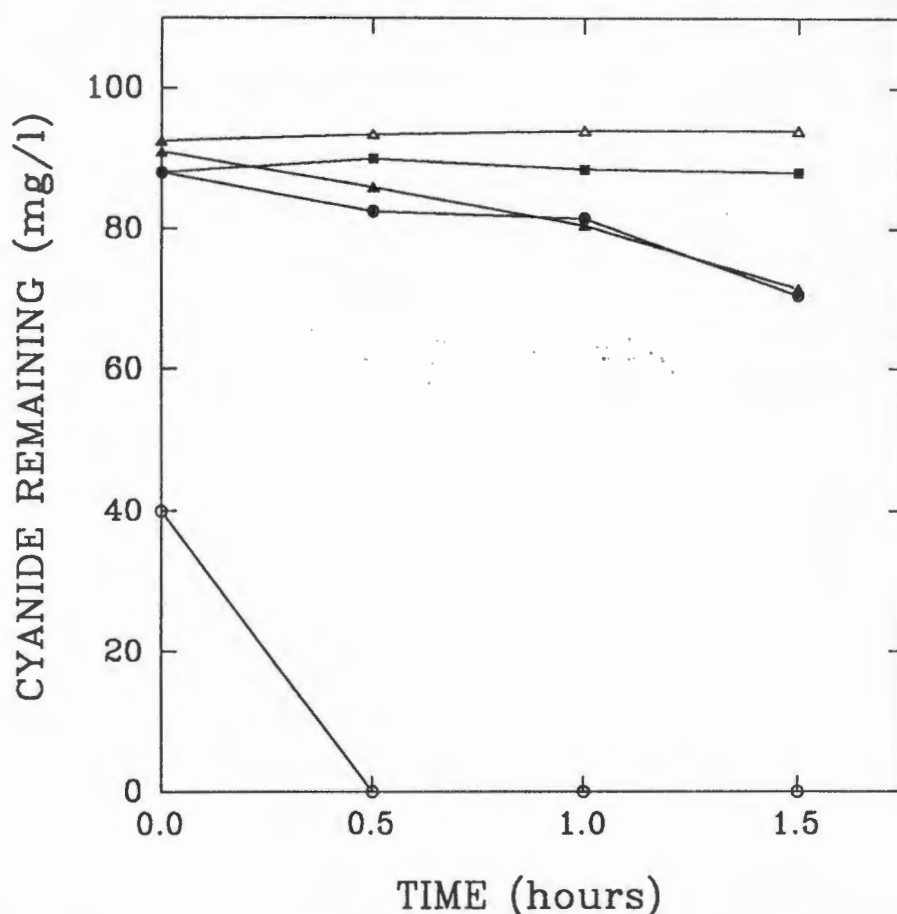


Fig. 2.7 The effects of  $\text{Cd}^{2+}$  and  $\text{Cr}^{3+}$  on the cyanide-degrading activity of *B. pumilus* C1. Experimental details are provided in the Materials and Methods (section 2.3.10). Oxoid nutrient broth in Millipore water with no added metal ions, (●); or with  $\text{Mn}^{2+}$ , (○);  $\text{Cd}^{2+}$ , (■); or  $\text{Cr}^{3+}$ , (▲) added to 0.2 mg/l. Cyanide control, (△), containing Millipore water and cyanide.

A more comprehensive experiment was undertaken to determine how late in the growth curve manganous ions could be added to still achieve high cyanide-degrading activity in the stationary phase of growth. Manganous ions were added at various stages in the growth curve to cultures growing in nutrient broth with a low endogenous manganese concentration. The cyanide-degrading activity of the cultures was determined at the end of a 15-h growth period (Fig. 2.9 and Table 2.13). All the cultures containing manganous ions displayed higher

cyanide-degrading activity than the control cultures containing no added manganese. However, the cultures which received manganese at the start of stationary phase displayed very low cyanide-degrading activity indicating that the manganese had been added after the optimal stage for its participation in the production of this activity. While it is possible that the 2 hours allowed between the addition of  $Mn^{2+}$  to cultures  $e_1$  and  $e_2$  and harvesting of the cells was insufficient to permit full induction of cyanide-degrading activity in these cultures, it should be noted that cyanide-degrading activity in cultures  $d_1$  and  $d_2$  (which received manganese 5 hours before harvesting) was lower than that in cultures which had received  $Mn^{2+}$  earlier in the growth curve. This suggested that the low activity in cultures  $e_1$  and  $e_2$  was a true indication of the inability of  $Mn^{2+}$  to promote cyanide-degrading activity late in the growth curve. Although it was evident that the addition of manganous ions could be delayed until as late as the late-exponential phase of growth with no significant reduction of cyanide-degrading activity in the stationary phase of growth, the highest activity was recorded with the cultures which received manganous ions before mid-exponential phase.

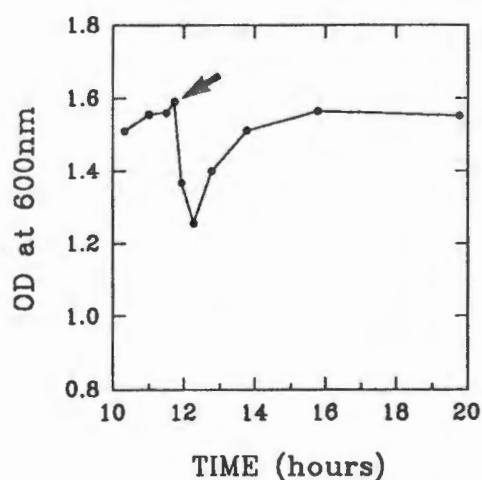


Fig. 2.8 Growth curve of a late-exponential phase culture of *B. pumilus* C1 showing changes in optical density on addition of  $Mn^{2+}$  to a final concentration of 0.22 mg/l. A 200-ml Oxoid nutrient broth (prepared in Millipore water) was inoculated with 100 ml of an overnight Oxoid nutrient broth culture and grown at 30 °C with shaking. The optical density of the culture was monitored during the late-exponential phase of growth. Manganese was added 11.73 h after inoculation (indicated by an arrow).

**Table 2.11 Induction of cyanide-degrading activity by  $Mn^{2+}$  in a culture of *B. pumilus* C1. The culture was grown at 30 °C with shaking in Oxoid nutrient broth prepared in Millipore water (i.e. with a low endogenous level of  $Mn^{2+}$ ) and  $Mn^{2+}$  was added in the late-exponential phase of growth. Incubation with shaking was continued and samples of the culture were removed at the indicated times for optical density measurements and for assaying cyanide-degrading activity (measured as the decrease in the absorbance at 520 nm,  $\Delta A_{520nm}$  in the cyanide assay). For the purposes of comparison, the  $\Delta A_{520nm}$  values were adjusted by division by the optical density at 600 nm of the culture at the time of sampling.**

| TIME SINCE<br>ADDITION OF<br>$Mn^{2+}$ (min) | OPTICAL<br>DENSITY OF<br>CULTURE<br>(600 nm) | DECREASE IN<br>AMOUNT OF<br>CYANIDE<br>( $\Delta A_{520nm}$ ) | $\Delta A_{520nm} \div$<br>$OD_{600nm}$ |
|----------------------------------------------|----------------------------------------------|---------------------------------------------------------------|-----------------------------------------|
| 0                                            | 1.592                                        | 0.028                                                         | 0.018                                   |
| 10                                           | 1.368                                        | -0.005                                                        | 0                                       |
| 30                                           | 1.256                                        | -0.004                                                        | 0                                       |
| 60                                           | 1.400                                        | 0.014                                                         | 0.010                                   |
| 120                                          | 1.512                                        | 0.100                                                         | 0.066                                   |
| 240                                          | 1.564                                        | 0.123                                                         | 0.079                                   |
| 480                                          | 1.552                                        | 0.057                                                         | 0.037                                   |

**Table 2.12 Induction of cyanide-degrading activity by  $Mn^{2+}$  in a cell-free extract of *B. pumilus* C1. A stationary-phase culture ( $OD_{600nm} = 1.308$  after 16.5 h at 30 °C with shaking in Oxoid nutrient broth prepared in Millipore water) was used for the preparation of the cell-free extract. The experimental method is described in the text. Cyanide-degrading activity was measured as the decrease in the absorbance at 520 nm ( $\Delta A_{520nm}$ ) in the cyanide assay.**

| TIME SINCE<br>ADDITION<br>OF $Mn^{2+}$<br>(min) | DECREASE IN CYANIDE ( $\Delta A_{520nm}$ ) |                                           |
|-------------------------------------------------|--------------------------------------------|-------------------------------------------|
|                                                 | CELL-FREE EXTRACT<br>+ 0.2 mg $Mn^{2+}$ /l | CELL-FREE EXTRACT<br>(NO $Mn^{2+}$ ADDED) |
| 0                                               | 0.063                                      | 0.049                                     |
| 10                                              | 0.055                                      | 0.063                                     |
| 20                                              | 0.078                                      | 0.046                                     |
| 40                                              | 0.075                                      | 0.061                                     |
| 80                                              | 0.057                                      | 0.049                                     |

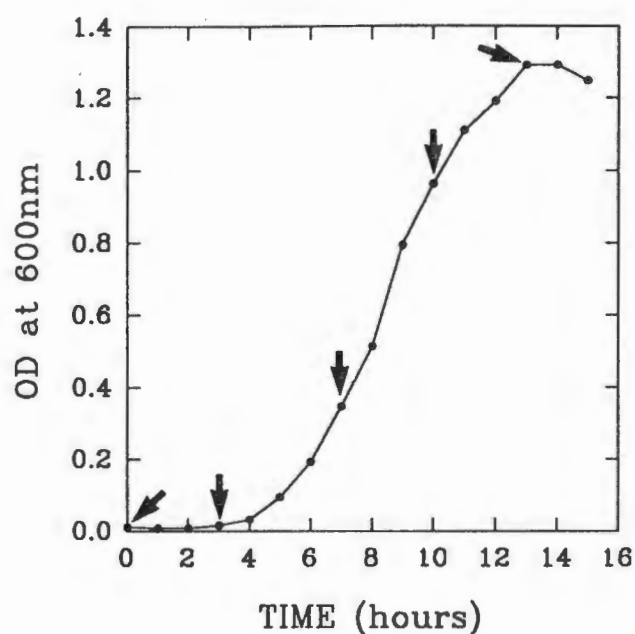


**Fig. 2.9** Growth curve of *B. pumilus* C1 in Oxoid nutrient broth (containing a low endogenous concentration of  $Mn^{2+}$ ) showing the approximate stages in the growth curve at which  $Mn^{2+}$  was added to pairs of cultures grown in parallel. Experimental details are provided in the Materials and Methods (section 2.3.11).

**Table 2.13** The effect of temporal addition of  $Mn^{2+}$  on the cyanide-degrading activity of *B. pumilus* C1 cultures grown in Oxoid nutrient broth prepared in Millipore water. Manganous ions were added to pairs of cultures at the times indicated. Cultures I and II served as negative controls for bacterial cyanide degradation.  $OD_{600nm}$  = optical density of the cultures at 600 nm. Cyanide-degrading activity was measured as the decrease in absorbance at 520 nm ( $\Delta A_{520nm}$ ) in the cyanide assay. For the purposes of comparison, the  $\Delta A_{520nm}$  values were adjusted by division by the corresponding  $OD_{600nm}$  values. Experimental details are provided in section 2.3.11.

| CULTURES       | TIME OF ADDITION OF $Mn^{2+}$ (h) | FINAL $OD_{600nm}$ OF CULTURES AT 15 h | CYANIDE DEGRADING ACTIVITY ( $\Delta A_{520nm}$ ) | AVERAGE $\Delta A_{520nm} \div OD_{600nm}$ |
|----------------|-----------------------------------|----------------------------------------|---------------------------------------------------|--------------------------------------------|
| I<br>II        | $Mn^{2+}$ not added               | 1.264<br>1.260                         | 0.024<br>0.023                                    | $0.019 \pm$<br>0.001                       |
| $a_1$<br>$a_2$ | 0                                 | 1.260<br>1.404                         | 0.233<br>0.276                                    | $0.191 \pm$<br>0.008                       |
| $b_1$<br>$b_2$ | 3                                 | 1.364<br>1.412                         | 0.233<br>0.238                                    | $0.170 \pm$<br>0.001                       |
| $c_1$<br>$c_2$ | 7                                 | 1.532<br>1.456                         | 0.313<br>0.267                                    | $0.194 \pm$<br>0.015                       |
| $d_1$<br>$d_2$ | 10                                | 1.452<br>1.420                         | 0.201<br>0.206                                    | $0.142 \pm$<br>0.005                       |
| $e_1$<br>$e_2$ | 13                                | 1.260<br>1.260                         | 0.045<br>0.066                                    | $0.044 \pm$<br>0.011                       |

#### 2.4.5 LOCATION OF CYANIDE-DEGRADING ACTIVITY

Cyanide-degrading activity appeared to be located within the cells as the culture supernatant from an overnight Oxoid nutrient broth culture displayed no ability to degrade cyanide (Table 2.14). The cells from this culture exhibited very high activity. A parallel experiment with cells grown for about 24 h in Spizizen *Bacillus* minimal medium gave similar results. The small amount of cyanide removed by the supernatant from the minimal medium culture may have been degraded by activity released into the medium by lysed cells. The boiled supernatant displayed no activity. These results showed that the cyanide-degrading activity was not secreted into the extracellular medium.

**Table 2.14 Comparison of cyanide-degrading activity in *B. pumilus* C1 cells and culture supernatants after overnight growth in Oxoid nutrient broth and Spizizen *Bacillus* minimal medium. The water + cyanide control served to determine the loss of volatile HCN during the experiment. The complete-medium + cyanide control served to determine the degree of reaction of cyanide with broth components. Experimental details are provided in section 2.3.9.**

| SAMPLE                                 | CYANIDE REMAINING (mg/l) |       |       |
|----------------------------------------|--------------------------|-------|-------|
|                                        | 0.5 h                    | 1 h   | 1.5 h |
| COMPLETE MEDIUM:<br>CELLS              | 4.5                      | 0.5   | 0     |
| COMPLETE MEDIUM:<br>SUPERNATANT        | 83.5                     | 86    | 85.5  |
| MINIMAL MEDIUM: CELLS                  | 0.5                      | 0     | 0     |
| MINIMAL MEDIUM:<br>SUPERNATANT         | 85                       | 76    | 68.5  |
| MINIMAL MEDIUM:<br>BOILED SUPERNATANT  | 97                       | 94.5  | 95    |
| CONTROL (WATER +<br>CYANIDE)           | 104                      | 101.5 | 103.5 |
| CONTROL (COMPLETE<br>MEDIUM + CYANIDE) | 75.5                     | 84    | 80.5  |

#### 2.4.6 REQUIREMENT FOR DE NOVO TRANSCRIPTION AND TRANSLATION

The effects of inhibiting transcription and translation on the production of cyanide-degrading activity were determined by adding rifampicin and chloramphenicol, respectively, to a mid-exponential-phase culture of *B. pumilus* C1 which had not yet acquired the ability to degrade cyanide. The minimum concentrations of these antibiotics which inhibited further growth of exponential-phase cultures were determined to be 40 µg/ml and 5 µg/ml for chloramphenicol and rifampicin, respectively (Fig. 2.10). The effect of addition of these antibiotics (to the minimum inhibitory concentrations) to a growing culture showed that the presence of either rifampicin or chloramphenicol prevented the appearance of cyanide-degrading activity in stationary phase (Fig. 2.11 and Table

2.15). However, the cyanide-degrading activity of a control culture increased from zero to a high level during the course of the experiment. These results indicated the requirement for both *de novo* transcription and translation for the development of cyanide-degrading activity in the stationary-phase of growth.

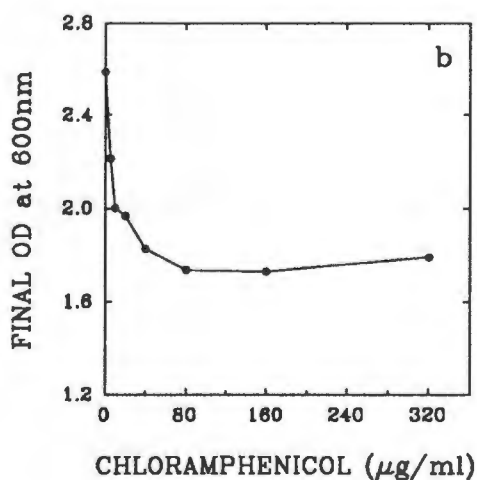
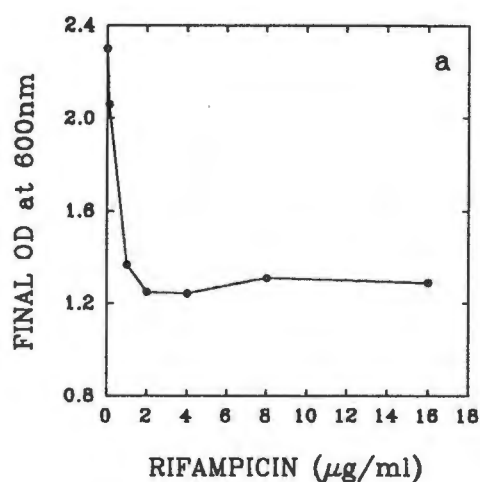


Fig. 2.10 The effect of varying concentrations of rifampicin (a) and chloramphenicol (b) on the further growth of a late-exponential-phase culture of *B. pumilus* C1. Experimental details are provided in the Materials and Methods (section 2.3.12). The optical densities of the cultures at 600 nm were 1.800 and 2.160 at the time of addition of rifampicin and chloramphenicol, respectively.

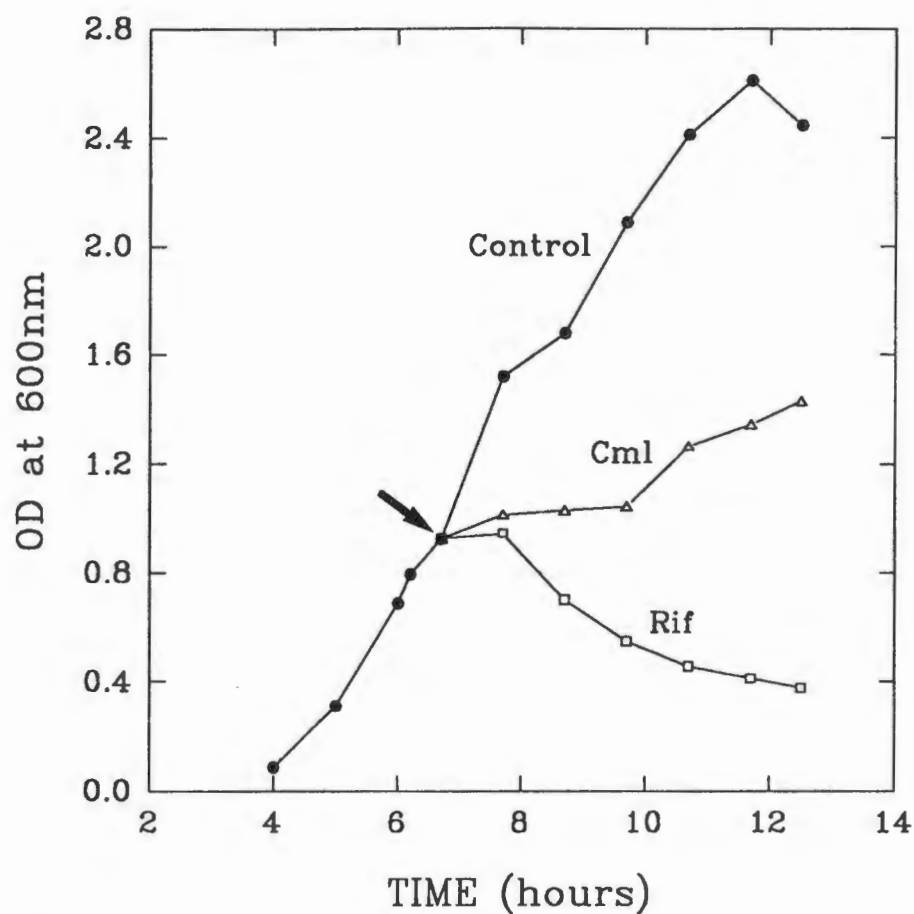


**Fig. 2.11** Growth of *B. pumilus* C1 before and after the addition of rifampicin and chloramphenicol to a mid-exponential-phase culture. The time of addition of the antibiotics is indicated by an arrow. Experimental details are provided in the Materials and Methods (section 2.3.13). Control culture, and culture before addition of antibiotics, (●); rifampicin culture, (□); and chloramphenicol culture, (△).

**Table 2.15** The effect of rifampicin and chloramphenicol on the production of cyanide-degrading activity by a growing culture of *B. pumilus* C1. Experimental details are provided in section 2.3.13. Cyanide-degrading activity in each cell-free extract was measured as the decrease in absorbance at 520 nm ( $\Delta A_{520\text{nm}}$ ) in the cyanide assay. For the purposes of comparison, the  $\Delta A_{520\text{nm}}$  value for each cell-free extract was adjusted by division by the amount of cell-free-extract protein used in the cyanide assay.

| TIME AFTER<br>ADDITION OF<br>ANTIBIOTIC | DECREASE<br>IN CYANIDE<br>( $\Delta A_{520\text{nm}}$ ) | AMOUNT<br>OF<br>PROTEIN<br>IN<br>ASSAY<br>(mg) | $\Delta A_{520\text{nm}}$<br>÷<br>mg<br>PROTEIN | PERCENTAGE<br>ACTIVITY |
|-----------------------------------------|---------------------------------------------------------|------------------------------------------------|-------------------------------------------------|------------------------|
| 0 h                                     | 0.004                                                   | 0.30                                           | 0.013                                           | <b>2.3</b>             |
| CONTROL + 1 h                           | 0.040                                                   | 0.35                                           | 0.114                                           | <b>20.6</b>            |
| RIF + 1 h                               | -0.038                                                  | 0.30                                           | 0                                               | <b>0</b>               |
| CML + 1 h                               | 0.025                                                   | 0.30                                           | 0.083                                           | <b>15.0</b>            |
| CONTROL + 5.8 h                         | 0.457                                                   | 0.83                                           | 0.554                                           | <b>100</b>             |
| RIF + 5.8 h                             | -0.020                                                  | 0.11                                           | 0                                               | <b>0</b>               |
| CML + 5.8 h                             | -0.006                                                  | 0.38                                           | 0                                               | <b>0</b>               |

#### **2.4.7 CYANIDE DEGRADATION BY CELL-FREE EXTRACTS OF *B. pumilus* C1**

Initial experiments with the low- and high-speed fractions of the cell-free extract indicated that there was little difference between them with respect to specific activity for cyanide degradation (results not shown). Subsequent experiments were therefore performed with the low-speed supernatant only. The cell-free extract displayed strong cyanide degrading activity (Fig 2.12) with a specific activity of  $0.27 \pm 0.08$   $\mu\text{moles cyanide removed/min/mg protein at } 30^\circ\text{C}$  (11 determinations).

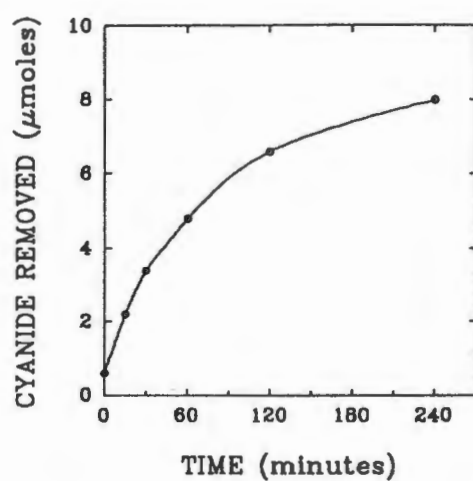


**Fig.2.12** Activity curve for cyanide degradation by a cell-free extract of *B. pumilus* C1 in 50 mM potassium phosphate 50 mM NaCl buffer, pH 7.4 (total protein = 0.4 mg; reaction volume = 2.04 ml). Experimental details are provided in Materials and Methods (section 2.3.6).

## 2.5 DISCUSSION

*B. pumilus* C1 is an aerobic, endospore-forming, Gram-positive bacterium which displays a remarkable ability to degrade free cyanide after overnight growth in a rich medium. Cyanide-degrading activity becomes detectable in the mid- to late-exponential phase of growth and persists well into stationary phase. Cyanide is not required to induce this activity. The production of cyanide-degrading activity appears to be closely linked to the phase of growth of the organism and could not be induced prematurely by cyanide or acetonitrile. The appearance of cyanide-degrading activity is also intimately linked to a requirement for *de novo* synthesis of mRNA and protein, indicating that the activity is not produced in an inactive form in the lag or early-exponential phase of growth and later activated in the mid- to late-exponential phase.

Manganoous ions ( $Mn^{2+}$ ) are essential for the appearance of cyanide-degrading activity, but the exact role they fulfil is obscure. This metal is required in very small amounts and as little as 0.01 mg  $Mn^{2+}$ /l is sufficient for the production of high cyanide-degrading activity. Manganese cannot induce activity *in vivo* when added to a late-exponential-phase culture grown with a low endogenous concentration of  $Mn^{2+}$  and exhibiting very low cyanide-degrading activity. Neither can it activate cyanide-degrading activity *in vitro* in a cell-free extract prepared from a similar low-activity culture grown with a low endogenous concentration of  $Mn^{2+}$ . It should be noted that although the experiment determining induction of cyanide-degrading activity in the early-exponential phase of growth by acetonitrile and cyanide was performed using glucose minimal medium, this medium did contain some added  $Mn^{2+}$ ; these data are therefore not the result of a low  $Mn^{2+}$  concentration in the medium.

These results, together with those showing that the presence of  $Mn^{2+}$  is required before the mid-exponential phase of growth for optimal cyanide degradation in the stationary phase, indicate that the effects of these ions are not exerted directly at the level of transcription, nor do they seem to exert a post-translational activation of a cyanide-degrading enzyme. There is thus good reason to believe that  $Mn^{2+}$  is required indirectly for the production of the cyanide-degrading activity through its involvement in, or control of, an early step in a multi-step process which culminates in the appearance of an ability to degrade cyanide in the mid- to late-exponential phase of growth.

The formation of endospores by members of the genus *Bacillus* is a developmental process controlled by an array of genes which are expressed in groups at different stages during sporulation (Losick *et al.*, 1986). The process is regulated by a cascade of sigma factors which control promoter recognition in stages between the initiation of the process and the appearance of an endospore (Losick and Pero, 1981; Losick *et al.*, 1986; Stragier and Losick, 1990). Manganese is essential for sporulation in *Bacillus* species (Charney *et al.*, 1951; Weinberg, 1964; Oh and Freese, 1976; Vasantha and Freese, 1979). Oh and Freese (1976) showed that growth of *B. subtilis* in a sporulation medium in the absence of manganous ions led to the incomplete metabolism of rapidly utilized carbon sources such as glucose and glycerol. Furthermore, growth was reduced, the cells accumulated 3-phosphoglyceric acid (3PGA), and normal sporulation was prevented. The subsequent addition of manganous ions to the medium resulted in the disappearance of 3PGA, the resumption of growth, and normal sporulation.

Manganous ions were shown to be required by phosphoglycerate phosphomutase (EC 5.4.2.1; PGA-mutase) (Oh and Freese, 1976; Watabe and Freese, 1979) which catalyzes the interconversion of 3-phosphoglyceric acid and 2-phosphoglyceric acid, and appears to be the only  $Mn^{2+}$ -requiring enzyme needed for sporulation in sporulation media (Vasantha and Freese, 1979). A manganese deficiency results in an inactive PGA-mutase which leads to an imbalance in the concentrations of intracellular metabolites and suppression of sporulation (i.e. the absence of sufficient manganese indirectly prevents cellular differentiation by causing an accumulation of 3PGA).

It may be speculated that the functions of manganous ions in *B. pumilus* C1 in sporulation and the production of cyanide-degrading activity are related. One possibility is that an early sporulation event (in the exponential phase of growth) triggers a sequence of events which results in, amongst others, the formation of cyanide-degrading activity. It may be noted in this regard that the *B. subtilis* RNA polymerase sigma factor,  $\sigma^H$ , which is required for the initiation of sporulation (and is encoded by the stage-0 gene, *spo0H*), also controls the recognition of supplemental promoters for the genes for the primary sigma factor,  $\sigma^A$  (*sigA*; Carter *et al.*, 1988) and fumarase (*citG*; Price *et al.*, 1989). However, while a pleiotropic role for manganous ions in *B. pumilus* C1 is possible (through an influence on the expression of sigma factors), this

hypothesis is as yet without proof (no attempt was made to compare spore yields in *B. pumilus* C1 grown in  $Mn^{2+}$ -supplemented and  $Mn^{2+}$ -deficient media).

It would be possible to test this hypothesis by creating sporulation mutants of *B. pumilus* C1 and testing whether they maintain their ability to degrade cyanide. The requirement for manganous ions in the earlier part of the growth curve suggests that it would be best to start with mutants in which sporulation is arrested at an early stage in the process (*spo0* mutants for example). The demonstration that a *spo* mutant was unable to degrade cyanide would lend strong support to the hypothesis that a close relationship exists between manganese, sporulation and cyanide-degrading activity in this organism.

The presence of sufficient  $Mn^{2+}$  in the growth medium appeared to exert a pronounced positive effect on the yield of *B. pumilus* C1 cells during overnight growth. When grown in Oxoid nutrient broth prepared in Millipore water (that is, in a medium containing only a low endogenous concentration of  $Mn^{2+}$ ) the cultures generally reached optical densities below 1.5, whereas in the presence of 0.01 mg  $Mn^{2+}$ /l (supplied as exogenous manganese) the cultures generally reached optical densities of over 2.5. This suggested that  $Mn^{2+}$  plays an integral part in the metabolism of this organism.

Cyanide-degrading activity in *B. pumilus* C1 does not appear to be exported into the extracellular medium. Its location within the cell was not determined, but it must occur either in the cytoplasm or attached to the surrounding membrane as Gram-positive bacteria do not have a periplasmic space. However, the ease with which highly active cell-free extracts could be prepared argue in favour of the activity being located in the cytoplasm in the form of a soluble enzyme.

The nature of the inhibition of cyanide degradation by Difco nutrient broth remains unresolved. The addition of manganous ions to this medium did not overcome the inhibitory effect, indicating that this effect was not simply the result of molecular antagonism of the role of  $Mn^{2+}$  by a component of the Difco medium. The observation that this medium supported relatively poor growth of *B. pumilus* C1, in addition to preventing high cyanide-degrading activity, suggests that the inhibitory effect is more general than simply the prevention of cyanide degradation.

The eight bacterial strains isolated from activated sewage sludge were identified as fluorescent species of *Pseudomonas* and have been shown, with reasonable confidence, to be strains of *P. fluorescens* and *P. putida*. However, the classification of these strains down to the biovar level should not be taken as necessarily correct because thorough biochemical testing was not undertaken and the mol% G + C of the DNA of each strain was not determined. Determination of the G + C content of the DNA is particularly important in distinguishing between biovars A and B of *P. putida* because they differ from each other in only a few phenotypic properties (Palleroni, 1984). In this regard it should be noted that there were a few discrepancies in the results of the biochemical tests which caused some confusion in the assignment of biovar type. Most strains of *P. putida* can be assigned to biovar A which is considered to be typical for this species.

The strains of *P. fluorescens* and *P. putida* displayed very poor cyanide-degrading activity by comparison with *B. pumilus* C1 and the other *B. pumilus* isolates and were thus not investigated any further. The ability of the pseudomonads to grow on glucose minimal agar with cyanide as the sole source of nitrogen suggests that they could utilize cyanide slowly for growth even though they were not able to rapidly remove cyanide from solution. In contrast, *B. pumilus* C1 grew very poorly, if at all, on minimal medium with cyanide as the sole nitrogen source.

## CHAPTER 3

THE ENZYMOLOGY OF CYANIDE DEGRADATION BY  
*B. pumilus* C1

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## CHAPTER 3

### THE ENZYMOLOGY OF CYANIDE DEGRADATION BY *B. pumilus* C1

#### 3.1 SUMMARY

A cyanide-degrading enzyme was purified from *B. pumilus* C1 by column chromatography and sucrose-gradient centrifugation, and characterized with respect to physico-chemical and kinetic properties. The enzyme consisted of three polypeptides of 41.2, 44.6 and 45.6 kDa; the molecular mass of the active enzyme by gel filtration was 417 kDa. Electron microscopy revealed a multimeric, rod-shaped protein of approximately  $9 \times 50$  nm in addition to many shorter structures. The enzyme rapidly degraded cyanide to formate and ammonia thereby exhibiting nitrilase-like activity. Activity was reduced under anaerobic conditions. Enzyme activity was optimal at 37 °C and pH 7.8 - 8.0. Activity was enhanced by azide, thiocyanate, formate, and acetate. Enhancement by azide was reversible, concentration dependent (with maximal enhancement at 4.5 mM azide), and increased with time. The enhancing effect of azide was not manifested under anaerobic conditions. Enzymatic activity was also enhanced by  $\text{Sc}^{3+}$ ,  $\text{Cr}^{3+}$ ,  $\text{Fe}^{3+}$  and  $\text{Tb}^{3+}$ ; enhancement was independent of metal-ion concentration above 5  $\mu\text{M}$ . Radiolabelling experiments with  $^{51}\text{Cr}^{3+}$  failed to demonstrate the binding of metal to the active enzyme. No activity was recorded with the cyanide substrate analogues  $\text{CNO}^-$ ,  $\text{SCN}^-$ ,  $\text{CH}_3\text{CN}$  and  $\text{N}_3^-$  and the possible degradation intermediate,  $\text{HCONH}_2$ . Kinetic studies indicated a  $K_m$  of  $2.56 \pm 0.48$  mM for cyanide and a  $V_{\max}$  of  $88.03 \pm 4.67$  mmoles cyanide/min/mg/l. The  $K_m$  increased approximately two-fold in the presence of 10  $\mu\text{M}$   $\text{Cr}^{3+}$  to  $5.28 \pm 0.38$  mM for cyanide and the  $V_{\max}$  to  $197.11 \pm 8.51$  mmoles cyanide/min/mg/l. The enzyme was tentatively named cyanide dihydratase.

### 3.2 INTRODUCTION

Enzymes which exhibit nitrile-hydratase or nitrilase activity towards cyanide (converting it to formamide and formate plus ammonia, respectively) have been described and characterized (Ingvorsen *et al.*, 1991; Wang *et al.*, 1992). However, there are few reports of such enzymes and it is unclear whether they are similar to the nitrile hydratases and nitrilases which act on organic nitriles, or whether they represent a sub-group of these nitrile-converting enzymes.

Purification of the cyanide-degrading enzyme from *B. pumilus* C1 was undertaken to characterize it and provide information on cyanide degradation at the molecular level in this organism. This information is important for determining the potential application of the enzyme in industry, as well as for extending the knowledge of this type of degradative microbiology. The characteristics of the enzyme determined in these investigations will assist in the determination of a catalytic mechanism for cyanide degradation. Studies at the protein level may also assist in the cloning of the gene or genes encoding the cyanide-degrading enzyme by means of the reverse genetics route.

### 3.3 MATERIALS AND METHODS

#### 3.3.1 MATERIALS

DEAE-cellulose anion-exchange matrix (DE52) was supplied by Whatman (England). Sephadex G-100, Sephadex G-200, and Sepharose CL-6B for gel-filtration were supplied by Pharmacia Fine Chemicals (Sweden). Bio-Gel A-5m gel-filtration matrix was from Bio-Rad Laboratories (U.K.). Spectra/Por dialysis tubing was supplied by Spectrum Medical Industries, Inc. (U.S.A.). Nicotinamide adenine dinucleotide (NAD, free acid) was supplied by E. Merck (Germany). Apoferritin,  $\beta$ -amylase and alcohol dehydrogenase for gel-filtration chromatography were supplied by the Sigma Chemical Co. (U.S.A.). Bovine serum albumin (fraction 5) was from Boehringer Mannheim GmbH (Germany). Formate dehydrogenase (F 5632) and lactoperoxidase (L-8257) were supplied by the Sigma Chemical Co., U.S.A. Iodo-gen® (1,3,4,6-tetrachloro-3 $\alpha$ ,6 $\alpha$ -diphenylglycouril) for chemically-mediated iodination of proteins was supplied by Pierce Chemical Co. (U.S.A.). Page Blue G90 was supplied by BDH Chemicals Ltd. (U.K.). Coomassie Brilliant Blue R250 was from Research Organics Inc. (U.S.A.). Manganese-54 (119.75 mCi/mg) was supplied as  $\text{MnCl}_2$  in 0.5 M HCl by New England Nuclear Research Products (U.S.A.) and chromium-51 (113.3 mCi/mg) was supplied as  $\text{CrCl}_3$  in 0.1 M HCl by Amersham (England). KCN (supplied by E. Merck, Germany) was used as the source of cyanide in all experiments. Chemicals for buffers, reagents, protein electrophoresis gels, and enzyme characterization experiments were of high purity. De-ionized water (filtered through a Millipore Milli-RO apparatus) was used throughout these studies.

#### 3.3.2 CULTURE CONDITIONS

*B. pumilus* C1 was grown in Oxoid nutrient broth supplemented with manganous ions (final concentration 0.01 mg  $\text{Mn}^{2+}$ /l) as described in section 2.3.6. Luria broth was later used as the growth medium after it was discovered that this medium generally supported good growth of the organism without the addition of exogenous manganese. However, it was still necessary to add manganese to some batches of the medium for maximal cyanide-degrading activity of the bacteria.

### 3.3.3 CELL-FREE EXTRACTS

Cell-free extracts of *B. pumilus* C1 were prepared in 10 mM Tris-HCl 50 mM NaCl (pH 8.0) as described in section 2.3.6.

### 3.3.4 PROTEIN DETERMINATIONS

Protein was assayed by the method of Lowry *et al.* (1951) as described in section 2.3.7.

### 3.3.5 ENZYME ASSAY

Enzymatic activity was determined in a volume of 120  $\mu$ l in 10 mM Tris-HCl 50 mM NaCl pH 8.0 in sealed tubes incubated for 1 h at 37 °C; one unit of activity was defined as the degradation of 1  $\mu$ mole of cyanide per min. Residual cyanide was determined by the absorption of the picric acid complex at 520 nm (section 2.3.4). Freshly purified enzyme was used for all characterization experiments.

### 3.3.6 PROTEIN PURIFICATION

All procedures were carried out at 4 °C or on ice. Although the protein purification was initially carried out with cell-free extract prepared in 50 mM potassium phosphate 50 mM NaCl (pH 7.4) this buffer was later changed to a 10 mM Tris-HCl 50 mM NaCl buffer (pH 8.0) and all experiments were conducted in this buffer unless otherwise indicated.

Whatman DE52 (DEAE-cellulose) anion-exchange matrix was prepared in 10 mM Tris-HCl 50 mM NaCl buffer (pH 8.0) according to the manufacturer's instructions. Undiluted cell-free extract (9 - 10 ml) was applied to a pilot anion-exchange column (1  $\times$  10 cm) equilibrated in 10 mM Tris-HCl 50 mM NaCl buffer (pH 8.0). The column was flushed with 2 column volumes of 10 mM Tris-HCl 50 mM NaCl (pH 8.0), followed by 2 - 3 column volumes each of three NaCl step-gradient solutions: 250 mM NaCl, 500 mM NaCl, and 1 M NaCl (all in 10 mM Tris-HCl, pH 8.0). The eluent from each of the four steps was collected and

dialysed against 10 mM Tris-HCl 50 mM NaCl (pH 8.0). The dialysed eluents were assayed for cyanide-degrading activity to determine the approximate NaCl concentration range in which cyanide-degrading activity was eluted from the column.

The anion-exchange purification step was scaled up using a  $1.6 \times 23$  cm column. A linear NaCl gradient (over the NaCl concentration range determined in the pilot experiment) was used for the elution of cyanide-degrading activity. The conditions for elution were optimized and the following anion-exchange procedure was developed: the anion-exchange column was equilibrated in 10 mM Tris-HCl 50 mM NaCl (pH 8.0) and 20 - 40 ml of cell-free extract (prepared from 400 - 800 ml of culture) was applied to the top of the column bed. The column was flushed with 10 mM Tris-HCl 250 mM NaCl (pH 8.0) until the absorbance at 280 nm of the eluent dropped to 0.1 or lower (approximately 5 h at a flow rate of 75 ml/h). A linear 250 - 360 mM NaCl gradient (in 10 mM Tris-HCl, pH 8.0) was used to elute bound proteins (starting with 245 ml of each gradient solution). One hundred and forty (140) 3.5-ml fractions were collected at a flow rate of about 52.5 ml/h. The flow rate was maintained using an Atto Perista peristaltic pump (SJ-1211). The absorbance at 280 nm was monitored continuously using an Instrumentation Specialties Co. (Isco) Type 6 Optical Unit connected to an Isco Model UA-5 Absorbance/Fluorescence Monitor (chart recorder). Every 4th fraction was assayed for cyanide-degrading activity by adding 20  $\mu$ l of a cold 611.8 mg CN<sup>-</sup>/l solution to 100- $\mu$ l samples in microfuge tubes (to give a final concentration of 102 mg CN<sup>-</sup>/l). All samples were incubated at 30 °C (subsequently 37 °C) for 1 h after which remaining cyanide was determined. Active fractions (exhibiting an  $A_{520\text{nm}}$  of 0.3 or lower in the assay) were pooled and concentrated approximately 50-fold in an Amicon 8400 ultrafiltration cell containing a PM-10 membrane.

Gel-filtration chromatography was performed in 10 mM Tris-HCl 50 mM NaCl (pH 7.4, later pH 8.0) with a maximum loading volume of 2 % of the matrix bed volume. Dry Sephadex G-100 polydextran gel-filtration matrix was prepared according to the manufacturer's instructions, degassed for 5 - 10 min at room temperature, and used to pack a  $1.6 \times 80$  cm column. The column was operated at 4 °C. Initial experiments with this column showed that the cyanide-degrading activity from cell-free extract eluted in the void volume of the column. Sepharose CL-6B and Bio-Gel A-5m gel-filtration matrices were tested for their ability to separate the cyanide-degrading activity from other *B. pumilus* C1 proteins and

were shown to have very similar properties in this regard. Degassed pre-swollen Bio-Gel A-5m was used to pack a  $1.6 \times 90$  cm column which was used in all subsequent gel-filtration experiments. Anion-exchange-purified protein (3.2 ml) was applied to the Bio-Gel A-5m column and eluted at a flow rate of approximately 6.5 ml/h. Every fourth fraction was assayed for cyanide-degrading activity as described for the fractions from anion-exchange chromatography. Active fractions were pooled and concentrated approximately 22-fold in an Amicon 8050 ultrafiltration cell containing an XM-50 membrane.

Cyanide-degrading enzyme, partially purified by anion-exchange and gel-filtration chromatography, was further purified by centrifugation on 12 - 30 % sucrose gradients (in 10 mM Tris-HCl 50 mM NaCl, pH 8.0). Gradients were prepared in  $1.4 \times 9.5$  cm Beckman Ultra-Clear™ centrifuge tubes at room temperature using a Gilson Minipuls 2 four-channel peristaltic pump and were then cooled at 4 °C for 1 - 2 h. Partially purified enzyme (700 - 800  $\mu$ l) was applied to the gradients and centrifuged in a Beckman SW40Ti rotor at 35 000 rpm ( $149\,800 \times g$  at  $r = 10.93$  cm) for 16 h at 4 °C. Gradients were fractionated into 60 5-drop fractions using an Isco Density Gradient Fractionator (Model 640). Fractions were diluted 1-in-10 in 10 mM Tris-HCl 50 mM NaCl buffer (pH 8.0) and assayed for cyanide-degrading activity as described for the fractions from anion-exchange chromatography.

Calibration of the gel-filtration column, to determine the molecular mass of the cyanide-degrading enzyme, was performed with proteins of known molecular mass (apo-ferritin, 443 kDa;  $\beta$ -amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; and bovine serum albumin, 66 kDa).

For the experiments involving manganese-54, four 200-ml Oxoid nutrient broths were prepared in 1-l conical flasks. Three broths were supplemented with non-radioactive manganous ions to a final concentration of 0.01 mg  $Mn^{2+}$ /l as usual. The fourth flask was supplemented with 91.43  $\mu$ Ci of  $^{54}Mn^{2+}$  and sufficient non-radioactive manganese to bring the concentration of manganous ions up to 0.01 mg  $Mn^{2+}$ /l. Each flask was inoculated with 20  $\mu$ l of an exponential-phase seed culture of *B. pumilus* C1 (grown in Oxoid nutrient broth at 30 °C with vigorous shaking) and the cultures were grown at 30 °C with vigorous shaking for 14 - 15 h as usual. The overnight cultures were harvested, washed and French pressed as described in section 2.3.6. The homogenate was centrifuged as described

previously and the cyanide-degrading activity was purified from the cell-free extract as described above.

For the experiments involving chromium-51, four 200-ml Luria broths were prepared in 1-l conical flasks and each was inoculated with 20  $\mu$ l of an exponential-phase seed culture of *B. pumilus* C1 (grown in Luria broth at 37 °C for 7 h with vigorous shaking). One of the cultures was supplemented with 87.69  $\mu$ Ci of  $^{51}\text{Cr}^{3+}$ . The other cultures were not supplemented with any metal ions (the endogenous concentration of  $\text{Mn}^{2+}$  ions in the Luria broth was assumed to be sufficient for the production of cyanide-degrading activity). The cultures were grown at 30 °C for 15 h with vigorous shaking and were then harvested, washed and a cell-free extract was prepared as described above for the manganese-54 experiment. The cyanide-degrading activity was purified from the cell-free extract as described above.

### 3.3.7 pH AND TEMPERATURE OPTIMA

Determination of the pH optimum was carried out by dilution of the purified enzyme into solutions of 600 mM Tris-HCl 50 mM NaCl buffered at pH values between pH 7.0 and pH 9.0. The resulting enzyme solutions were prewarmed for 6 min at 37 °C and then mixed with an unbuffered KCN solution (final concentration 2 mM) in a total volume of 120  $\mu$ l. Shadow experiments, in which the components were mixed in the same proportions, showed that the pH was controlled at the expected value. Triplicate enzyme samples at each pH were incubated at 37 °C for 1 h after which the residual cyanide was determined. The set of samples showing the greatest decrease (average) in absorbance at 520 nm (relative to duplicate controls at the same pH) was taken as 100 % activity and the changes in absorbance at 520 nm of all the other samples were expressed as a percentage of the most active sample.

Determination of the temperature optimum for enzymatic activity was carried out by incubating triplicate samples of purified enzyme, buffered at pH 8.0, for 10 minutes at the stated temperatures before adding buffered KCN and assaying the enzymatic activity at the same temperature for a further 1 h. Residual cyanide was measured as described above. The set of samples showing the greatest decrease (average) in absorbance at 520 nm (relative to a control incubated at the same temperature) was taken as 100 % activity.

The thermal stability of the enzyme was determined by diluting purified enzyme into 10 mM Tris-HCl 50 mM NaCl buffer (pH 8.0) and incubating triplicate samples at temperatures from 4 - 80 °C for 30 min. Samples were placed on ice for about 1 min and were then prewarmed for 5 min at 37 °C. Cyanide was added to each sample and incubation at 37 °C was continued for 1 h. Remaining cyanide was assayed as described previously. The set of samples showing the greatest decrease (average) in absorbance at 520 nm (relative to a control incubated at the same temperature) was taken as 100 % activity.

### 3.3.8 ANAEROBIC CYANIDE DEGRADATION

Freshly prepared cell-free extract in 10 mM Tris-HCl 50 mM NaCl (pH 8.0) was dispensed into microfuge tubes in 320- $\mu$ l volumes. Samples of the cell-free extract containing 0.1 %  $\text{NaN}_3$  were similarly dispensed into microfuge tubes. Samples of the cell-free extract, the cell-free extract containing 0.1 % azide, and the buffer, were placed (with lids open) in an Oxoid gas jar containing pellets of palladium metal. The jar was sealed, flushed with a mixture of 95 %  $\text{CO}_2$  5 %  $\text{H}_2$  for 30 min, and allowed to stand at room temperature for 27.5 h so that the samples could become anaerobic. Similar samples were used as a set of aerobic controls and were allowed to stand at room temperature under aerobic conditions for the same length of time.

The gas jar was placed in an anaerobic incubator and half of the microfuge tubes containing anaerobic cell-free extract (with and without azide) were microfuged for 12 min as the protein solutions had become turbid. Supernatants (245  $\mu$ l) were transferred to clean tubes and 5  $\mu$ l of aerobic 250 mM KCN solution was added to the protein and buffer samples (attempts to make the cyanide solution anaerobic resulted in complete loss of cyanide from solution). Samples were incubated anaerobically at 35 °C for 2 h and then duplicate 50- $\mu$ l volumes were removed from each sample and pipetted into picric acid reagent (100  $\mu$ l) in glass test tubes. The test tubes were removed from the anaerobic incubator and the cyanide assay was continued under aerobic conditions.

The anaerobic cell-free extract samples which had not been microfuged were removed from the anaerobic incubator, allowed to become aerobic, and then microfuged for 12 min at room temperature together with the aerobic cell-free

extract control samples. The anaerobic cell-free extract samples all exhibited large brown pellets after microfuging, whereas the aerobic control samples exhibited only very small brown pellets. Supernatants (245  $\mu$ l) were transferred to clean microfuge tubes and 5  $\mu$ l of the aerobic 250 mM KCN solution was added. The samples were incubated aerobically at 35 °C for 2 h and assayed for remaining cyanide as described previously. The decrease in absorbance at 520 nm (relative to a buffer control) of the cell-free extract sample assayed under aerobic (i.e. standard) conditions was taken as 100 % activity. The activities of the other samples were expressed as a percentage of this activity.

### 3.3.9 THE EFFECTS OF THIOCYANATE, FORMATE, ACETATE AND AZIDE

Formate was prepared by diluting concentrated formic acid ( $pK_a = 3.74$  at 25 °C) into 10 mM Tris-HCl 50 mM NaCl (pH 8.0) and adjusting the pH to 8.0 with 10 M NaOH. Acetate was similarly prepared from glacial acetic acid ( $pK_a = 4.74$ ). The effects on enzymatic activity of varying concentrations of thiocyanate, formate and acetate were determined by incubating the purified enzyme at 37 °C for 1 h in the presence of 5 mM KCN and increasing concentrations of the relevant test compounds. Residual cyanide was determined as described previously. The decrease in absorbance at 520 nm of each set of samples (relative to appropriate buffer controls) was expressed as a percentage of the decrease in absorbance at 520 nm of the enzyme controls in each case.

The effect of varying azide concentrations was determined in a similar way. A 3 M stock solution of  $NaN_3$  in 10 mM Tris-HCl 50 mM NaCl (pH 8.0) was prepared and the pH was adjusted to 8.0. Freshly prepared cyanide-degrading enzyme was diluted into 10 mM Tris-HCl 50 mM NaCl (pH 8.0) containing  $NaN_3$  at final concentrations of 0.3 mM to 1.5 M (samples were prepared in triplicate). A set of corresponding control azide samples containing no enzyme was prepared in duplicate. All samples were incubated at 4 °C for 16 h, allowed to warm up to room temperature for 20 - 25 min, and then prewarmed for 8 min at 37 °C. Cyanide was added to each sample to a final concentration of 5 mM from KCN stock solutions containing the appropriate concentration of  $NaN_3$  (to prevent dilution of the azide on addition of the substrate). Incubation was continued at 37 °C for 1 h and the residual cyanide was determined. The decrease (average) in absorbance at 520 nm of an enzyme control sample containing no azide (relative to an azide-free buffer control) was taken as 100 % activity. The changes in

absorbance at 520 nm (relative to azide-containing buffer controls) for all other samples were expressed as a percentage of the enzyme control activity.

### 3.3.10 THE EFFECTS OF METAL IONS

For the initial experiments, purified enzyme (5 ml) was dialysed twice against 5 l of 10 mM Tris-HCl 50 mM NaCl 5 mM EDTA buffer (pH 8.0), and then once against 5 l of 10 mM Tris-HCl 50 mM NaCl 0.1 mM EDTA buffer (pH 8.0) at 4 °C. Dialysed enzyme was diluted into 10 mM Tris-HCl 50 mM NaCl (pH 8.0) containing the indicated metal ions at a final free-metal-ion concentration of 238  $\mu\text{M}$  (taking into account the metal-chelating action of EDTA). Duplicate samples were incubated at room temperature for 1 h and were then prewarmed at 37 °C for 5 min. Cyanide was added to a final concentration of 5 mM and incubation was continued at 37 °C for 1 h. Remaining cyanide was assayed as described in section 2.3.4. The decrease (average) in absorbance at 520 nm of a control enzyme sample, containing no added metal ions (relative to a metal-free buffer control) was taken as 100 % activity. The decrease in absorbance at 520 nm of the metal-containing enzyme samples (relative to the appropriate metal-containing buffer controls) were expressed as a percentage of the activity of the enzyme control sample. In subsequent experiments the enzyme was not dialysed against EDTA and there was no pre-incubation of the enzyme with metal ions (200  $\mu\text{M}$ ) before assaying cyanide-degrading activity.

### 3.3.11 OTHER ASSAYS

Ammonia production was assayed by the absorption of the nitroprusside complex at 570 nm (Fawcett and Scott, 1960) in 100 mM phosphate 50 mM NaCl pH 8.0 as Tris-HCl prevented colour development. Samples (100  $\mu\text{l}$ ) in microfuge tubes were mixed with 100  $\mu\text{l}$  water followed by the immediate addition of 200  $\mu\text{l}$  sodium phenate, 300  $\mu\text{l}$  0.01 % sodium nitroprusside, and 300  $\mu\text{l}$  0.02 M sodium hypochlorite (Appendix A), in that order. The mixtures were incubated at 37 °C for 15 min and the absorbance was measured against a water blank. A linear calibration curve was obtained over the range of standard  $\text{NH}_4\text{Cl}$  solutions tested (4 - 20  $\mu\text{g NH}_4^+/\text{ml}$ ). To determine the presence of ammonia in solution after cyanide degradation, cell-free extract (in 100 mM sodium phosphate 50 mM NaCl buffer, pH 8.0) was diluted in the same buffer and 120- $\mu\text{l}$

samples were incubated with 6 mM KCN for 1 h at 37 °C (controls showed that these conditions allowed complete degradation of the cyanide). Samples were assayed directly for ammonia as described above with the exception that only 80  $\mu$ l water was added to bring the sample volume up to 200  $\mu$ l before the addition of the assay reagents.

Cyanate was assayed by the absorption of the dicyanatodipyridine copper(II) complex at 680 nm (Martin and McClelland, 1951). Samples (200  $\mu$ l) in microfuge tubes were mixed with 400  $\mu$ l of a 0.138 M  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  2.1 M pyridine solution (Appendix A) and incubated at 25 °C for 10 min. The dicyanatodipyridine copper(II) complex was extracted with four 200- $\mu$ l volumes of chloroform. For each extraction the aqueous and organic phases were mixed in the microfuge tube by inversion for 2 min. The phases were separated by microfuging for 50 - 60 s. The lower chloroform phase was removed after each extraction and transferred to a clean microfuge tube. Chloroform was added to the pooled chloroform extracts to bring the volume up to 1 ml and the solution was mixed. The absorbance of each sample was measured against a blank containing chloroform. Protein-containing samples exhibited a thin disc of denatured protein at the aqueous-organic interface after each chloroform extraction, but this did not interfere with the removal of the lower chloroform phase. A linear calibration curve was obtained over the range of standard NaOCN solutions tested (10 - 20 mM). To determine whether the cyanide-degrading enzyme could degrade cyanate, freshly purified enzyme was diluted in 10 mM Tris-HCl 50 mM NaCl buffer (pH 8.0). The enzyme sample and a control (containing only buffer) were pre-warmed for 12 min at 37 °C and NaOCN was added to 20 mM (a 50 mM NaOCN stock solution was prepared in 10 mM Tris-HCl 50 mM NaCl buffer, pH 8.0, and the pH was re-adjusted to 8.0). Incubation was continued at 37 °C and duplicate 200- $\mu$ l volumes were removed from the enzyme and control samples at 0, 1, 2 and 4 h for the cyanate assay described above. A control experiment showed that the enzyme could degrade 20 mM KCN within 2 h under these conditions.

Thiocyanate was determined by the absorption at 490 nm of the ferric complex using a modification of the method of Bailly (1957). Samples (50  $\mu$ l) in microfuge tubes were mixed with a solution containing 200  $\mu$ l 15 mM ferric ammonium sulphate, 130  $\mu$ l water, 20  $\mu$ l 2 M HCl, 200  $\mu$ l acetone, and 400  $\mu$ l 2-butanone (Appendix A) and the absorbance was measured against a water blank. A linear calibration curve was obtained over the range of standard NaSCN solutions

tested (4 - 12 mM). The ability of the cyanide-degrading enzyme to degrade thiocyanate was tested as described above for the cyanate-degradation assay, with the exception that the enzyme and control samples were incubated with 6 mM NaSCN and duplicate 50- $\mu$ l samples were removed at the indicated times for the thiocyanate assay. A fresh blank containing 10 mM Tris-HCl 50 mM NaCl buffer (pH 8.0) was prepared at each time interval. A control experiment showed that the enzyme could degrade 6 mM KCN within 2 h under the conditions of the experiment.

Formamide was converted to hydroxamic acid and assayed colourimetrically by determining the absorption at 570 nm of the resulting ferric complex (Fry and Millar, 1972; Ingram, 1960). Samples (200  $\mu$ l) in microfuge tubes were mixed with 400  $\mu$ l of a 1:1 mixture of 3.5 M NaOH and 2.3 M  $\text{NH}_2\text{OH}\cdot\text{HCl}$  (Appendix A), and incubated at 60 °C for 10 min. The solutions were allowed to cool to room temperature and then 200  $\mu$ l 4 M HCl and 200  $\mu$ l 1.23 M  $\text{FeCl}_3$  (in 0.075 M HCl) were added. The absorbance was measured against a water blank as soon as possible after the addition of the  $\text{FeCl}_3$  solution (i.e. within 5 - 10 min). A linear calibration curve was obtained over the range of standard  $\text{HCONH}_2$  solutions tested (2 - 8 mM). To determine the presence of formamide in solution after cyanide degradation, purified cyanide-degrading enzyme was diluted in 10 mM Tris-HCl 50 mM NaCl (pH 8.0) and incubated at 37 °C with 3.6 mM KCN for 1 h (conditions which permitted complete degradation of the cyanide). Controls containing only enzyme, and enzyme plus 3.9 mM formamide, were incubated under the same conditions. Samples were assayed directly for formamide as described above. A formamide-degradation assay was performed as described for the cyanate-degradation assay, with the exception that the enzyme and control samples were incubated with 6 mM formamide. A control experiment showed that the enzyme could degrade 6 mM KCN within 2 h under the conditions of the experiment.

Azide was measured by its absorbance at 250 nm using a blank of 10 mM Tris-HCl 50 mM NaCl buffer. A linear calibration curve was obtained over the range of standard  $\text{NaN}_3$  solutions tested (0.5 - 5 mM). The ability of the cyanide-degrading enzyme to degrade  $\text{NaN}_3$  was assayed as described for the cyanate-degradation assay, with the exception that the enzyme and control samples contained 2 mM  $\text{NaN}_3$  and the duration of the assay was 2 h. The control samples (containing 10 mM Tris-HCl 50 mM NaCl, pH 8.0, and azide) were blanked against the Tris-HCl NaCl buffer, whereas the enzyme samples were

blanked against the Tris-HCl NaCl buffer containing the same concentration of enzyme.

The ability of the cyanide-degrading enzyme to degrade acetonitrile was assayed as described for the cyanate-degradation assay with the exception that purified enzyme was diluted into 100 mM sodium phosphate 50 mM NaCl buffer (pH 8.0) and incubated with 10 mM  $\text{CH}_3\text{CN}$ . Duplicate 100- $\mu\text{l}$  volumes were removed from the enzyme sample and a phosphate-buffer control at 0, 0.25, 0.5, 1, 2 and 4 h and assayed for ammonia as described above.

Formate was determined by the reduction of  $\text{NAD}^+$  using formate dehydrogenase (Quayle, 1966). An extinction co-efficient ( $\epsilon$ ) at 340 nm of  $6.22 \times 10^3 \text{ l.mol}^{-1}\text{.cm}^{-1}$  was used to calculate the amount of NADH formed. To test for the presence of formate in solution after cyanide degradation, freshly purified cyanide-degrading enzyme was diluted into 10 mM Tris-HCl 50 mM NaCl (pH 8.0) and incubated with 5 mM KCN in a volume of 120  $\mu\text{l}$  for 1 h at 37 °C (conditions which allowed complete degradation of the cyanide). The initial amount of KCN was 0.6  $\mu\text{mole}$ . The 120- $\mu\text{l}$  samples from the cyanide-degradation step were each mixed with 100  $\mu\text{l}$  10 mM  $\text{NAD}^+$  and 730  $\mu\text{l}$  100 mM sodium phosphate 50 mM NaCl buffer (pH 7.43). A control sample (to determine any interference by the cyanide-degrading enzyme) was prepared in the same phosphate buffer and had a similar composition to the experimental samples, but contained 0.6  $\mu\text{mole}$  sodium formate instead of the cyanide-degradation product. A similar formate-containing solution was prepared as a blank. All samples were prewarmed for 6 min at 37 °C and formate dehydrogenase (50  $\mu\text{l}$  of a 10 U/ml solution in the phosphate buffer) was added to the experimental and control samples which were thoroughly mixed, and incubated at 37 °C for 1 h. The blank received no formate dehydrogenase. The absorbances at 340 nm of the experimental and control samples were measured against the blank.

The effect on enzymatic activity of different buffer solutions was determined by diluting the purified cyanide-degrading enzyme in 10 mM Tris-HCl 50 mM NaCl (pH 8.0) and dialysing samples once against 2 l each of 50 mM sodium phosphate 50 mM NaCl (pH 8.0), 50 mM borate 50 mM NaCl (pH 8.0), 50 mM glycine 50 mM NaCl (pH 8.0), and 10 mM triethanolamine 50 mM NaCl (pH 8.0). The absorbance at 280 nm of the dialysed samples was measured before assaying cyanide-degrading activity. The enzymatic activity of each sample after dialysis

was expressed as the decrease in absorbance at 520 nm ( $\Delta A_{520\text{nm}}$ ) in the cyanide assay divided by the measured protein absorbance at 280 nm.

The fate of the radiolabel after degradation of  $K^{14}\text{CN}$  was determined by incubating triplicate samples of the cyanide-degrading enzyme with 6 mM KCN (containing 1  $\mu\text{Ci}$   $K^{14}\text{CN}$ ) for 1 h at 37 °C. Control experiments showed that these conditions were sufficient for complete degradation of the cyanide. Radioactivity in 1- $\mu\text{l}$  volumes of the samples was determined using a Beckman LS1701 Liquid Scintillation System before addition of the enzyme, after the period of incubation, and then again after the incubated samples had been allowed to stand uncovered at room temperature in a fumecupboard for 1 h.

### 3.3.12 CHEMICAL MODIFICATION OF AMINO ACIDS

Carboxymethylation of cysteinyl residues with iodoacetic acid ( $\text{ICH}_2\text{COOH}$ ) was performed according to the method described by Gurd (1967). A stock iodoacetic acid solution was prepared by dissolving iodoacetic acid crystals in 10 mM Tris-HCl 50 mM NaCl (pH 8.0). Purified enzyme was mixed with iodoacetic acid in molar ratios of 1:1 to 1:10<sup>6</sup> and incubated at room temperature for 2 h and then at 4 °C overnight. A control containing only enzyme, and controls containing buffer and iodoacetic acid were treated similarly. All samples were diluted in the Tris-HCl NaCl buffer and assayed for cyanide-degrading activity.

The effect on enzymatic activity of converting cysteinyl residues to S- $\beta$ -(4-pyridylethyl)-L-cysteine was performed according to Friedman *et al.* (1970). Purified enzyme was mixed with HPLC-purified 4-vinylpyridine in molar ratios of 1:100 and 1:1000 and incubated in the dark at room temperature for 2.75 h. Samples were dialysed once against 2 l of the Tris-HCl NaCl buffer at 4 °C and assayed for cyanide-degrading activity.

Iodination of tyrosine residues was performed according to the method of Sodoyez *et al.* (1975). Stock solutions of KI and  $\text{H}_2\text{O}_2$  were prepared in 10 mM Tris-HCl 50 mM NaCl buffer (pH 8.0). Purified enzyme (120  $\mu\text{l}$ ) was mixed at room temperature with 10  $\mu\text{l}$  0.1 M KI, 10  $\mu\text{l}$  0.1 M  $\text{H}_2\text{O}_2$ , and 10  $\mu\text{l}$  of a 1 mg/ml solution of lactoperoxidase in Tris-HCl NaCl buffer, in that order. Three subsequent additions of KI,  $\text{H}_2\text{O}_2$  and lactoperoxidase were made after 4, 8 and 12 min. Control enzyme samples were left untreated, or received only KI and

H<sub>2</sub>O<sub>2</sub>, H<sub>2</sub>O<sub>2</sub> and lactoperoxidase, or KI and lactoperoxidase. The enzyme sample to which KI, H<sub>2</sub>O<sub>2</sub> and lactoperoxidase were added turned a light brown colour indicating the formation of I<sub>2</sub>. All samples were dialysed once against 2 l of the Tris-HCl NaCl buffer at 4 °C and then assayed for cyanide-degrading activity.

The effect on enzymatic activity of carbethoxylation of histidyl residues with diethylpyrocarbonate (DEPC) was performed according to the method of Miles (1977). DEPC was diluted in absolute ethanol, acetonitrile or dimethyl sulphoxide and its concentration was determined accurately by reaction with 10 mM imidazole in phosphate-buffered saline (pH 7.4). The absorbance at 230 nm of the N-carbethoxyimidazole product ( $\epsilon_{230\text{nm}} = 3.0 \times 10^3 \text{ l.mol}^{-1}\text{.cm}^{-1}$ ) allowed calculation of the DEPC concentration. Nucleophiles such as H<sub>2</sub>O and Tris promote the decomposition of DEPC. Purified cyanide-degrading enzyme was diluted in 100 mM sodium phosphate 50 mM NaCl buffer (pH 8.0), mixed with 0.1 - 40 mM of DEPC and incubated at room temperature for 2 h before determining the cyanide-degrading activity of the samples.

### 3.3.13 POLYACRYLAMIDE GEL ELECTROPHORESIS

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 15 % polyacrylamide slab gels (20 × 20 cm) according to the method of Laemmli (1970) (Appendix A). Protein-containing samples (20 - 120 µg protein) were mixed with an equal volume of sample-application buffer (Appendix A), applied to the top of polyacrylamide gels, and electrophoresed at room temperature in 24.8 mM Tris-HCl 200 mM glycine 0.1 % SDS tank buffer at 100 V (constant voltage) for approximately 18 h. The electrophoresis apparatus was cooled by blowing air onto the surface of the front gel plate. Proteins were stained with Page Blue G90 (Appendix A). Destaining of gels was accomplished in 7 % acetic acid. The relative molecular masses of the enzyme polypeptides were determined by comparison with the mobilities of standard proteins of known molecular mass (phosphorylase b, 97.4 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; lysozyme, 14.3 kDa; cytochrome c, 12.4 kDa).

### 3.3.14 ELECTROELUTION OF POLYPEPTIDES FROM GEL SLICES

Purified cyanide-degrading enzyme (300 - 350  $\mu$ l) was dispensed into a microfuge tube coated with a thin film of Iodo-gen® (according to the manufacturer's instructions) and mixed with 1  $\mu$ l Na<sup>125</sup>I (containing approximately 30  $\mu$ Ci of radioactivity). The sample was incubated at room temperature in a lead container for 1 h, mixed with an equal volume of sample-application buffer, and applied across the top of an SDS polyacrylamide gel. Proteins were electrophoresed as described in section 3.3.13 and stained with Coomassie Brilliant Blue R250 (electroelution appeared to be more efficient when proteins were stained with this dye). Destaining was accomplished using 7 % acetic acid 25 % methanol. Strips of gel containing the proteins of interest were excised using a scalpel and the radioactivity in each gel slice was quantitated using a Packard 5220 Auto-Gamma Scintillation Spectrometer. Electroelution of polypeptides from polyacrylamide gel slices was performed by Dr George Lindsey of the Department of Biochemistry, University of Cape Town, using a Schleicher & Schüell BIOTRAP according to the manufacturer's instructions. A potential difference of 200 V was applied across the sample chamber (approximately 10 V/cm) and polypeptides were eluted overnight at room temperature into 24.8 mM Tris-HCl 200 mM glycine 0.1 % SDS buffer (the same buffer used for polyacrylamide-gel electrophoresis). Samples were freeze-dried, taken up in water, acetone precipitated, and finally resuspended in water for NH<sub>2</sub>-terminal sequencing.

### 3.3.15 AUTOMATED NH<sub>2</sub>-SEQUENCING OF POLYPEPTIDES

Automated gas-phase sequencing of polypeptides by the Edman degradation with phenylisothiocyanate (PITC) was performed by Prof. Wolf Brandt of the Department of Biochemistry, University of Cape Town (Hewick *et al.*, 1981; Brandt *et al.*, 1984).

### 3.3.16 KINETICS

The kinetic properties of the enzyme were investigated in 10 mM Tris-HCl 50 mM NaCl (pH 8.0) at 37 °C in a volume of 500  $\mu$ l. The enzyme concentration was kept constant and the initial rates of reaction with varying KCN concentrations

(up to 10 mM) were measured by removing samples at intervals after the addition of substrate. The total assay period in each case was kept short (3 - 18 min) to reduce the influence of the loss of volatile HCN on the rate measurements. Samples were prewarmed for 10 - 12 min before the addition of the substrate and initial reaction rates were determined twice for each cyanide concentration. Samples removed from the reaction mixtures were pipetted into picric-acid reagent in test tubes on ice and were assayed for remaining cyanide after all samples for a particular experiment had been collected.

Two sets of kinetic data were determined. The first set was determined in the presence of 1 mM EDTA so as to remove any metal ions from solution (EDTA chelates  $M^{2+}$  ions and probably did not remove  $M^{3+}$  ions very effectively). Total protein used in EDTA experiments was 0.0025 mg (in a reaction volume of 500  $\mu$ l). Samples were incubated for 30 min on ice after the addition of EDTA and the initial rate of cyanide degradation was measured as described above. The second data set was determined in the presence of 10  $\mu$ M  $Cr^{3+}$  to measure the effect on kinetic activity of  $M^{3+}$  ions ( $Cr^{3+}$  was taken as representative of  $M^{3+}$  ions in general). Total protein used in  $Cr^{3+}$  experiments was 0.00069 mg (in a reaction volume of 500  $\mu$ l). No pre-incubation period with the metal ions was allowed before prewarming the samples and adding substrate.

Initial-rate data were regressed using linear regression and plotted according to the method of Lineweaver and Burk (1934). The maximal reaction rate,  $V_{max}$ , and the Michaelis constant,  $K_m$ , (in the presence of EDTA and  $Cr^{3+}$ ) were determined from the data plots. These values were checked using the EZ-FIT computer software developed by F. W. Perrella (E. I. DuPont de Nemours & Co., U.S.A.) for analysing kinetic data.

### 3.3.17 ELECTRON MICROSCOPY

Fractions 32 - 38 from a sucrose-gradient run were pooled and dialysed once against 2 l of 10 mM Tris-HCl 50 mM NaCl (pH 8.0) at 4 °C to remove the sucrose. The dialysed sample was concentrated by dialysis against dry polydextran (Sephadex G-200) at 4 °C, applied to formvar-carbon-coated grids, rinsed with distilled water, and stained with phosphotungstic acid (1 %), pH 6.2. The prepared samples were examined using a Hitachi-600 electron microscope.

### 3.4 RESULTS

#### 3.4.1 PURIFICATION OF A CYANIDE-DEGRADING ENZYME

Initial experiments with ammonium-sulphate fractionation of cell-free extracts showed that the addition of  $(\text{NH}_4)_2\text{SO}_4$  to 35 % saturation precipitated only a small amount of the cyanide-degrading activity; increasing the  $(\text{NH}_4)_2\text{SO}_4$  concentration of this solution to 55 % saturation precipitated most of the cyanide-degrading activity. A comparison of the specific activities of the cell-free extract before and after ammonium-sulphate fractionation showed that this treatment increased the specific activity approximately two- to three-fold. Since this represented only a small increase in the purity of the cyanide-degrading activity, and furthermore entailed a lengthy dialysis procedure, this step was not incorporated into the overall purification procedure.

Incubation of cell-free extracts at room temperature or 4 °C under anaerobic conditions for 21 - 25 h resulted in the precipitation of 13 - 29 % of the protein, but not the cyanide-degrading activity. Although this suggested an anaerobic incubation step could be incorporated into the enzyme purification scheme, this approach was abandoned as a result of the lengthy incubation period required for the cell-free extract samples to become anaerobic.

A purification procedure was developed for the cyanide-degrading enzyme using column chromatography and sucrose-gradient centrifugation as described in section 3.3.6 (Figs. 3.1a, 3.1b, 3.1c). The cyanide-degrading activity was purified 42-fold by this procedure with an overall yield of 2.3 %; the purification data are summarized in Table 3.1. The enzyme responsible for this activity had a molecular mass of approximately 417 kDa by gel filtration chromatography. Analysis of the purified protein by SDS-PAGE, however, indicated that the active enzyme consisted of three polypeptides of 41.2, 44.6 and 45.6 kDa (Fig. 3.1d) which co-purified with the same ratio to one another throughout the purification procedure suggesting that the enzyme was a multimeric complex. The small difference in molecular mass between the larger two polypeptides made their separation difficult on SDS polyacrylamide gels under the conditions described in section 3.3.13; on many occasions these polypeptides were not completely resolved. If the purification was carried out without stringent temperature control, or if the enzyme had been subjected to freezing and thawing, SDS-PAGE of the purified enzyme revealed, in addition to the normal three bands, a further

three faster migrating bands of markedly lower intensity but with the same relative pattern of staining intensity. This reinforced the proposal that the holoenzyme consisted of multiples of a basic structure containing three polypeptide chains and therefore suggested that the original bands did not arise from proteolysis of a larger monomeric protein.

Examination of the purified enzyme using electron microscopy (Fig. 3.2) revealed particles of heterogenous sizes and shapes. Tightly packed spiral structures of approximately 9 nm diameter and 50 nm length could be readily observed on the phosphotungstic-acid-stained grid (Fig. 3.2a). Many shorter tightly packed spiral structures were also visible (Fig. 3.2b) in addition to some smaller particles (Figs. 3.2b, 3.2c). In addition, arrays of six of the smallest particles, in close association (Fig. 3.2b), were visible. These possibly arose by fragmentation of the spiral structure on application to the grid. The smallest particle (Fig. 3.2c), which appeared to be less tightly packed, appeared to consist of more than one component. These components may correspond to the three polypeptides seen on SDS-PAGE.

The structure of the enzyme revealed by electron microscopy may explain some of the behaviour of this protein in solution. Application of small amounts of enzyme to anion-exchange or gel-filtration columns resulted in a loss of cyanide-degrading activity (for example, no cyanide-degrading activity could be recovered from the  $1.6 \times 23$  cm anion-exchange column after application to the column of the cell-free extract from a single 200-ml culture, and elution of the protein under the normal operating conditions described above). This suggested that the enzyme was sensitive to dilution and emphasizes the importance of purifying the enzyme under the conditions described in section 3.3.6. Enzyme activity may be lost in dilute solutions as a result of dissociation of the holoenzyme into its constituent polypeptides.

Although enzyme activity could be routinely purified by the method described, periods of anomalous behaviour were recorded during which the cyanide-degrading activity from 40 ml of cell-free extract could not be recovered from the anion-exchange column. Flushing the column with a 1 M NaCl solution (in which the enzyme is active) did not elute the activity. Furthermore, the yield of activity after anion-exchange chromatography varied. Table 3.1 shows that approximately 80 % of the cyanide-degrading activity was lost during the anion-

exchange step. Earlier work showed that the recovery of cyanide-degrading activity during this step was as high as 70 %.

Dialysis of the purified enzyme against low-ionic-strength solutions, such as 1 mM Tris-HCl 10 mM NaCl (pH 8.0), resulted in the loss of at least 80 % of enzymatic activity. Although low-ionic-strength solutions reduced enzymatic activity, increasing the ionic strength by adding 10 mM Tris-HCl 50 mM NaCl (pH 8.0) actually increased enzymatic activity despite diluting the enzyme (Table 3.2). Thus increasing the ionic composition of the buffer from 1.9 mM Tris-HCl 14 mM NaCl to 5.5 mM Tris-HCl 30 mM NaCl resulted in significantly higher activity despite diluting the enzyme 1-in-2. Although the 1-in-5 dilution of the enzyme had the highest ionic strength, and still exhibited higher activity than the undiluted enzyme, this activity was slightly lower than that of the 1-in-2-diluted sample demonstrating the effect of a lower enzyme concentration. This suggested that partial restoration of enzymatic activity was possible after exposure to conditions of low ionic strength.

Although separation of the constituent polypeptide chains could be achieved by SDS-PAGE, only the middle band of the three protein bands could be recovered from the gel by electroelution. This polypeptide was subjected to gas-phase  $\text{NH}_2$ -terminal sequencing and the following amino-acid sequence was generated:

Thr Ser Ile Tyr Pro Lys Phe X Ala Ala X Val Gln.

C18 reverse-phase HPLC in 0.1 % trifluoroacetic acid was unsuccessful in separating the protein into its constituent polypeptide chains with all the bands co-eluting at 60 % acetonitrile. Interestingly, when the entire protein was subjected to gas-phase sequencing only one sequence was generated:

Thr Ser Ile Tyr Pro Lys <sup>Lys</sup>Phe Phe Ala Ala X.  
Ala

Although there was some uncertainty about the identity of the amino acid at position 7, the  $\text{NH}_2$ -terminal sequence for the whole enzyme appeared to be identical to that generated for the middle protein band alone. Possible explanations for the generation of only a single sequence are that two of the three polypeptide chains are blocked at the amino terminus, that the three chains arose by gene duplication and have identical amino termini, or that the three chains

are transcribed from the same gene but are subjected to different post-translational modifications.

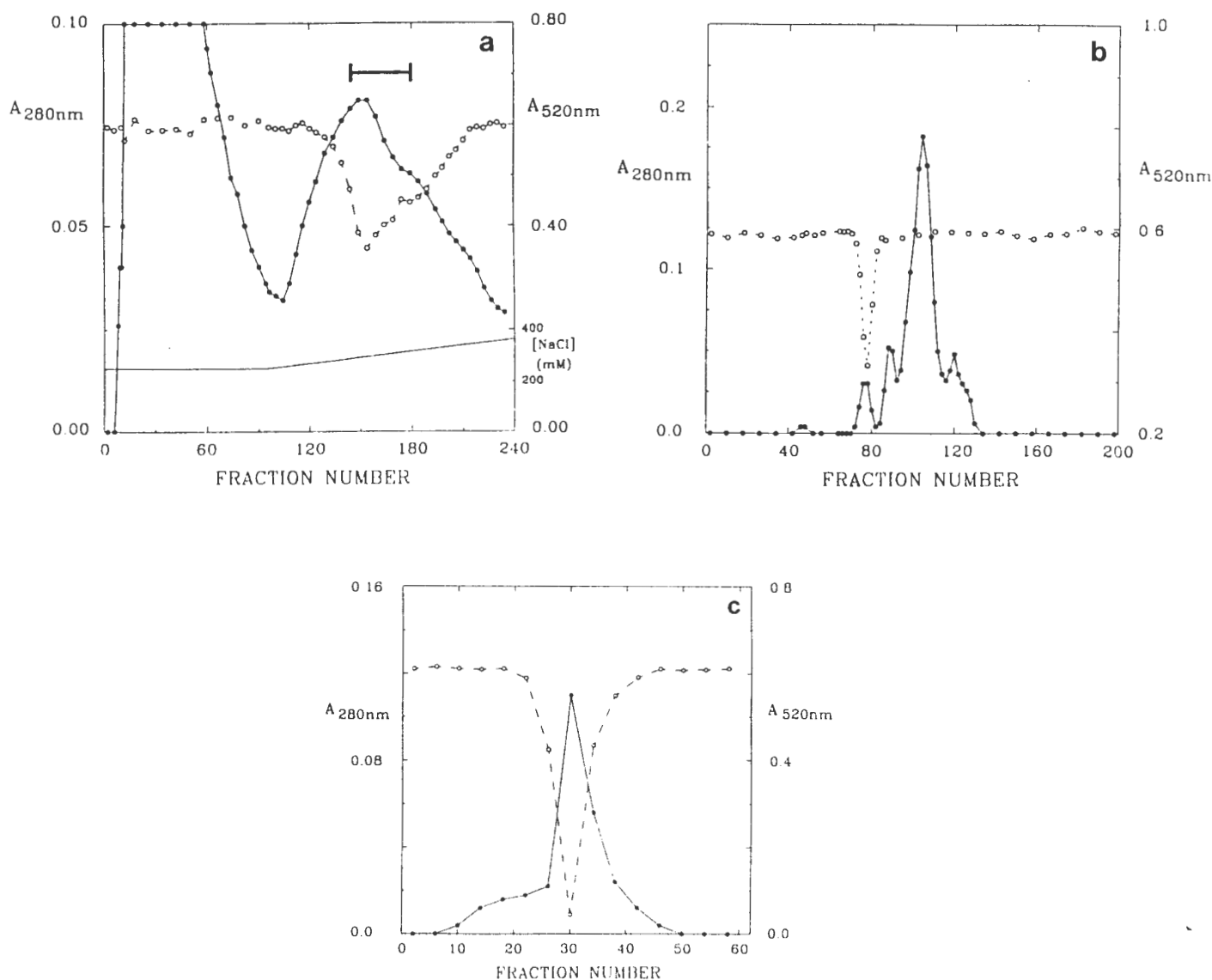


**Fig.3.1 (a)-(c) Purification of the cyanide-degrading enzyme from *B. pumilus* C1 by column chromatography and sucrose-gradient centrifugation. (a) Anion-exchange chromatography of cell-free extract. Active fractions (144 to 179, bar) were pooled, concentrated by ultrafiltration and applied to the Bio-Gel A-5m column. (b) Gel-filtration chromatography of the protein concentrate from the anion-exchange column. Active fractions (63 to 76) were pooled, concentrated by ultrafiltration and applied to a sucrose gradient. (c) Sucrose-gradient centrifugation of the protein concentrate from the gel-filtration column. Protein, (●); activity, (○). Experimental methods are described in section 3.3.6.**

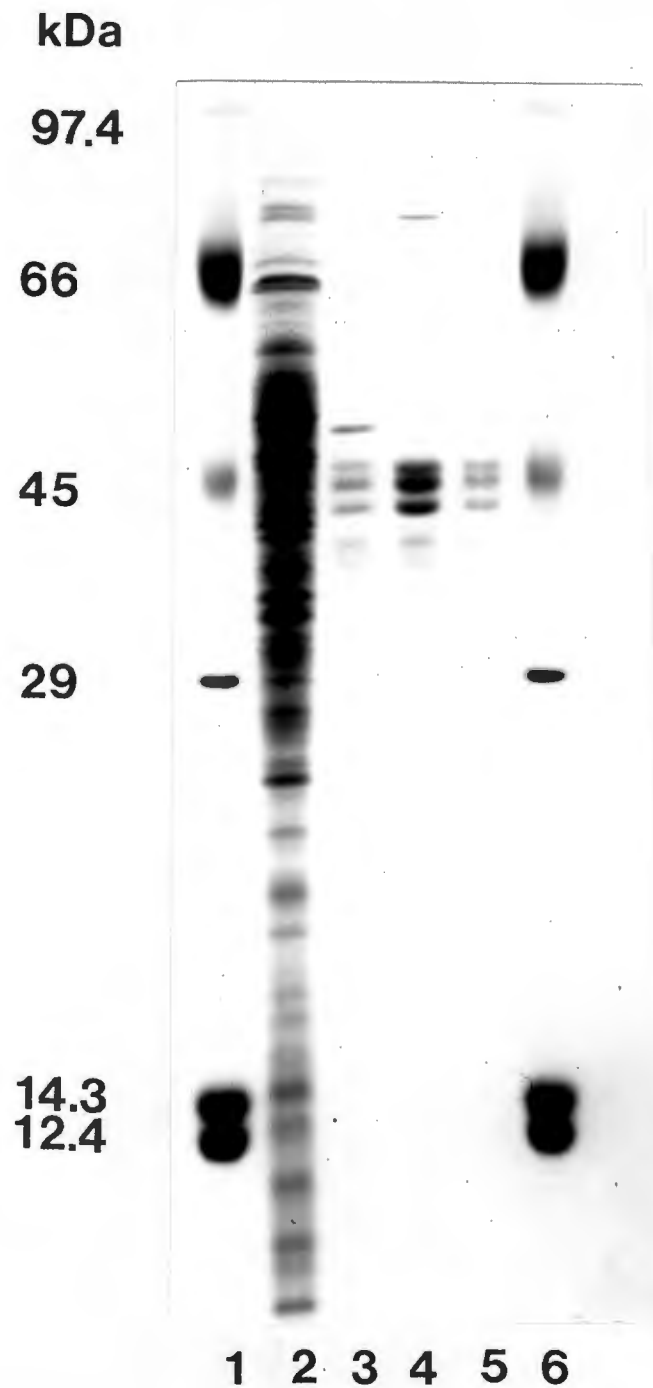


**Fig.3.1 (d) SDS-PAGE of the steps in the purification of the cyanide-degrading enzyme. Lanes 1 & 6, molecular-weight markers (in order of decreasing molecular weight: phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, lysozyme, and cytochrome c); lane 2, cell-free extract; lane 3, protein concentrate from DE52 anion-exchange column; lane 4, protein concentrate from A-5m gel-filtration column; lane 5, pooled active fractions from sucrose gradient.**

Table 3.1 Purification table for the cyanide-degrading enzyme from *B. pumilus* C1.

| PURIFICATION STEP | VOLUME<br>(ml)   | TOTAL<br>PROTEIN<br>(mg) | TOTAL<br>ACTIVITY<br>(U) | SPECIFIC<br>ACTIVITY<br>(U/mg) | FOLD<br>PURIFICATION | % YIELD |
|-------------------|------------------|--------------------------|--------------------------|--------------------------------|----------------------|---------|
| CELL-FREE EXTRACT | 15               | 225.0                    | 272.9                    | 1.2                            | 1                    | 100     |
| ANION EXCHANGE    | 5.4 <sup>a</sup> | 3.0                      | 48.6                     | 16.4                           | 13.5                 | 17.8    |
| GEL FILTRATION    | 1.4 <sup>b</sup> | 0.7                      | 20.7                     | 30.0                           | 24.8                 | 7.6     |
| SUCROSE GRADIENT  | 1.8              | 0.1                      | 6.3                      | 50.9                           | 41.9                 | 2.3     |

<sup>a</sup> Volume of pooled, concentrated active fractions from the DE52 column.

<sup>b</sup> Volume of pooled, concentrated active fractions from the A-5m column.

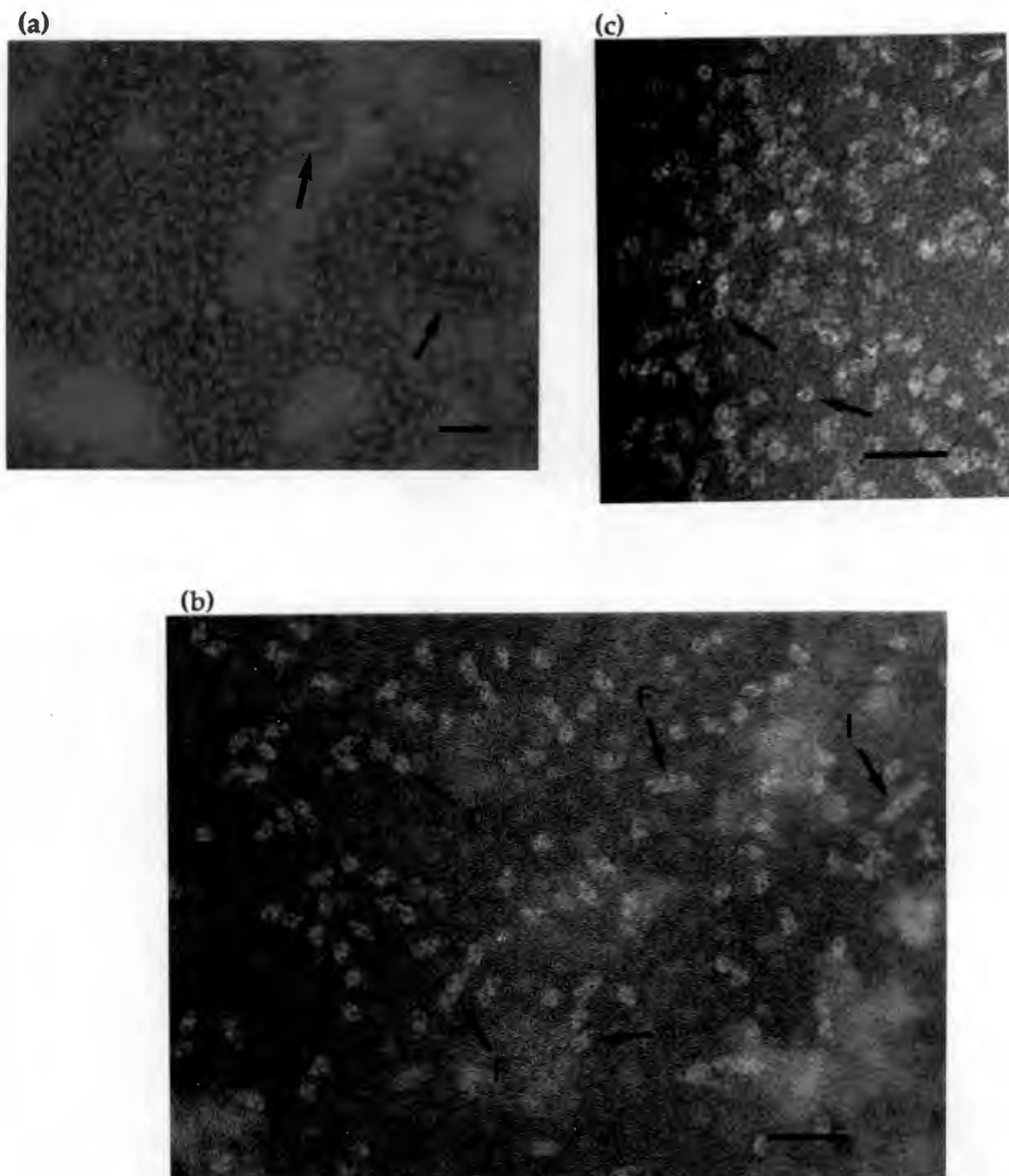


**Fig.3.2** Electron microscopy of phosphotungstate-stained cyanide dihydratase after sucrose-gradient purification. The bar in each micrograph represents 50 nm. (a) An area of the grid is shown that contained several tightly packed spiral structures (arrowed). Magnification:  $\times 151\,500$ . (b) An area of the grid is shown that contained predominantly fragmented structures (F→) although one intact tightly packed spiral structure (I→) is visible. Monomers ( $\odot$ →) and a hexameric array of monomeric particles are also visible. These latter structures may have arisen by fragmentation of the putative native tightly packed spiral structure; one such hexamer is arrowed (→). Magnification:  $\times 244\,000$ . (c) An area of the grid is shown that contained predominantly monomeric structures. Three such monomers are arrowed. Magnification:  $\times 247\,000$

**Table 3.2** The influence of ionic strength on cyanide-degrading activity. The enzyme was dialysed against 1 mM Tris-HCl 10 mM NaCl (pH 8.0) and then diluted in 10 mM Tris-HCl 50 mM NaCl (pH 8.0). Cyanide was added in 10 mM Tris-HCl 50 mM NaCl (pH 8.0). Concentrations of Tris-HCl and NaCl were calculated for the samples after the addition of cyanide. Activity was measured as the decrease in absorbance at 520 nm ( $\Delta A_{520\text{nm}}$ ) in the cyanide assay.

| IONIC COMPOSITION OF<br>ASSAY SOLUTION | ENZYME<br>DILUTION | $\Delta A_{520\text{nm}}$ |
|----------------------------------------|--------------------|---------------------------|
| 1.9 mM Tris-HCl 14 mM NaCl             | Undiluted          | 0.088                     |
| 5.5 mM Tris-HCl 30 mM NaCl             | 1-in-2             | 0.327                     |
| 8.2 mM Tris-HCl 42 mM NaCl             | 1-in-5             | 0.267                     |

### 3.4.2 DEGRADATION OF CYANIDE AND RELATED COMPOUNDS

Since the ultimate sucrose-gradient purification step increased the specific activity only 1.7-fold but resulted in the loss of 70 % of the material, most characterization and all kinetic experiments were carried out using the A-5m column eluate. The purified cyanide-degrading enzyme was shown to function as a nitrilase, converting cyanide to formate and ammonia according to the equation:



The production of formate (assayed by the formate-dehydrogenase-catalysed reduction of  $\text{NAD}^+$ ) was equivalent to 83.8 % of the decrease in the cyanide concentration. Although ammonia was present after cyanide degradation the amount detected was not stoichiometric with the amount of formate produced, suggesting that most of the ammonia was stripped from solution (only about 6 % of the expected amount of ammonia was detected). Degradation of  $\text{K}^{14}\text{CN}$  resulted in > 95 % of the radioactivity remaining in solution thereby eliminating  $\text{CO}_2$  as a reaction product (Table 3.3). The increase in the average counts per minute during the course of the experiment is probably due to pipetting error as 1- $\mu\text{l}$  samples were used for counting the radioactivity.

**Table 3.3** The fate of the radiolabel from  $K^{14}CN$  after enzymatic degradation of the cyanide. Experimental details are provided in section 3.3.11.

| STAGE IN EXPERIMENT                                 | AVERAGE COUNTS PER MINUTE |
|-----------------------------------------------------|---------------------------|
| BEFORE ADDITION OF ENZYME                           | 17 035 $\pm$ 322          |
| AFTER INCUBATION WITH ENZYME                        | 19 713 $\pm$ 1670         |
| AFTER INCUBATION AND 1 h UNCOVERED IN FUME CUPBOARD | 20 145 $\pm$ 1396         |

Since the addition of only one molecule of water to cyanide would result in the formation of formamide, the relationship between the cyanide-degrading enzyme and formamide was investigated. A small quantity of formamide (0.4 mM, corrected for absorbance due to enzyme protein alone) was detected after cyanide degradation; this was equivalent to 11 % of the amount of cyanide degraded. The assay detected 4 mM formamide in the enzyme control containing 3.9 mM formamide, indicating some inaccuracy in the assay. Despite the small amount of formamide detected after cyanide degradation, the purified enzyme was unable to degrade formamide and the presence of formamide during the degradation of cyanide had no effect on the rate of cyanide removal (Table 3.4). The enzyme was also unable to hydrolyze cyanate ( $CNO^-$ ) and thiocyanate ( $SCN^-$ ), and displayed no activity towards azide. No ammonia could be detected during incubation of the enzyme with 10 mM acetonitrile, suggesting that this compound does not serve as a substrate for the enzyme. Whole cells of *B. pumilus* C1 did not display any activity towards the metal-cyanide complexes, ferric cyanide and manganese cyanide (Pravin Gokool, National Chemical Products, personal communication). The enzyme was tentatively named cyanide dihydratase to indicate its substrate specificity and mode of action.

The effect of the presence of thiocyanate on cyanide degradation was investigated to determine whether this structurally similar compound was a competitive inhibitor. Surprisingly, thiocyanate concentrations of up to 20 mM

slightly enhanced enzymatic activity; above this concentration thiocyanate inhibited the enzyme (Table 3.5).

The kinetics of cyanide degradation by the cyanide dihydratase could be described using the Michaelis-Menten equation. The initial velocity of cyanide degradation by cyanide dihydratase in 10 mM Tris-HCl 50 mM NaCl 1 mM EDTA pH 8.0 at 37 °C was investigated as described in section 3.3.16. A Lineweaver-Burk plot (Fig. 3.3) showed a linear relationship between substrate concentration and reaction velocity over the range of concentrations tested; the Michaelis constant ( $K_m$ ) was  $2.56 \pm 0.48$  mM and the maximal velocity ( $V_{max}$ ) was  $88.03 \pm 4.67$  mmol/min/mg protein/l.

**Table 3.4** The effect of formamide concentration on cyanide degradation by the *B. pumilus* C1 enzyme. Formamide was added to a constant amount of enzyme to the indicated final concentrations and the samples were prewarmed at 37 °C for 5 min. Cyanide was added to a final concentration of 4 mM and incubation was continued for 1 h at 37 °C. The decrease in absorbance at 520 nm ( $\Delta A_{520nm}$ ) of each sample (relative to the appropriate formamide-containing controls) was expressed as a percentage of the  $\Delta A_{520nm}$  of an enzyme control containing no formamide.

| FORMAMIDE<br>CONCENTRATION (mM) | PERCENTAGE<br>ACTIVITY |
|---------------------------------|------------------------|
| 0                               | 100                    |
| 2                               | 112.8                  |
| 4                               | 105.8                  |
| 8                               | 105.5                  |
| 16                              | 105.5                  |
| 32                              | 108.3                  |

**Table 3.5** The effect of thiocyanate concentration on cyanide dihydratase activity. Experimental details are provided in section 3.3.9.

| THIOCYANATE<br>CONCENTRATION (mM) | PERCENTAGE<br>ACTIVITY |
|-----------------------------------|------------------------|
| 0                                 | 100                    |
| 2.5                               | 114.7                  |
| 5                                 | 113.1                  |
| 10                                | 110.4                  |
| 20                                | 104.0                  |
| 40                                | 99.2                   |
| 80                                | 92.4                   |
| 160                               | 62.5                   |

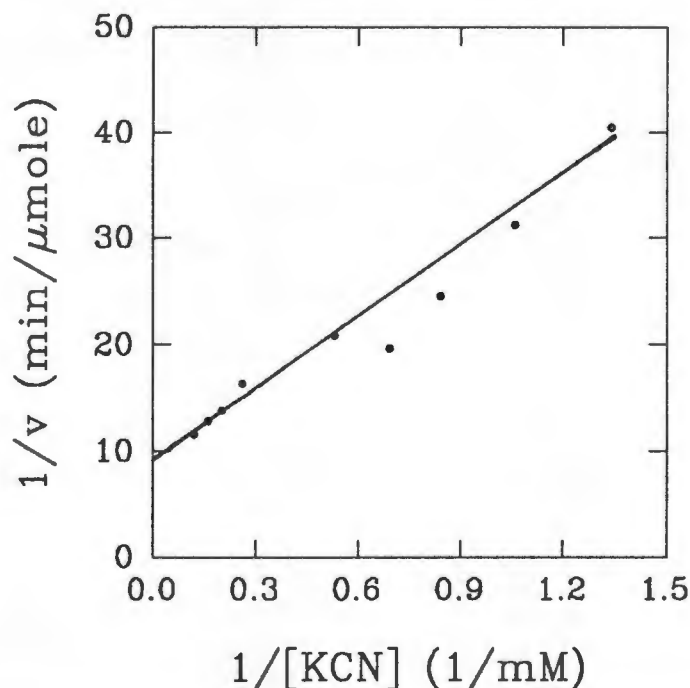


**Fig. 3.3** Lineweaver-Burk plot of the reciprocals of initial velocity versus substrate concentration for cyanide dihydratase in the presence of 1 mM EDTA with cyanide as substrate (at 37 °C, pH 8.0; total protein = 0.0025 mg, reaction volume = 500  $\mu$ l). The data shown are the averages of two independent experiments with all measurements carried out in duplicate. A Bio-Gel A-5m eluate enzyme preparation was used for these determinations. Experimental details are provided in section 3.3.16.

### 3.4.3 REQUIREMENT FOR $Mn^{2+}$ AND THE EFFECTS OF OTHER METAL IONS

*B. pumilus* C1 required manganous ions in the growth medium for the production of cyanide-degrading activity, with maximal activity being exhibited when at least 0.01 mg  $Mn^{2+}$ /l (0.18  $\mu M$   $Mn^{2+}$ ) was present; the presence of  $Mn^{2+}$  was also required for the bacterium to grow well (section 2.4.4). To investigate whether cyanide dihydratase was a manganese-containing metallo-enzyme the purified enzyme was dialysed twice against 5 l 10 mM Tris-HCl 50 mM NaCl 5 mM EDTA, pH 7.4, and once against 10 mM Tris-HCl 50 mM NaCl 0.1 mM EDTA, pH 7.4, to remove any bound metal ions. This treatment resulted in a small decrease in enzymatic activity. Re-addition of  $Mn^{2+}$  to the dialysed enzyme restored full enzyme activity (Table 3.6). Similar results were obtained when the experiment was repeated using the stronger chelating agent, diethylenetriamine penta-acetic acid (DTPA) at 2 mM, and then 0.1 mM. While this suggested some involvement of manganous ions in enzymatic activity, purification of cyanide dihydratase from bacteria grown in the presence of  $^{54}Mn^{2+}$  showed that despite the uptake of 97.7 % (89.3  $\mu Ci$ ) of the isotope by the bacteria, no radioactivity was associated with the purified enzyme (as determined using a Packard 5220 Auto-Gamma Scintillation Spectrometer and autoradiography of the electrophoresed purified enzyme) (Fig. 3.4).

Since a wide variety of metal ions are known to interact with cyanide, forming both simple salts and multi-ligand complexes, the effect of some metal ions on the rate of cyanide degradation by cyanide dihydratase was investigated. Table 3.7 summarizes the results for the various metal ions tested. It was clear that the trivalent metal cations  $Sc^{3+}$ ,  $Fe^{3+}$ ,  $Cr^{3+}$  and  $Tb^{3+}$  all enhanced enzymatic activity approximately 2-fold whereas  $Hg^{2+}$  and to a lesser extent  $Pb^{2+}$  and  $Ag^{+}$  were inhibitory. A comparison of the effects of  $Hg^{2+}$  and  $Pb^{2+}$  at concentrations below 200  $\mu M$  (Table 3.8) showed that  $Hg^{2+}$  was a potent inhibitor of enzymatic activity even at low concentrations. Inhibition by  $Hg^{2+}$  suggested the presence of a sulphydryl moiety at or near the active site. However, inactivation of the enzyme by chemical modification of cysteinyl residues with iodoacetic acid or 4-vinylpyridine could not be demonstrated. Table 3.9 shows that the presence of a 5-fold molar excess of  $\beta$ -mercaptoethanol over  $Hg^{2+}$  protected the enzyme from the effects of  $Hg^{2+}$ . Since 200  $\mu M$   $Cr^{3+}$  caused a significant enhancement of enzymatic activity, the relationship between this metal ion and cyanide dihydratase was investigated.

**Table 3.6** The effect of  $\text{Mn}^{2+}$  on the activity of purified cyanide dihydratase before and after dialysis against 5 mM EDTA.

| TREATMENT                                                 | PERCENTAGE ACTIVITY |
|-----------------------------------------------------------|---------------------|
| UNDIALYSED ENZYME                                         | 100                 |
| UNDIALYSED ENZYME + 1 mM $\text{Mn}^{2+}$                 | 104.9               |
| EDTA-DIALYSED ENZYME                                      | 86.9                |
| EDTA-DIALYSED ENZYME + 1 mM $\text{Mn}^{2+}$ (free metal) | 101.6               |

**Fig. 3.4 (a)**

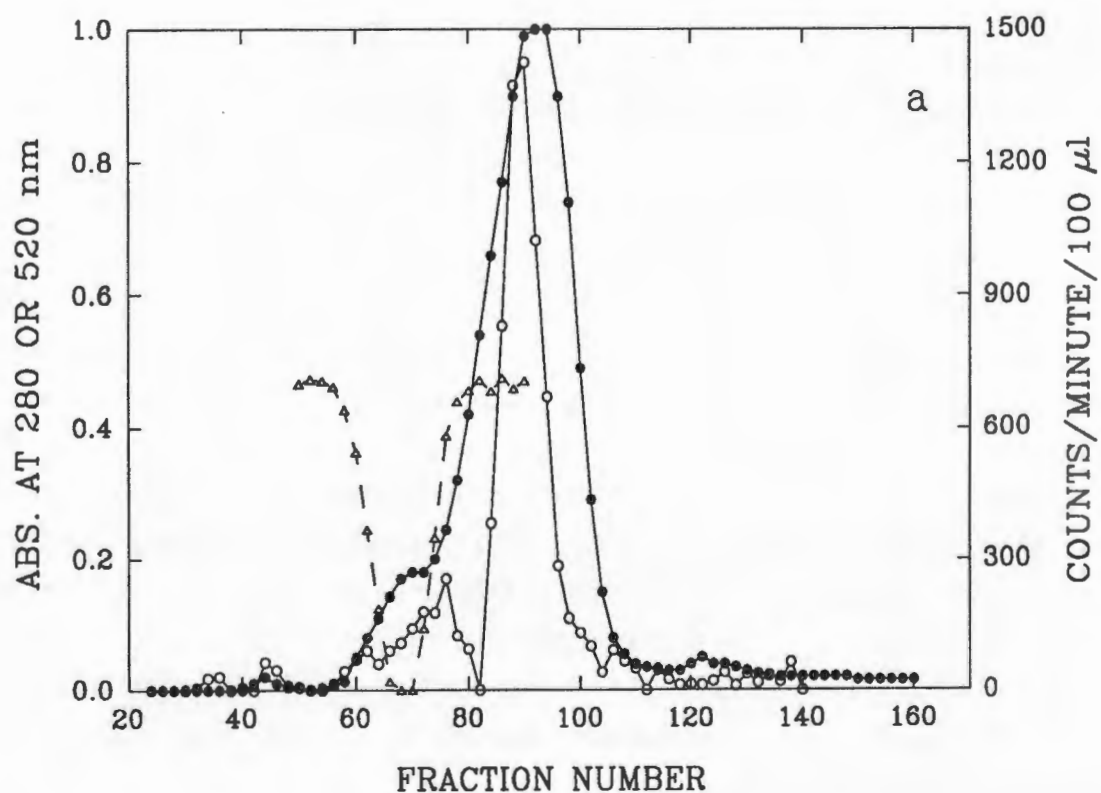


Fig. 3.4 (b)

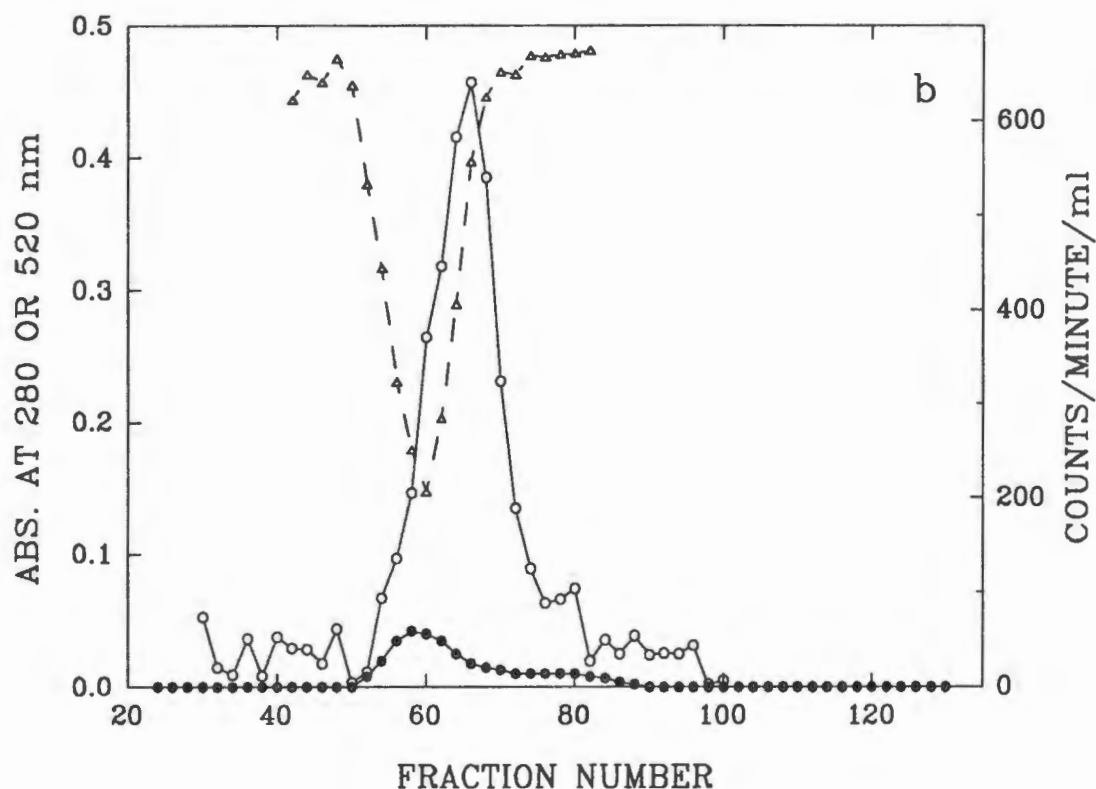


**Fig.3.4** Protein, activity, and radioactivity profiles of the cyanide dihydratase after purification from *B. pumilus* C1 cultures grown in the presence of  $^{54}\text{Mn}^{2+}$ . Activity was measured as the decrease in absorbance at 520 nm in the standard column-fraction assay described in section 3.3.6. (a) Profiles from the gel-filtration column. (b) Active fractions from the column in (a) were pooled and re-applied to the same column. Protein, (●); activity, (Δ); and radioactivity, (○).

Enzyme activity was assayed over a range of  $\text{Cr}^{3+}$  concentrations (1 - 500  $\mu\text{M}$ ) to determine if the enhancing effect of these ions was catalytic (implying a direct involvement of the ions in enzymatic catalysis) or chemical (implying enhanced cyanide removal by an indirect mechanism dependent on the chromium concentration). As little as 5  $\mu\text{M}$   $\text{Cr}^{3+}$  was sufficient for maximal enhancement of enzymatic activity when freshly purified enzyme was used in the assay (Fig. 3.5). However, the amount of  $\text{Cr}^{3+}$  required for maximal enhancement was higher when the enzyme preparation was not fresh. Chromium at 0.026 mg  $\text{Cr}^{3+}$ /l (0.48  $\mu\text{M}$   $\text{Cr}^{3+}$ ) and  $\text{Fe}^{3+}$  at 0.028 mg  $\text{Fe}^{3+}$ /l (0.50  $\mu\text{M}$   $\text{Fe}^{3+}$ ) could not replace  $\text{Mn}^{2+}$  as the inducer of cyanide-degrading activity during overnight growth of *B. pumilus* C1 (Table 3.10).

**Table 3.7 The effects of various metal ions on cyanide dihydratase activity. Metal ions were added to a final concentration of 200  $\mu\text{M}$ . The experimental method is described in section 3.3.10.**

| METAL ION        | PERCENTAGE ACTIVITY |
|------------------|---------------------|
| None             | 100                 |
| $\text{Sc}^{3+}$ | 190.1               |
| $\text{Fe}^{3+}$ | 189.6               |
| $\text{Cr}^{3+}$ | 177.0               |
| $\text{Tb}^{3+}$ | 174.2               |
| $\text{Co}^{2+}$ | 86.9                |
| $\text{Cd}^{2+}$ | 65.5                |
| $\text{Hg}^{2+}$ | 6.7                 |
| $\text{Pb}^{2+}$ | 23.0                |
| $\text{Ag}^{+}$  | 53.8                |

**Table 3.8 Inhibition of cyanide dihydratase activity by low concentrations of  $\text{Hg}^{2+}$  and  $\text{Pb}^{2+}$ . Samples were not dialysed against EDTA before addition of the metal ions.**

| METAL                              | PERCENTAGE ACTIVITY |
|------------------------------------|---------------------|
| No metal added                     | 100                 |
| 10 $\mu\text{M}$ $\text{Hg}^{2+}$  | 5.0                 |
| 10 $\mu\text{M}$ $\text{Pb}^{2+}$  | 63.7                |
| 100 $\mu\text{M}$ $\text{Pb}^{2+}$ | 53.8                |

**Table 3.9** Protection of cyanide dihydratase activity from the effects of  $\text{Hg}^{2+}$  by  $\beta$ -mercaptoethanol. Samples were incubated on ice for 30 min after the addition of  $\beta$ -mercaptoethanol,  $\text{Hg}^{2+}$ , or both ( $\beta$ -mercaptoethanol was added to the samples before  $\text{Hg}^{2+}$  was added). All samples were allowed to warm up at room temperature for 10 min and were then prewarmed at 37 °C for 6 min before adding cyanide and incubating at 37 °C for a further 1 h. Activity was expressed as the decrease in absorbance at 520 nm ( $\Delta A_{520\text{nm}}$ ) in each sample (relative to the appropriate  $\beta$ -mercaptoethanol- and  $\text{Hg}^{2+}$ -containing controls). The  $\Delta A_{520\text{nm}}$  of the enzyme control (no additions) was taken as 100 % activity.

| ENZYME TREATMENT                                                                   | PERCENTAGE ACTIVITY |
|------------------------------------------------------------------------------------|---------------------|
| No additions                                                                       | 100                 |
| + 10 $\mu\text{M}$ $\text{Hg}^{2+}$                                                | 2.9                 |
| + 20 $\mu\text{M}$ $\beta$ -Mercaptoethanol                                        | 105.8               |
| + 50 $\mu\text{M}$ $\beta$ -Mercaptoethanol                                        | 100.5               |
| + 20 $\mu\text{M}$ $\beta$ -Mercaptoethanol<br>+ 10 $\mu\text{M}$ $\text{Hg}^{2+}$ | 5.3                 |
| + 50 $\mu\text{M}$ $\beta$ -Mercaptoethanol<br>+ 10 $\mu\text{M}$ $\text{Hg}^{2+}$ | 100.5               |

To investigate whether cyanide dihydratase was a chromium-containing metallo-enzyme, the enzyme was purified from *B. pumilus* C1 cells grown in Luria broth containing  $^{51}\text{Cr}^{3+}$ . Although the cells took up 48 % (42.1  $\mu\text{Ci}$ ) of the radioactivity, there was no radioactivity associated with the purified enzyme as measured in a Packard 5220 Auto-Gamma Scintillation Spectrometer (Fig. 3.6). Moreover, the purified enzyme failed to bind  $^{51}\text{Cr}^{3+}$  by equilibrium dialysis (Table 3.11).

Analysis of the catalytic parameters for the enzyme in the presence of 10  $\mu\text{M}$   $\text{Cr}^{3+}$  (Fig. 3.7) showed that the kinetics of cyanide degradation could still be described using the Michaelis-Menten equation. The  $K_m$  for cyanide increased by a factor of 2.1 to  $5.28 \pm 0.38$  mM cyanide, indicating a decreased affinity of the enzyme for its substrate in the presence of 10  $\mu\text{M}$   $\text{Cr}^{3+}$ . Under these conditions

the  $V_{\max}$  increased by a factor 2.2 to  $197.11 \pm 8.51$  mmol cyanide/min/mg protein/l.

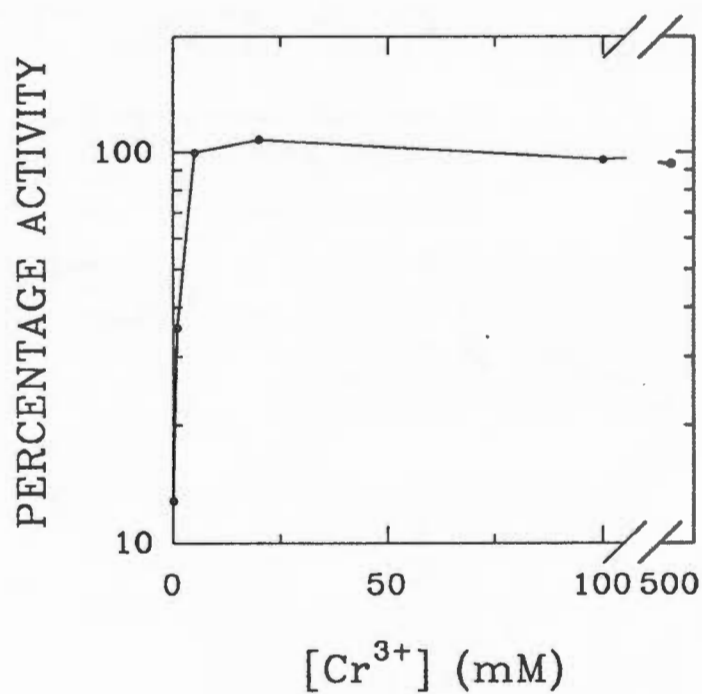


Fig. 3.5 Plot of cyanide dihydratase activity as a function of  $[\text{Cr}^{3+}]$ .

**Table 3.10** The effect of  $\text{Cr}^{3+}$  and  $\text{Fe}^{3+}$  on the production of cyanide-degrading activity during overnight growth of *B. pumilus* C1 in Oxoid nutrient broth prepared in Millipore water. Cultures were supplemented with  $\text{Cr}^{3+}$  or  $\text{Fe}^{3+}$  to the concentrations indicated in the text (or with  $\text{Mn}^{2+}$  to 0.014 mg  $\text{Mn}^{2+}$ /l) and grown overnight at 30 °C with shaking. Cultures were harvested by centrifugation, resuspended in 10 ml sterile Millipore water and prewarmed at 37 °C for 10 min. Cyanide was added to each sample and incubation at 37 °C was continued for a further 10 min, after which 140  $\mu\text{l}$  of cell suspension was removed from each sample and microfuged for 1.5 min. Duplicate 50- $\mu\text{l}$  volumes of each supernatant were used for the cyanide assay. Activity was expressed as the decrease in absorbance at 520 nm ( $\Delta A_{520\text{nm}}$ ) relative to a control containing only water and cyanide. To compare the activities of cultures containing different cell densities, the  $\Delta A_{520\text{nm}}$  value for each culture was divided by the optical density at 600 nm of that culture (column 4).

| CULTURE MEDIUM                                               | OPTICAL DENSITY AT 600 nm | DECREASE IN CYANIDE ( $\Delta A_{520\text{nm}}$ ) | $\Delta A_{520\text{nm}} \div \text{OD}_{600\text{nm}}$ | PERCENTAGE ACTIVITY |
|--------------------------------------------------------------|---------------------------|---------------------------------------------------|---------------------------------------------------------|---------------------|
| OXOID BROTH IN MILLIPORE WATER (A)                           | 1.452                     | 0.007                                             | 0.005                                                   | 3.5                 |
| (A) + $\text{Mn}^{2+}$                                       | 3.280                     | 0.468                                             | 0.143                                                   | 100                 |
| (A) + $\text{Fe}^{3+}$                                       | 1.484                     | 0.002                                             | 0.001                                                   | 0.7                 |
| (A) + $\text{Cr}^{3+}$                                       | 1.464                     | 0.008                                             | 0.005                                                   | 3.5                 |
| (A) + $\text{Mn}^{2+}$ + $\text{Fe}^{3+}$                    | 3.324                     | 0.375                                             | 0.113                                                   | 79.0                |
| (A) + $\text{Mn}^{2+}$ + $\text{Cr}^{3+}$                    | 3.332                     | 0.434                                             | 0.130                                                   | 90.9                |
| (A) + $\text{Fe}^{3+}$ + $\text{Cr}^{3+}$                    | 1.528                     | 0.000                                             | 0                                                       | 0                   |
| (A) + $\text{Mn}^{2+}$ + $\text{Fe}^{3+}$ + $\text{Cr}^{3+}$ | 3.392                     | 0.369                                             | 0.109                                                   | 76.2                |

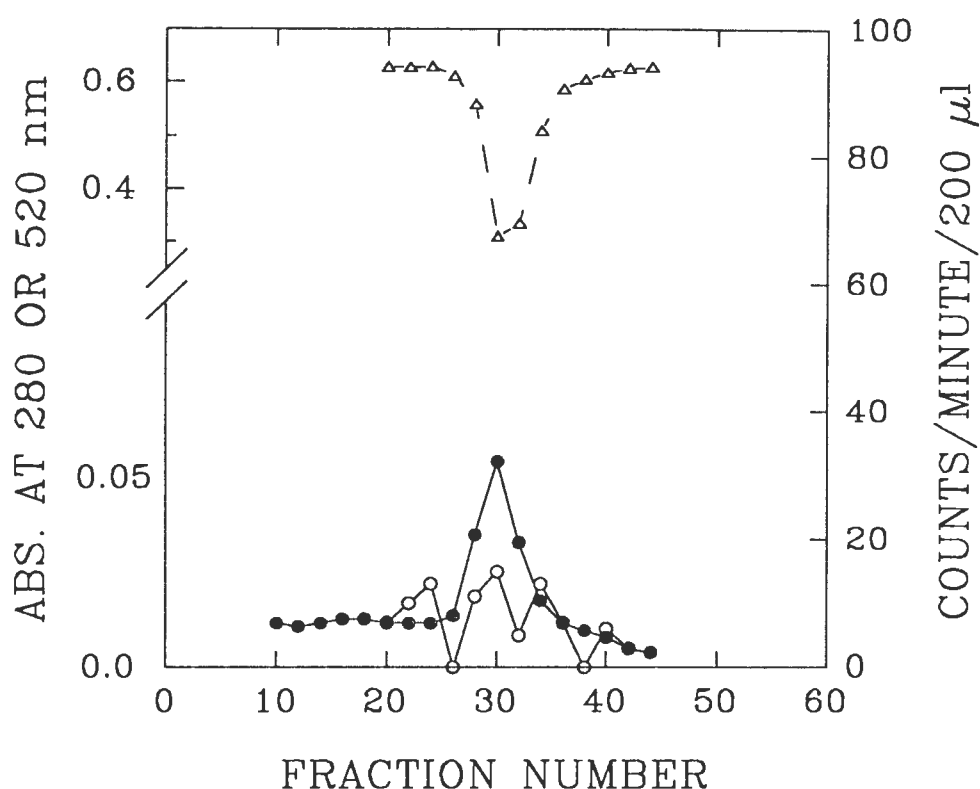


Fig.3.6 Protein, activity, and radioactivity profiles after purification of the cyanide dihydratase from *B. pumilus* C1 cultures grown in the presence of  $^{51}\text{Cr}^{3+}$ . Experimental details are provided in section 3.3.6. Activity was measured as the decrease in absorbance at 520 nm in the standard column-fraction assay described in section 3.3.6. Protein, (●); activity, (Δ); and radioactivity, (○).

Table 3.11 Comparison of the radioactivity associated with the purified cyanide dihydratase and the buffer after dialysis of 1 ml of purified enzyme (0.13 mg protein) against 500 ml of 10 mM Tris-HCl 50 mM NaCl buffer (pH 8.0) containing 9.2 nCi  $^{51}\text{Cr}^{3+}$ /ml. Dialysis was performed at 4 °C for 17 h. The radioactivity in duplicate 100-μl samples was determined using a Beckman LS1701 Liquid Scintillation System.

| STAGE IN EXPERIMENT                                         | AVERAGE COUNTS PER MINUTE |
|-------------------------------------------------------------|---------------------------|
| $^{51}\text{Cr}^{3+}$ -CONTAINING BUFFER<br>BEFORE DIALYSIS | 491 ± 23                  |
| AFTER DIALYSIS: INSIDE<br>DIALYSIS BAG                      | 489 ± 7                   |
| OUTSIDE<br>DIALYSIS BAG                                     | 477 ± 18                  |

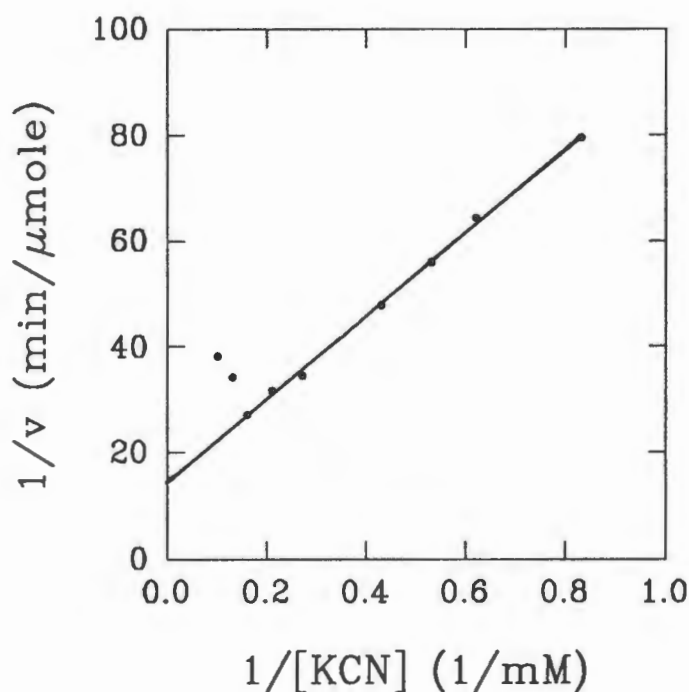


**Fig. 3.7** Lineweaver-Burk plot of the reciprocals of initial velocity versus substrate concentration for the cyanide dihydratase in the presence of  $10 \mu\text{M}$   $\text{Cr}^{3+}$  with cyanide as substrate (at  $37^\circ\text{C}$ , pH 8.0; total protein =  $0.00069 \text{ mg}$ , reaction volume =  $500 \mu\text{l}$ ). The data shown are the averages of two independent experiments with all measurements carried out in duplicate. A Bio-Gel A-5m eluate enzyme preparation was used for these determinations. Experimental details are provided in section 3.3.16.

### 3.4.4 CHARACTERIZATION

Optimal enzymatic activity was shown to occur between pH 7.8 and 8.0 (Fig. 3.8); increasing the pH above pH 8.0 had a dramatic effect on the activity with no activity being exhibited above pH 8.4. The presence of the trivalent metal ions  $\text{Cr}^{3+}$  or  $\text{Tb}^{3+}$  at a concentration of  $200 \mu\text{M}$ , however, protected the enzyme against the inhibitory effect of increasing the pH (Table 3.12). Thus the enzyme displayed a similar level of activity in the presence of these metal ions at pH 8.0 and pH 8.6.

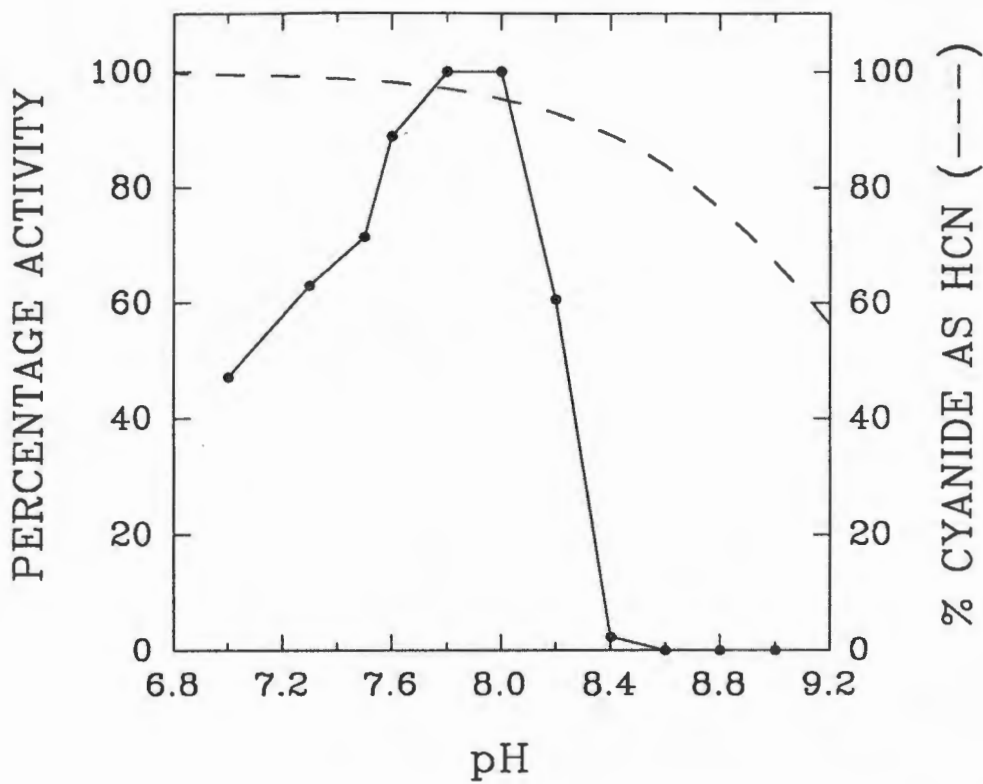


Fig. 3.8 Activity of the cyanide dihydratase with increasing pH. The dashed line represents the calculated proportion of cyanide in the form of HCN as a function of pH. Experimental details are provided in section 3.3.7.

Table 3.12 Comparison of the activity of cyanide dihydratase at pH 8.0 and 8.6 in the absence of added metal ions, and in the presence of 200  $\mu\text{M}$   $\text{Cr}^{3+}$  or  $\text{Tb}^{3+}$ . The decrease in absorbance at 520 nm ( $\Delta A_{520\text{nm}}$ ) of the enzyme control at each pH (relative to the appropriate buffer controls) was taken as 100 % activity. The  $\Delta A_{520\text{nm}}$  value for the metal-containing samples at each pH (relative to appropriate metal-containing buffer controls) was expressed as a percentage of the  $\Delta A_{520\text{nm}}$  value for the enzyme controls.

| ENZYME<br>TREATMENT                      | pH 8.0                    |            | pH 8.6                    |            |
|------------------------------------------|---------------------------|------------|---------------------------|------------|
|                                          | $\Delta A_{520\text{nm}}$ | % ACTIVITY | $\Delta A_{520\text{nm}}$ | % ACTIVITY |
| ENZYME<br>CONTROL<br>(No metal<br>added) | 0.342                     | 100        | 0.044                     | 100        |
| + 200 $\mu\text{M}$<br>$\text{Cr}^{3+}$  | 0.411                     | 120.2      | 0.420                     | 954.5      |
| + 200 $\mu\text{M}$<br>$\text{Tb}^{3+}$  | 0.411                     | 120.2      | 0.307                     | 697.7      |

The optimum temperature for cyanide degradation was shown to be 37 °C (Fig. 3.9); activity was rapidly lost above 40 °C. The enzyme was most stable at about 22 °C, a temperature considerably lower than the activity optimum (Fig. 3.10). Enzymatic activity was reduced to 50 % by 0.5 M urea, and was completely inhibited by 0.02 % sodium dodecyl sulphate. The enzyme activity was unaffected by NaCl concentrations of up to 2.94 M. Dialysis of the purified enzyme (in 10 mM Tris-HCl 50 mM NaCl, pH 8.0) against buffers of different composition showed that the activity was not significantly different in the Tris-HCl and borate buffers, but appeared to decrease in the phosphate, glycine and triethanolamine buffers (Table 3.13).

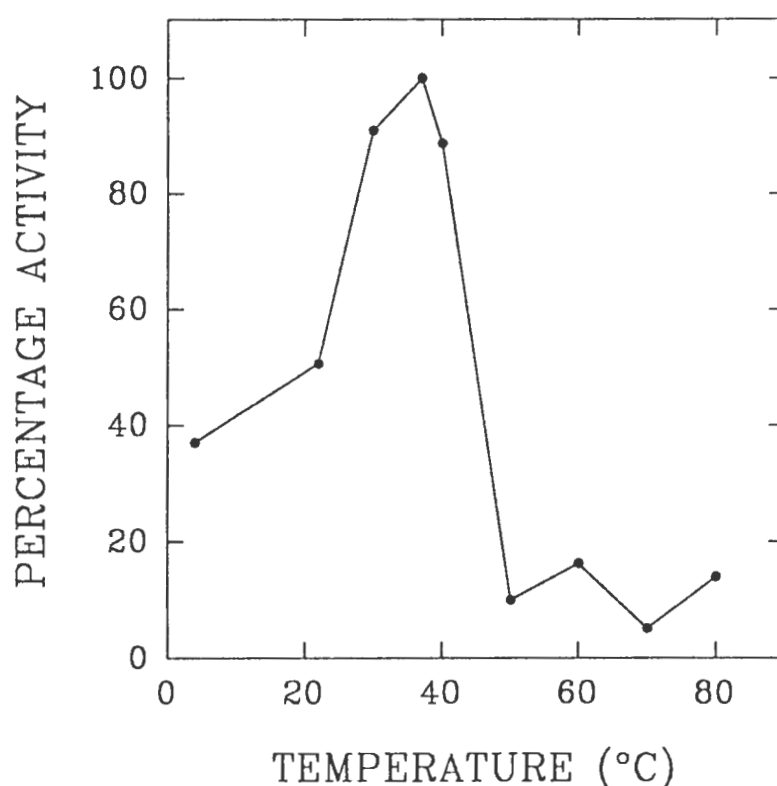


**Fig. 3.9** Activity of cyanide dihydratase at increasing temperatures. Experimental details are provided in section 3.3.7.

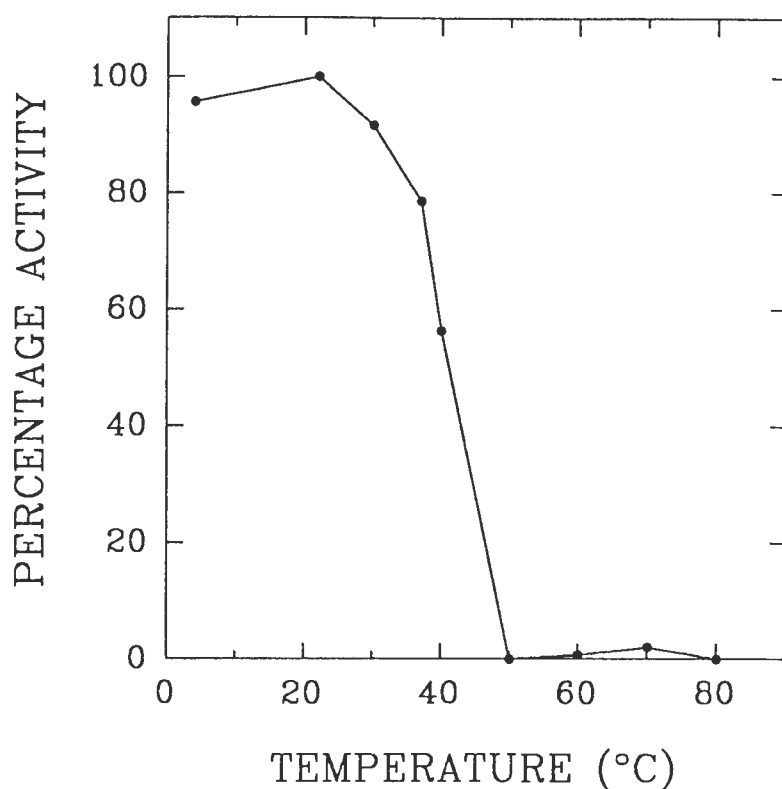


**Fig. 3.10 Thermal stability of cyanide dihydratase.** Experimental details are provided in section 3.3.7.

Addition of 0.02 % sodium azide as a bacteriostatic agent to solutions containing cyanide dihydratase was shown to increase the specific activity of the enzyme. This enhancement increased gradually over a period of days (Table 3.14) and was concentration dependent (Table 3.15) with maximal stimulation observed with 4.5 mM azide. Interestingly, the small enhancement of specific activity brought about by thiocyanate did not show any time-dependent increase (Table 3.14) and in fact decreased by approximately the same amount as the enzyme control during the course of the experiment. This suggested that thiocyanate could no longer exert its enhancing effect when the enzyme activity had deteriorated. Removal of azide from the enzyme by dialysis restored the specific activity to its original level; re-addition of azide once again increased the specific activity (Table 3.16). High concentrations of azide (150 mM and above) inhibited the enzyme (Table 3.15).

**Table 3.13 Comparison of cyanide dihydratase activity in buffers of different composition. The activity of each sample was measured as the decrease in cyanide in 1 h at 37 °C divided by the absorbance at 280 nm of the sample before the start of the assay (column 4). Experimental details are provided in section 3.3.11.**

| BUFFER                                      | $\Delta A_{520\text{nm}}$ | $A_{280\text{nm}}$ | $\frac{\Delta A_{520\text{nm}}}{A_{280\text{nm}}}$ | % ACTIVITY |
|---------------------------------------------|---------------------------|--------------------|----------------------------------------------------|------------|
| 10 mM Tris-HCl<br>50 mM NaCl<br>(CONTROL)   | 0.184                     | 0.019              | 9.684                                              | 100        |
| 50 mM glycine<br>50 mM NaCl                 | 0.133                     | 0.018              | 7.389                                              | 76.3       |
| 50 mM borate<br>50 mM NaCl                  | 0.046                     | 0.005              | 9.200                                              | 95.0       |
| 50 sodium<br>phosphate<br>50 mM NaCl        | 0.070                     | 0.010              | 7.000                                              | 72.3       |
| 10 mM<br>triethanol-<br>amine<br>50 mM NaCl | 0.107                     | 0.021              | 5.095                                              | 52.6       |

**Table 3.14 The effect of the addition of azide and thiocyanate on cyanide dihydratase activity as a function of time. Azide and thiocyanate were added to the indicated concentrations and enzymatic activity was assayed (as described in section 3.3.16) after incubation at 4 °C for 7.5 h and 72.5 h (5 mM KCN was used in the specific activity assays).**

| ENZYME TREATMENT          | SPECIFIC ACTIVITY<br>( $\mu\text{moles CN}^-/\text{min}/\text{mg protein}$ ) |        |
|---------------------------|------------------------------------------------------------------------------|--------|
|                           | 7.5 h                                                                        | 72.5 h |
| CONTROL<br>(No additions) | 30.3                                                                         | 26.52  |
| 5 mM $\text{NaN}_3$       | 68.3                                                                         | 78.0   |
| 10 mM NaSCN               | 32.07                                                                        | 26.1   |

**Table 3.15** The effect of increasing concentrations of sodium azide on cyanide dihydratase activity. Experimental details are provided in section 3.3.9.

| [NaN <sub>3</sub> ]<br>(mM) | PERCENTAGE ACTIVITY |
|-----------------------------|---------------------|
| 0                           | 100                 |
| 0.015                       | 89.7                |
| 0.15                        | 117.9               |
| 0.45                        | 155.6               |
| 1.5                         | 259.0               |
| 4.5                         | 360.7               |
| 15                          | 338.5               |
| 45                          | 142.7               |
| 150                         | 25.6                |
| 1500                        | 0                   |

**Table 3.16** The effect of azide addition, removal, and re-addition on cyanide dihydratase activity. Azide was added to 5 mM at two stages in the experiment and the experimental and control samples were incubated on ice for 4.5 h before assaying cyanide degrading activity as described in section 3.3.16. No period of incubation on ice was needed for the cyanide assay performed on the samples after the dialysis step. A small volume of each sample was diluted and used for the activity assay at each stage in the experiment (5 mM KCN was used in the specific activity assays). Dilutions and cyanide additions for the activity assays on azide-containing samples were performed using azide-containing buffer and KCN solutions so as not to dilute the azide in the samples.

| ENZYME TREATMENT                     | ENZYME CONTROL<br>(No NaN <sub>3</sub> ) | ENZYME<br>+ 5 mM NaN <sub>3</sub> |
|--------------------------------------|------------------------------------------|-----------------------------------|
| BEFORE DIALYSIS                      | 28.3                                     | 60.9                              |
| AFTER DIALYSIS                       | 23.1                                     | 24.3                              |
| AZIDE RE-ADDED TO<br>DIALYSED SAMPLE | 26.7                                     | 54.2                              |

Although the results presented in section 3.4.2 indicated that enzymatic cyanide degradation was independent of oxygen, assays for cyanide degradation under anaerobic conditions indicated a small reduction of activity at reduced oxygen concentrations; activity could be restored by allowing the anaerobic samples to become aerobic again (Table 3.17). The activity recorded under anaerobic conditions might have been lower if it had been possible to use an anaerobic cyanide solution to initiate the cyanide-degradation assay. Interestingly, azide did not appear to enhance cyanide degradation under anaerobic conditions and the azide-containing anaerobic samples displayed a similar reduction in activity to the samples without azide (Table 3.17).

**Table 3.17 Comparison of the cyanide-degrading activity of a *B. pumilus* C1 cell-free extract under aerobic and anaerobic conditions, with and without azide. Experimental details are provided in section 3.3.8.**

| ASSAY CONDITIONS                                                      | PERCENTAGE ACTIVITY |
|-----------------------------------------------------------------------|---------------------|
| CELL-FREE EXTRACT ASSAYED <b>ANAEROBICALLY</b>                        | 77.3                |
| CELL-FREE EXTRACT + 0.1 % $\text{NaN}_3$ ASSAYED <b>ANAEROBICALLY</b> | 75.9                |
| CELL-FREE EXTRACT ASSAYED <b>AEROBICALLY</b>                          | 100                 |
| CELL-FREE EXTRACT + 0.1 % $\text{NaN}_3$ ASSAYED <b>AEROBICALLY</b>   | 108.6               |
| <b>ANAEROBIC</b> CELL-FREE EXTRACT ASSAYED <b>AEROBICALLY</b>         | 92.6                |

Formate and acetate in the millimolar concentration range also stimulated cyanide dihydratase activity (Table 3.18).

**Table 3.18** The effect of increasing concentrations of (a) formate and (b) acetate on cyanide dihydratase activity. Experimental details are provided in section 3.3.9.

(a)

| [HCOO <sup>-</sup> ] (mM) | PERCENTAGE ACTIVITY |
|---------------------------|---------------------|
| 0                         | 100                 |
| 2.5                       | 80.2                |
| 5                         | 86.4                |
| 10                        | 97.5                |
| 20                        | 126.5               |
| 40                        | 155.6               |
| 80                        | 222.8               |
| 160                       | 261.1               |
| 320                       | 358.6               |

(b)

| [CH <sub>3</sub> COO <sup>-</sup> ] (mM) | PERCENTAGE ACTIVITY |
|------------------------------------------|---------------------|
| 0                                        | 100                 |
| 5                                        | 124.1               |
| 10                                       | 125.9               |
| 40                                       | 127.1               |
| 160                                      | 177.1               |
| 320                                      | 231.3               |

Attempts to show a relationship between chemical modification of tyrosyl and histidyl residues and loss of enzymatic activity were unsuccessful. The enzyme appeared to be sensitive to oxidation by hydrogen peroxide which is required for the lactoperoxidase-mediated iodination of tyrosyl residues. The effect of iodination of these residues could thus not be determined. The effect on enzymatic activity of carbethoxylation of histidyl residues could not be

determined as the solvents (absolute ethanol, acetonitrile or dimethyl sulphoxide) required for dilution of the active reagent, diethyl pyrocarbonate, inactivated the enzyme.

### 3.5 DISCUSSION

The cyanide-degrading activity from *B. pumilus* C1 was purified and characterized, and a large multimeric enzyme was shown to be responsible for this activity. The enzyme exhibited a nitrilase-like mode of action and converted the cyanide carbon atom to formate; the cyanide nitrogen atom appeared to be converted to ammonia, although the evidence for this was not convincing. However, an alternate fate for the cyanide nitrogen atom seems unlikely in view of the fact that the purified enzyme required no added co-factors for activity under the experimental conditions used. It is thus likely that ammonia is the other product of enzymatic cyanide degradation. Stripping of ammonia from solution remains the most plausible explanation for the low amounts of ammonia measured after cyanide degradation. Although the enzyme displayed no activity towards formamide, and formamide did not appear to affect enzymatic activity toward cyanide, formamide could nevertheless be a reaction intermediate which does not leave the active site during the dihydration of cyanide to formate. The enzyme exhibited no activity towards the cyanide structural analogues, thiocyanate, cyanate, azide, and acetonitrile, suggesting that it is specific for cyanide.

The decrease in enzymatic activity under anaerobic conditions is not understood, but the effect of reducing conditions on activity appears to be indirect since the lack of oxygen did not completely abolish enzymatic activity, and the nitrilase-like mode of action furthermore suggests that the reaction is independent of oxygen. Although the mode of action of the *B. pumilus* C1 enzyme is similar to that of the cyanidase described from the Gram-negative bacterium, *A. xylosoxidans* subsp. *denitrificans* (Ingvorsen *et al.*, 1991), the latter enzyme has been developed by Novo Nordisk A/S (Denmark) as a biocatalyst and has been patented under the name Cyanidase®. This precludes designation of the *B. pumilus* C1 enzyme as a cyanidase and the enzyme has accordingly been tentatively named cyanide dihydratase to indicate both its mode of action and substrate specificity.

Interestingly, the *B. pumilus* C1 cyanide dihydratase and the cyanidase from *A. xylosoxidans* subsp. *denitrificans* have some physico-chemical properties in common. Both enzymes have a molecular mass above 300 000 Da and are composed of subunits. They also have similar pH optima around pH 8.0 and retain activity at very high ionic strength (Basheer *et al.*, 1992). Both enzymes

display nitrilase activity exclusively to cyanide with a  $K_m$  of about 3 mM, and convert cyanide to formate and ammonia in a single-step reaction. The two enzymes, however, are clearly different proteins in that the *B. pumilus* C1 cyanide dihydratase consists of three polypeptide chains whereas the cyanidase contains only two. Moreover, the amino-terminal sequences bear no resemblance to one another and the enzymes have different temperature optima: 37 °C for the *B. pumilus* C1 enzyme compared with 26 °C for the cyanidase. This may account for the seemingly substantially higher  $V_{max}$  for the *B. pumilus* C1 enzyme:  $88.03 \pm 4.67$  mmol/min/mg/l compared with  $0.4 \pm 0.01$  mmol cyanide/l/min/g for the Cyanidase® biocatalyst (Basheer *et al.*, 1992). However, a very low cyanidase-to-support ratio in the Cyanidase® biocatalyst could also account for the much lower  $V_{max}$  value reported.

Other microbial cyanide-degrading enzymes that have been described include the formamide hydrolyases from the fungi, *S. loti* (Fry and Millar, 1972) and *G. sorghi* (Wang *et al.*, 1992). These were also reported to be large proteins with molecular masses of (at least) 600 000 and 300 000 Da, respectively, and displayed  $K_m$ s for cyanide of 25 mM and 12 mM, respectively. In contrast to these large cyanide-degrading enzymes, two small enzymes of 17 000 and 40 000 Da were reported in the fungus, *Fusarium solani* (Shimizu and Taguchi, 1968; Shimizu *et al.*, 1969). The *F. solani* enzymes degraded cyanide to ammonia, indicating that these were not formamide hydrolyases (the fate of the cyanide carbon atom was not determined).

One interpretation of the electron microscopy data for the *B. pumilus* C1 cyanide dihydratase is that the active holoenzyme is a rod-shaped, hexameric protein composed of identical monomeric particles arranged in a helix, the hexamer being readily dissociated. Evidence in favour of this hypothesis is that the enzyme displayed an unusual sensitivity to low-ionic-strength solutions and to dilution, with the catalytic activity being suddenly lost (section 3.4.1). If this interpretation is correct, the molecular mass of the active enzyme determined by gel-filtration chromatography may be an over-estimate since the molecular mass determined by exclusion chromatography assumes that the protein is globular rather than rod-shaped. An alternative interpretation of the electron microscopy data is that the rod-shaped structures visible in the electron micrographs were formed by association of the smallest particle and that the active enzyme is actually a smaller multimer than that indicated by the rod-shaped structures. In this regard it has been suggested that the tendency of the subunits of some

organic nitrilases (EC 3.5.5.1) to associate into very large aggregates of several hundred kilodaltons may have led to an over-estimate of the molecular mass of the holoenzyme (Goldlust and Bohak, 1989). Although the data preclude distinguishing between these interpretations, the association of a basic structure would be expected to have led to a range of active species of increasing molecular mass which should have appeared as several active protein peaks during gel-filtration chromatography and sucrose-gradient centrifugation. Elucidation of the true tertiary structure of the cyanide dihydratase will require more sophisticated electron-microscopy techniques in addition to subunit cross-linking studies.

The enhancement of enzymatic activity of the *B. pumilus* C1 enzyme by a variety of trivalent metal ions could possibly be due to these ions acting as cyanide carriers to present the substrate to the catalytic site in a more favourable manner. Chromium(III), however, which was also shown to enhance enzymatic activity, is known to be relatively inert with respect to ligand exchange reactions in solution (Cotton and Wilkinson, 1980). It is therefore unlikely that cyanide could replace  $\text{H}_2\text{O}$  in  $[\text{Cr}(\text{H}_2\text{O})_6]^{3+}$  during the course of a normal enzyme-activity assay (1 h at 37 °C). Alternatively, enhancement of enzymatic activity by these cations could be due to changes in protein structure brought about by the presence of these ions. This latter explanation is more likely as the presence of  $\text{Cr}^{3+}$  was shown to increase the  $K_m$  for cyanide by a factor of two, and  $\text{Cr}^{3+}$  and  $\text{Tb}^{3+}$  were also shown to extend the pH range of enzymatic activity. In the presence of  $\text{Cr}^{3+}$  and  $\text{Tb}^{3+}$ , the enzyme was shown to function at pH 8.6 (where no activity was recorded in the absence of trivalent metal ions) with approximately the same activity as at pH 8.0, the pH optimum. It is expected that similar kinetic and pH data would be obtained with those trivalent metal ions not tested in these assays (i.e.  $\text{Sc}^{3+}$  and  $\text{Fe}^{3+}$ ). Trivalent metal ions may either maintain a favourable active site conformation by electrostatic interactions with negatively-charged residues or may influence the stability of the holoenzyme since numerous incomplete structures were visible on the electron micrographs. This putative interaction was not caused by tight binding of the metal ions to the protein since no association of  $^{51}\text{Cr}^{3+}$  with cyanide dihydratase could be demonstrated for the enzyme purified from bacteria grown in the presence of this isotope. Binding of  $^{51}\text{Cr}^{3+}$  to the enzyme could also not be demonstrated after equilibrium dialysis of the enzyme against  $^{51}\text{Cr}^{3+}$ -containing buffer. Stabilization of enzymatic activity at pH 8.6 by trivalent metal ions, despite the fact that these ions do not appear to bind to the enzyme, is intriguing; at pH 8.6 lysine residues are partially

deprotonated and it is possible that their role in salt bridges is being assisted or replaced by trivalent metal ions as the pH increases above the optimum recorded in the absence of these ions. The relationship between these metal ions and the holoenzyme remains unclear, although the enzyme does not appear to be a metallo-enzyme or a metal-activated enzyme. It is extremely unlikely that the metal-ion effect is related to changes in ionic strength, as these metal ions were used at concentrations below 1 mM. Electron microscopy was not performed on the purified cyanide dihydratase in the presence of trivalent metal ions, as the changes in enzymatic activity brought about by these ions are likely to have been caused by minor conformational changes in the protein which would not have been detectable under the electron microscope.

Enzyme activity was also enhanced by azide, thiocyanate, formate and acetate, but the reason for the enhancement by these anions is unknown. Although activity enhancement by formate might suggest product stimulation of the enzyme this is unlikely as the enzyme was also stimulated by acetate. It seems possible that the stimulation of activity by the inorganic ions occurs by a different mechanism from that of the organic ions. Formate and acetate enhanced activity over a wide range of concentrations with activity increasing with organic-anion concentration. Azide and thiocyanate enhanced enzymatic activity only over a limited range of concentrations, and inhibition of activity was observed at high concentrations of these anions. Optimal enhancement of enzymatic activity by these inorganic ions occurred at relatively low concentrations (4.5 mM for azide and 2.5 mM for thiocyanate) suggesting that their effects were not the result of a favourable increase in ionic strength. It should be noted that enzymatic activity was not influenced by a wide range of ionic strengths above 50 mM NaCl and all characterization experiments were conducted in 10 mM Tris-HCl 50 mM NaCl. The enzyme only displayed sensitivity to ionic strength in solutions of very low ionic strength (e.g. 1 mM Tris-HCl 10 mM NaCl).

Inhibition of enzymatic activity by  $\text{Hg}^{2+}$ ,  $\text{Pb}^{2+}$  and  $\text{Ag}^{+}$  suggested the involvement of a cysteinyl sulphhydryl group in catalysis, although this could not be confirmed using two other sulphhydryl-modifying reagents. It may be noted that the cyanide hydratase from *G. sorghi* was susceptible to inhibition by  $\text{Ag}^{+}$ , mercury acetate and *p*-chloromercuric benzoate (all at 1 mM), but was not inhibited by iodoacetamide at 5 mM (Wang *et al.*, 1992). This enzyme was shown to be a multimeric protein composed of a number of 45-kDa subunits. Although

there is insufficient data to make possible a detailed comparison between the few characterized cyanide-degrading enzymes and the corresponding organic nitrilases and nitrile hydratases, the cyanide-degrading enzymes appear to be superficially similar to their organic-nitrile-degrading counterparts with respect to being multimeric, sulphydryl enzymes.

The high specific activity of cyanide dihydratase from *B. pumilus* C1 together with its ease of purification, stability, and kinetic properties make it a worthy candidate for investigation as a biocatalyst for an industrial process for the detoxification of cyanide. However, its apparent lack of activity towards metal-cyanide complexes and to thiocyanate would require the use of additional methods to completely remove these ions from gold-mining cyanidation wastewaters.

## CHAPTER 4

### STUDIES ON THE CLONING IN *E. coli* OF THE GENES FOR CYANIDE DIHYDRATASE

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## CHAPTER 4

### STUDIES ON THE CLONING IN *E. coli* OF THE GENES FOR CYANIDE DIHYDRATASE

#### 4.1 SUMMARY

A genomic library of *B. pumilus* C1 chromosomal DNA in the vector, pEcoR251, was screened in *E. coli* strain LK111 by a colony-Western-blot technique using antibodies to cyanide dihydratase. Failure to isolate a cyanide-degrading *E. coli* clone prompted the construction of a second genomic library of *B. pumilus* C1 DNA in the *E. coli*-*Bacillus subtilis* shuttle vector, pEB1. The pEB1 genomic library was screened in *E. coli* strain LK111 by two different procedures. The first involved a differential-growth method in which the fastest-growing *E. coli* genomic-library transformants, under cyanide-containing sloppy-agar overlays, were selected and assayed for cyanide-degrading activity. The second screening procedure explored the hypothesis that cyanide-degrading *E. coli* clones would be able to reduce 2,3,5-triphenyltetrazolium chloride (TTC) in the presence of cyanide. This latter method involved plating transformants on a cyanide-containing indicator medium containing TTC. Neither of these screening procedures produced cyanide-degrading *E. coli* clones, suggesting that the cyanide dihydratase gene, or genes, are not expressed in *E. coli*, or that the genes are not linked on the *B. pumilus* C1 chromosome and could not be cloned as a unit by the cloning strategy employed. A Southern-hybridization technique was attempted using a synthetic oligomeric DNA probe corresponding to the predicted DNA coding region for the NH<sub>2</sub>-terminal amino-acid sequence of cyanide dihydratase. Initial experiments, however, showed that the oligonucleotide probe was not sufficiently specific to *B. pumilus* C1 chromosomal DNA to warrant screening the pEB1 genomic library by a colony-Southern-hybridization procedure.

## 4.2 INTRODUCTION

The study of cyanide degradation in microorganisms has concentrated predominantly on understanding the process at the cellular level and the molecular biology has remained largely unexplored. Investigations into the enzymology of cyanide degradation in both bacteria and fungi have been reported, but these studies remain preliminary and no reaction mechanism has yet been proposed for any cyanide-degrading enzyme.

Information on the genetic aspects of cyanide degradation is limited to one paper reporting the cloning and sequencing of the cyanide-hydratase gene (*cht*) from *G. sorghi* (Wang and VanEtten, 1992), and an entry in the EMBL database for the sequence of the *chy1* gene encoding the cyanide hydratase of *F. lateritium* (Cluness *et al.*, 1993; accession number: M99046). The cloning and sequencing of the *chy1* gene has not been reported in the literature (*F. lateritium* is the organism on which ICI Bioproducts' Cycleclear preparation is based).

This chapter describes a number of attempts to clone the gene (or genes) for the cyanide dihydratase of *B. pumilus* C1 in *E. coli*, and discusses possible requirements for cloning these genes from *B. pumilus* C1.

*E. coli* was chosen as the cloning host for these studies as genetic systems for this organism are well established and it is unable to degrade cyanide.

### 4.3 MATERIALS AND METHODS

#### 4.3.1 MATERIALS

Tryptone and yeast extract for Luria broth was supplied by Biolab Diagnostics (Pty) Ltd., South Africa. Brain-heart broth was supplied by E. Merck (Germany). Tryptose Blood Agar Base was supplied by Oxoid (U.K.). Restriction endonucleases, restriction-enzyme buffers and T4 ligase were supplied by Boehringer Mannheim GmbH (Germany). Ampicillin was supplied by the Sigma Chemical Co. (U.S.A.). 2,3,5-Triphenyltetrazolium chloride (Art. 8380) was supplied by E. Merck (Germany). Goat-anti-rabbit antibody conjugated to alkaline phosphatase (A 8025), 5-bromo-4-chloro-3-indolyl phosphate (*p*-toluidine salt, B 8503) and nitro blue tetrazolium (N 6876) were supplied by the Sigma Chemical Co. (U.S.A.). Chemicals for buffers, solutions and reagents were of high purity. Water filtered through a Millipore Milli-RO apparatus was used throughout these studies. HATF nitrocellulose filter discs (85 mm diameter, 0.45  $\mu$ m) for colony Western blots were supplied by the Millipore Corporation (U.S.A.). A TE Series Transphor Electrophoresis Unit, supplied by Hoefer Scientific Instruments (U.S.A.), was used for electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose membranes. Hybond-N+ membranes (Amersham, U.K.) were used in Southern-hybridization analysis.

#### 4.3.2 ORGANISMS, PLASMIDS AND CULTURE CONDITIONS

*E. coli* strains LK111 (F<sup>-</sup> *thi-1 thr-1 leuB6 lacY1 tonA21 supE44*  $\lambda^-$  *r\_K^- m\_K^+ lacIq lacZ*  $\Delta$ M15; Zabeau and Stanley, 1982) and K514 (F<sup>-</sup> *thi-1 thr-1 leuB6 lacY1 tonA21 supE44*  $\lambda^-$  *r\_K^- m\_K^+*; an *r\_K^- m\_K^+* derivative of strain C600; Appleyard, 1954; Wood, 1966) were cultured in Luria broth at 37 °C with vigorous shaking. Plasmid pEcoR251 (M. Zabeau, Plant Genetic Systems, Ghent, Belgium) is a positive-selection vector containing the pBR322 origin of replication, an ampicillin-resistance gene, and the *E. coli* Eco RI endonuclease gene under the control of the bacteriophage- $\lambda$  rightward promoter, P<sub>R</sub> (Appendix C). This vector is lethal in *E. coli* hosts (e.g. strain LK111) which do not express the bacteriophage- $\lambda$  cI repressor protein (which suppresses transcription from P<sub>R</sub>). Recombinant plasmids can be selected in such hosts if the Eco RI endonuclease gene has been insertionally inactivated by cloning DNA into a restriction site within this gene.

Plasmid pEB1, constructed by Lin *et al.* (1990), is an *E. coli*-*B. subtilis* shuttle vector (Appendix C); the same cloning strategy is employed with pEB1 as with pEcoR251. *B. pumilus* C1 was cultured in Luria broth supplemented with 0.01 mg Mn<sup>2+</sup>/l (as described in section 2.4.4). *B. subtilis* strain 1A297 (*aspT trpC2 amyE*; Bacillus Genetic Stock Center, Department of Microbiology, Ohio State University, U.S.A.) was maintained on brain-heart broth solidified with 1.5 % agar, and cultured at 37 °C in brain-heart broth supplemented with 0.5 % yeast extract.

#### **4.3.3 RESTRICTION-ENDONUCLEASE DNA DIGESTIONS**

DNA was routinely digested with restriction endonucleases in a volume of 20 µl in sterile microfuge tubes as described by Sambrook *et al.* (1989).

#### **4.3.4 AGAROSE-GEL ELECTROPHORESIS**

DNA was routinely examined by electrophoresis in 0.8 % agarose gels in TBE buffer (Appendix A) as described by Sambrook *et al.* (1989).

#### **4.3.5 PREPARATION OF COMPETENT CELLS OF *E. coli***

Cells of *E. coli* strains LK111 and K514, which were competent to take up DNA, were prepared using the dimethyl sulfoxide (DMSO) method of Chung and Miller (1988), described in Appendix B.

#### **4.3.6 CONSTRUCTION OF A GENOMIC LIBRARY OF *B. pumilus* C1 CHROMOSOMAL DNA**

##### **4.3.6.1 PREPARATION OF *B. pumilus* C1 CHROMOSOMAL DNA**

Chromosomal DNA of *B. pumilus* C1 was isolated from a 200-ml overnight culture of the organism according to a modification of the method of Marmur (1961) (Appendix B).

#### 4.3.6.2 PARTIAL DIGESTION OF *B. pumilus* C1 CHROMOSOMAL DNA

*B. pumilus* C1 chromosomal DNA was partially digested with *Sau* 3AI. This restriction enzyme was chosen as it recognizes a 4-bp DNA sequence and is expected to restrict chromosomal DNA at many sites. The partially-digested DNA was extracted with an equal volume of Tris-buffered phenol, followed by two extractions with chloroform/isoamyl alcohol (24:1). The DNA was precipitated and resuspended in TE buffer, pH 8.0 (2 × 500 µl).

#### 4.3.6.3 SUCROSE-GRADIENT FRACTIONATION OF PARTIALLY-DIGESTED CHROMOSOMAL-DNA FRAGMENTS

Sucrose-gradient fractionation of partially-digested *B. pumilus* C1 chromosomal DNA was performed as described by Sambrook *et al.* (1989). The gradient solutions were fractionated into fifty fractions (0.5 - 1 ml) and examined by electrophoresis in a 0.5 % agarose gel. Fractions containing DNA fragments of 9 - 14 kb were pooled, as were the fractions containing DNA fragments of 6 - 10 kb, and the fractions containing fragments of 3 - 6 kb. The DNA was precipitated and resuspended in a small volume of TE buffer, pH 8.0 (15 - 30 µl).

#### 4.3.6.4 PREPARATION OF pEB1 PLASMID DNA

Plasmid DNA was extracted from 800 ml of *E. coli* strain K514, containing pEB1, using the method for large-scale isolation of plasmid DNA described in Appendix B (section B.2). *Bgl* II was chosen as the restriction endonuclease to prepare pEB1 DNA for the genomic-library ligations, as it cuts pEB1 only once (within the *EcoRI* gene, Appendix C) and generates sticky ends which are compatible with those generated by *Sau* 3AI, thereby making possible ligation of plasmid DNA with *Sau* 3AI-digested *B. pumilus* C1 chromosomal DNA.

#### 4.3.6.5 LIGATION OF *pEB1* DNA WITH *B. pumilus* C1 CHROMOSOMAL DNA

A ratio of vector (plasmid) to insert (chromosomal) DNA of 0.25 pmole: 0.25 pmole was chosen for the genomic-library ligations. *pEB1* has a length of 6.267 kb (Appendix C) and *B. pumilus* C1 chromosomal-DNA fragments of 6 - 10 kb were used for the genomic-library ligations. Ligated DNA was transformed into *E. coli* strain LK111; a total of 12,915 transformants was collected during the construction of the genomic library.

Thirty-six (36) transformants were selected at random during the course of these ligation/transformation experiments and plasmid DNA was extracted from them as described in the method for small-scale isolation of plasmid DNA (Appendix B). The isolated DNAs were restricted with *Cla* I and examined by agarose-gel electrophoresis. Twenty (20) transformants definitely contained chromosomal DNA and another 9 may have contained chromosomal DNA. Thus the actual number of transformants in the gene bank containing chromosomal DNA was estimated to be 55.6 - 80.6 % (20 - 29 out of every 36 transformants). It was apparent that non-lethal religation of linear *pEB1* DNA without insert DNA had occurred, indicating that the *EcoRI* gene was somehow inactivated during digestion and religation.

The number of transformants required to obtain a genomic library, with a given probability of representing the genome of an organism, can be calculated from the formula:

$$P = 1 - (1 - f)^N$$

Re-arranging to make *N* the subject of the formula:

$$N = \frac{\ln(1 - P)}{\ln(1 - f)}$$

where *P* is the probability (e.g. 99 %) that any given DNA sequence is present amongst *N* transformants, and *f* is the fraction of the total genome represented by each fragment of insert DNA (Clarke and Carbon, 1976).

If the *B. pumilus* C1 chromosome is assumed to have a similar size to the *B. subtilis* chromosome (4250 kbp; Piggot *et al.*, 1990), then approximately 4890 transformants are required to ensure that the genomic library has a 99.9 % probability of representing the *B. pumilus* C1 chromosome (assuming that the transformants contain 6-kb fragments of *B. pumilus* C1 DNA). The 12,915 transformants obtained during the construction of the genomic library are sufficient to ensure this probability even if only 55 % of the transformants are assumed to contain chromosomal DNA (55 % of the total number of genomic-library transformants is 7103 transformants, giving a probability of 99.99 % that the genomic library represents the *B. pumilus* C1 chromosome).

#### 4.3.6.6 ISOLATION OF GENOMIC-LIBRARY PLASMID DNA

The transformants were combined into two pools and the genomic-library plasmid DNA was isolated from these pools using the large-scale DNA isolation procedure described in Appendix B. The isolated DNA was not purified by CsCl-gradient centrifugation however, but after resuspension in TE buffer, pH 8.0, (Appendix B, section B.2) was treated with RNase. The solutions were extracted with phenol:chloroform:isoamyl alcohol (25:25:1) and then with chloroform. The DNA was precipitated and resuspended in TE buffer (pH 8.0).

#### 4.3.7 PREPARATION OF ANTIBODIES TO *B. pumilus* C1 CYANIDE DIHYDRATASE

Purified cyanide dihydratase from the A-5m gel-filtration column (1 ml containing approximately 1 mg protein; section 3.3.6) was mixed with 1 ml of Freund's incomplete adjuvant and used to immunize a young, male rabbit. The injection was repeated twice at weekly intervals followed by a booster injection in the sixth week. Nine serum samples were collected at weekly intervals from 4 weeks after the first injection and were stored at 4 °C. Serum sample IV was used for Western blot analysis of proteins.

#### 4.3.8 WESTERN BLOTTING

Western blotting was performed as described by Sambrook *et al.* (1989).

#### **4.3.9 IMMUNOLOGICAL SCREENING OF A *B. pumilus* C1 GENOMIC LIBRARY IN *E. coli***

A genomic library of *B. pumilus* C1 chromosomal DNA in pEcoR251 (constructed by Mr Stuart Glaun) was screened with the cyanide-dihydratase antibodies (section 4.3.7) by a colony-Western-blot procedure described by Sambrook *et al.* (1989).

Cyanide-dihydratase antibodies (serum IV) were used at a dilution of 1-in-1000 in blocking buffer. Goat-anti-rabbit antibody conjugated to alkaline phosphatase was used at a dilution of 1-in-5000 in blocking buffer. The processed nitrocellulose filters were immersed in alkaline-phosphatase substrate buffer (Appendix A) containing 0.112 mg NBT/ml and 0.075 mg BCIP/ml (Appendix A) and incubated at room temperature for up to 30 min to allow the development of an intense purple colour at the sites of antibody-antigen complexes.

Positive clones were subcultured on Luria/Ap agar, inoculated into 5 ml of sterile Luria broth containing 100 µg Ap/ml and grown in standard containers at 37 °C for 14 - 15 h with shaking. A 1-ml sample of each culture was pipetted into a microfuge tube and microfuged at room temperature for 2 min. The supernatant was removed by aspiration, the cell pellet was resuspended in 0.5 ml of sterile Millipore water, and cyanide was added (final concentration 102 mg CN<sup>-</sup>/l). Samples were incubated at 37 °C in a waterbath for 1 h, microfuged for 3 min to pellet the cells, and duplicate 50-µl volumes of supernatant were assayed for remaining cyanide (section 2.3.4).

#### **4.3.10 SCREENING OF A *B. pumilus* C1 GENOMIC LIBRARY IN *E. coli* USING CYANIDE SLOPPY-AGAR OVERLAYS**

This screening procedure relies on the hypothesis that an *E. coli* clone expressing the *B. pumilus* C1 cyanide dihydratase would be more resistant to the effects of cyanide than the other transformants and would therefore grow faster under a thin, cyanide-containing sloppy-agar overlay. Thus by transforming a genomic library of *B. pumilus* C1 DNA into competent cells of *E. coli*, plating them on Luria/Ap agar medium, and overlaying these plates with sloppy agar containing

sufficient cyanide to suppress the growth of normal *E. coli* cells, the fastest-growing colonies can be selected and assayed for cyanide-degrading activity.

To determine the lowest inhibitory concentration of cyanide for these experiments, competent cells of *E. coli* strain LK111 were transformed with pUC18 DNA and plated in 100- $\mu$ l aliquots on Luria/Ap agar medium. Sterile, half-strength Tryptose Blood Agar Base (prepared according to the manufacturer's instructions) was dispensed in 3-ml aliquots into sterile test tubes (12  $\times$  100 cm) prewarmed to 55 °C in a heating block. The molten sloppy agar was mixed with cyanide at concentrations of up to 400 mg CN<sup>-</sup>/l, and poured over the plated transformants (ensuring that the entire surface of the plate was covered). When the agar overlays had set, the plates were sealed with Parafilm and incubated in a sealed container at 37 °C for approximately 16 h. These results showed that a cyanide concentration of 240 mg CN<sup>-</sup>/l in the agar overlay prevented the formation of colonies by most transformants during overnight growth.

The genomic library of *B. pumilus* C1 DNA in pEB1 was screened in *E. coli* by this procedure with 240 mg CN<sup>-</sup>/l in the sloppy agar. All plates were sealed with Parafilm and incubated at 37 °C in a sealed container for 20 - 22 h. The largest colonies on each plate were transferred to fresh Luria/Ap agar medium using sterile toothpicks and incubated overnight at 37 °C. These clones were assayed for cyanide-degrading activity (as described in section 4.3.9).

#### **4.3.11 SCREENING OF A *B. pumilus* C1 GENOMIC LIBRARY IN *E. coli* GROWN ON CYANIDE INDICATOR-MEDIUM**

2,3,5-Triphenyltetrazolium chloride (TTC) is used in tests for the germinating power of seeds and in microbiology to determine whether cells are respiring. When bacteria, such as *E. coli*, are grown on medium containing TTC the respiring cells reduce the colourless TTC compound to an insoluble, dark red triphenylformazan derivative and the bacterial colonies appear dark red.

The use of TTC has been incorporated into the taxonomic test to distinguish between members of the Enterobacteriaceae which can grow in the presence of 0.025 % KCN (e.g. *Citrobacter*, *Klebsiella*, *Enterobacter*) and those which cannot (e.g.

*Salmonella*) (Munson, 1974, and references quoted therein). The presence of an indicator in the medium facilitates interpretation of the results of this test (Munson, 1974).

When *E. coli* strain LK111 was grown on Luria agar containing 0.0055 % v/v TTC at 37 °C, red colonies appeared during overnight incubation. However, when this organism was grown on the same medium containing a low concentration of cyanide (e.g. 2 mM KCN), growth at 37 °C was slow and very small white colonies, which could not reduce TTC, appeared within 30 h. When white colonies from the TTC-cyanide plates were restreaked onto fresh TTC medium without cyanide the organism once again grew strongly at 37 °C forming dark red colonies. Furthermore, white *E. coli* colonies growing on TTC-cyanide plates turned dark red with standing at room temperature as the cyanide was gradually lost from the medium as volatile HCN (the process took 10 - 14 days for plates which were not sealed with Parafilm, indicating that the cyanide concentration had to drop to a very low level before TTC could be reduced). *E. coli* streaked from an anaerobic Luria agar plate (without TTC) onto fresh, anaerobic Luria-TTC agar medium (without cyanide) grew well during overnight incubation at 37 °C and produced dark red colonies. These observations showed that cyanide prevented reduction of TTC by *E. coli* under aerobic conditions and suggested that cyanide induced the synthesis of a cyanide-insensitive oxido-reductase which could not reduce TTC. It may be that this alternate oxido-reductase was a cyanide-insensitive terminal oxidase in the aerobic electron-transport chain of *E. coli* (i.e. these results may have revealed a fork in the electron-transport chain).

This suggested that TTC might be incorporated into a cyanide-containing agar-indicator-medium on which *E. coli* strain LK111, transformed with DNA from a genomic library of *B. pumilus* C1, could be plated and grown. Since *E. coli* is incapable of reducing TTC in the presence of even low concentrations of cyanide, it was proposed that any clone expressing active *B. pumilus* C1 cyanide dihydratase might reduce the cyanide concentration in the vicinity of the positive clone sufficiently to allow reduction of TTC, thereby producing a red colony amongst white colonies.

As it was shown that *B. pumilus* C1 streaked on Luria agar, containing 0.0054 % TTC and 4 mM KCN, formed red colonies during overnight incubation at 37 °C, it was decided to use this higher concentration of cyanide in the hope that the higher concentration would reduce the growth of non-cyanide-degrading clones

relative to any cyanide-degrading clones. The genomic library of *B. pumilus* C1 chromosomal DNA in pEB1 was screened in *E. coli* strain LK111 by transforming genomic-library DNA into competent cells of this strain and plating the transformants in 100- $\mu$ l aliquots on Luria agar containing 0.0054 % TTC, 100  $\mu$ g Ap/ml, and 4 mM KCN (Appendix A). The sterile cyanide indicator-medium was poured approximately 1 h before use in order to minimize the loss of volatile HCN before plating the transformants. The plates were thus very wet at the time of use. Transformed cells were plated onto the wet medium, the plates were sealed with Parafilm, and incubated in a sealed container at 37 °C for 3 - 4 days (whereafter the plates were left at room temperature in a fumecupboard for an additional period of 7 - 9 days). Tiny white colonies appeared on the cyanide indicator-medium within 30 h at 37 °C. In each screening experiment, cells transformed with a known amount of pUC18 DNA (and plated on Luria agar containing 100  $\mu$ g Ap/ml and 0.0054 % TTC) served as a control for the ability of transformed cells to reduce TTC (and also allowed measurement of the transformation frequency). In addition, 20  $\mu$ l of the cells from each of the genomic-library-transformation mixtures (after incubation at 37 °C in TSB/20 mM glucose; Appendix A) were plated onto Luria/Ap to allow a crude estimate to be made of how many transformants had been screened on the cyanide indicator-plates in each screening experiment.

Colonies appearing red or dark pink after incubation at 37 °C were transferred to fresh Luria/Ap plates using sterile toothpicks. The plates were incubated overnight at 37 °C and the clones were assayed for cyanide-degrading activity as described in section 4.3.9 (with the exception that the cell pellets from 1-ml volumes of the overnight cultures were resuspended in 1 ml of sterile Millipore water and were assayed for the ability to degrade 5 mM KCN).

#### 4.3.12 SYNTHESIS OF AN OLIGOMERIC DNA PROBE

A mixture of oligomeric DNA probes was synthesized by Professor Dawie Botes of the Department of Biochemistry (University of Cape Town) according to the following sequence predicted for the NH<sub>2</sub>-terminal amino-acid sequence generated for cyanide dihydratase (section 3.4.1):

Ile Tyr Pro Lys Phe X Ala Ala  
 5' ATI TAT<sup>T</sup>/<sub>C</sub> CCI AAG<sup>G</sup>/<sub>A</sub> TTT<sup>T</sup>/<sub>C</sub> III GCI GC 3'

#### 4.3.13 SOUTHERN HYBRIDIZATION

Double digestions of chromosomal DNA from *E. coli* strain LK111, *B. subtilis* strain 1A297, and *B. pumilus* C1 (approximately 30 µg of DNA per double digestion) were performed in 60-µl volumes using *Hind* III/*Eco*R V (Boehringer Mannheim buffer B) and *Pst* I/*Eco*R I (Boehringer Mannheim buffer H). Chromosomal DNA was prepared as described in section 4.3.6.1. Southern-hybridization analysis of the chromosomal DNA with synthetic oligonucleotides (section 4.3.13) was performed as described by Sambrook *et al.* (1989).

Radiolabelled oligonucleotide probe was prepared by T4-phage polynucleotide-kinase-mediated 5'-end-labelling of the oligonucleotide mixture as described by Sambrook *et al.* (1989). The single-stranded synthetic oligonucleotides were assumed to have an absorbance at 260 nm such that 1 absorbance unit  $\equiv$  20 µg DNA/ml. The radiolabelled oligonucleotides were not separated from unincorporated label and the efficiency of the oligonucleotide radiolabelling was not determined. Hybridization was performed at 37 °C in Church hybridization buffer (Appendix A). Washing was performed with Wash Buffer A (Appendix A).

## 4.4 RESULTS

### 4.4.1 SCREENING THE *pEcoR251* GENOMIC LIBRARY

Western blots of cell-free extracts of *B. pumilus* C1, *B. subtilis* and *E. coli* showed that although the antibodies did not react with *E. coli* proteins to any extent, they did react with some low-molecular-weight proteins in the *B. subtilis* cell-free extract, and with several of the proteins in the *B. pumilus* C1 cell-free extract. A very strong reaction was recorded between the serum-IV antibodies and purified cyanide dihydratase proteins (Fig. 4.1). These results showed that the antisera contained antibodies to a number of *B. pumilus* C1 proteins present in the partially-purified enzyme preparation used to immunize the rabbit. Pre-adsorption of serum-IV antibodies against a lysate of *B. pumilus* C1, prepared from a culture grown with a low concentration of manganous ions (and which was unable to degrade to cyanide), reduced the intensity of the alkaline-phosphatase colour reaction at the sites of antibody-antigen complexes on Western blots, but did not remove all of the unwanted antibodies from the antiserum.

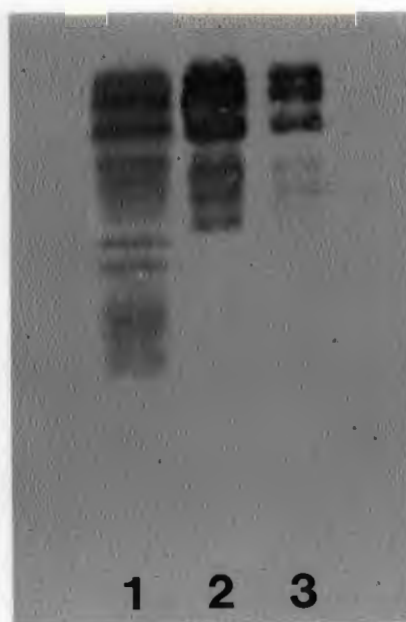


Fig. 4.1 Western-blot analysis of *B. pumilus* C1 proteins using the serum-IV cyanide-dihydratase antibodies. Cyanide dihydratase was purified as described in section 3.3.6. Lane 1, cell-free extract; lane 2, A-5m-gel-filtration protein concentrate; lane 3, sucrose-gradient-purified cyanide dihydratase.

The pEcoR251 genomic library was screened in *E. coli* strain LK111 by the immunological method described in section 4.3.9. As expected, the mixture of antibodies in the rabbit serum led to the generation of many false positive reactions during the screening experiments. Several thousand colonies were screened by this method and those colonies showing the strongest colour reaction in the alkaline-phosphatase assay were tested for cyanide-degrading activity (as described in section 4.3.9). None of the 172 clones tested displayed any ability to degrade cyanide. Although these cyanide non-degrading clones may have been expressing an inactive component of cyanide dihydratase, they could also have been expressing non-cyanide-dihydratase proteins; further investigation of these clones was not undertaken.

#### 4.4.2 SCREENING THE *pEB1* GENOMIC LIBRARY

Since many false positives were generated by screening the pEcoR251 genomic library by the colony-Western-blot procedure, it was proposed that the pEB1 library be screened in *E. coli* by a different method; a cyanide sloppy-agar-overlay procedure was chosen. In preparation for screening the new genomic library, the minimum concentration of cyanide, which inhibited growth of *E. coli* strain LK111, was determined as described in section 4.3.10.

The library was screened in this host by the cyanide-sloppy-agar-overlay procedure and the fastest-growing (largest) colonies from each transformation experiment were tested for cyanide-degrading activity (as described in section 4.3.9). None of the 126 clones tested displayed any cyanide-degrading activity.

The development of a cyanide-TTC indicator-medium (Fig. 4.2a, b, c) prompted the screening of the pEB1 library in *E. coli* strain LK111 once more, as this technique represented the best and most direct method for isolating cyanide-degrading clones. More than 30 000 transformants were screened and 81 clones displaying a red or pink colony phenotype were assayed for cyanide-degrading activity (as described in section 4.3.11). None of these clones displayed any cyanide-degrading activity. Three clones, designated 3I4a, 3I4b, and 3I4c (isolated from a single cyanide-TTC plate), formed dark red colonies and best exhibited the phenotype expected for a positive clone (even though they could not degrade cyanide). Restriction analysis of the plasmid DNA from these clones revealed that they all contained the same DNA insert. A cell-free extract of clone

3I4b in 10 mM Tris-HCl 50 mM NaCl, pH 8.0 (prepared as for *B. pumilus* C1 cell-free extracts, section 2.3.6) was also incapable of degrading cyanide.

The red phenotype of clone 3I4b on cyanide-TTC indicator-medium was shown to be plasmid-encoded as *E. coli* retransformed with the clone DNA exhibited red colonies on cyanide-TTC medium (Fig. 4.2e). Some of the 3I4b transformants, however, were white and formed larger colonies than those exhibiting the red, TTC-reducing phenotype (Fig. 4.2e). This phenomenon was reproducible.

(a)

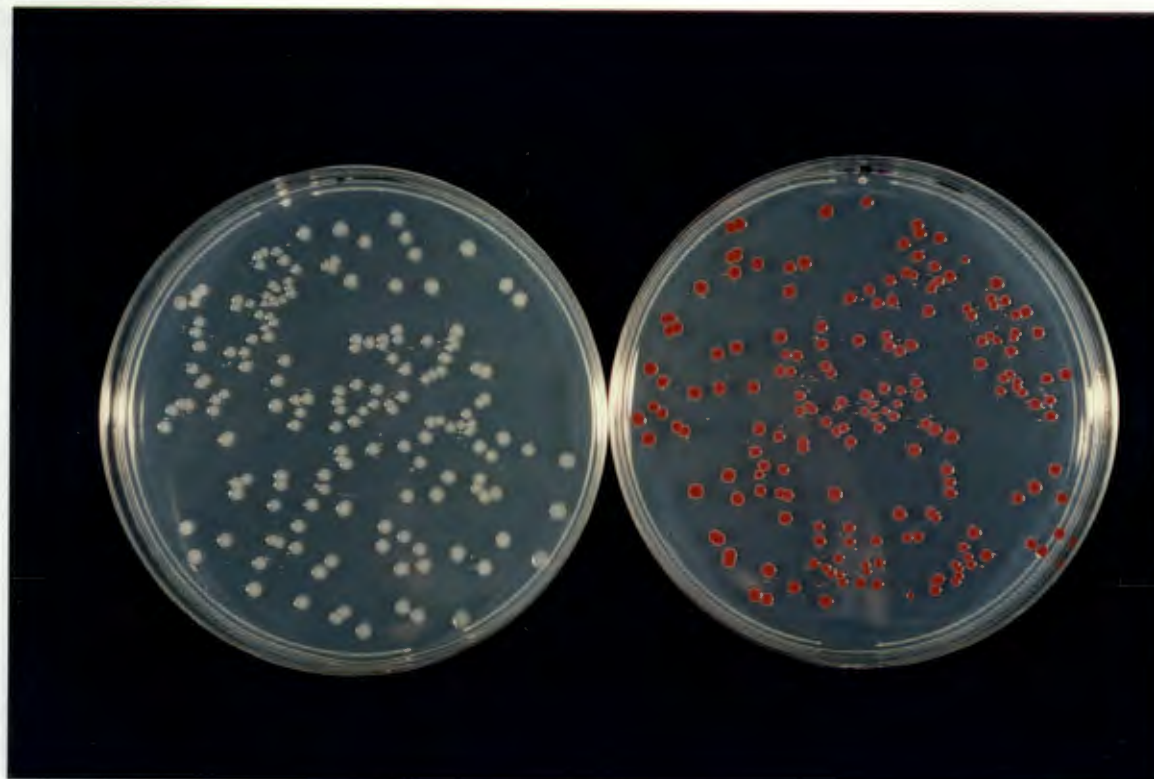


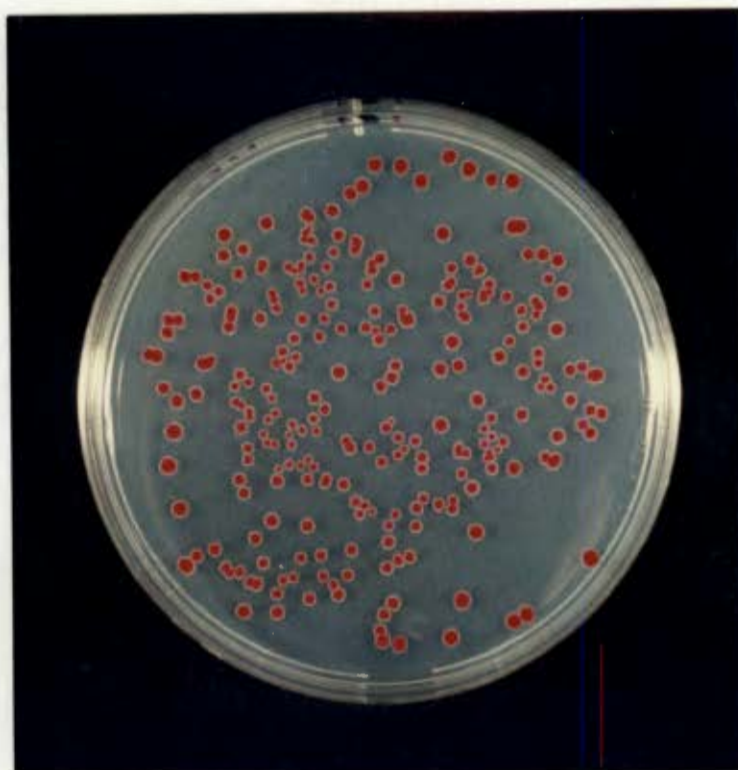
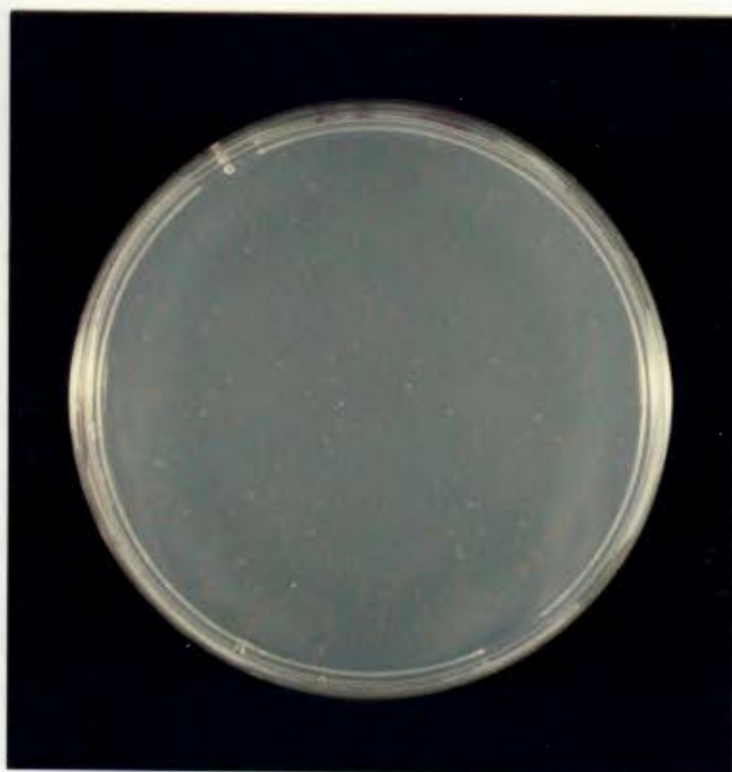
**Fig. 4.2** Photographs showing the phenotype exhibited by pUC18 and clone-3I4b transformants on TTC indicator medium with and without cyanide. (a) pUC18 transformants on Luria/Ap (left) and Luria/Ap/TTC (right). (b) pUC18 transformants on Luria/Ap/TTC/4 mM cyanide. (c - e) Clone 3I4b transformants on Luria/Ap (c), Luria/Ap/TTC (d), and Luria/Ap/TTC/cyanide (e). Luria/Ap and Luria/Ap/TTC plates were incubated at 37 °C for 18 - 20 h. Luria/Ap/TTC/cyanide plates were incubated at 37 °C for approximately 4 days.

**Fig. 4.2 (b)**



**(c)**



**Fig. 4.2 (d)****(e)**

#### 4.4.3 SOUTHERN HYBRIDIZATION OF AN OLIGOMERIC DNA PROBE TO *B. pumilus* C1 CHROMOSOMAL DNA

An experiment to determine whether the synthetic oligonucleotide-probe mixture hybridized specifically to *B. pumilus* C1 chromosomal DNA showed that the probe hybridized to *B. pumilus* C1 DNA, as well as to the negative controls (chromosomal DNA from *E. coli* strain LK111 and *B. subtilis* strain 1A297) (Fig. 4.3). Increasing the stringency of the washing conditions during the procedure decreased the intensity of the signals in the *B. pumilus* C1 and negative-control lanes on the autoradiograph. Neither of the genomic libraries was screened by colony-Southern-hybridization.



Fig. 4.3 Southern-hybridization analysis of *B. pumilus* C1 DNA using synthetic oligonucleotides corresponding to the NH<sub>2</sub>-terminal sequence of cyanide dihydratase. Hybridization was carried out at 37 °C; washing steps were carried out at room temperature (2 × 10 min) and 37 °C (1 × 10 min) using Wash Buffer A. Other details are provided in sections 4.3.12 and 4.3.13. Lanes 1, 4, 6 & 7, no DNA; lanes 2 & 14, λ *Pst* I standards; lane 3, pEB1 library DNA (pool 1); lane 5, pEB1 library DNA (pool 2); lanes 8 - 10, *Hind* III/*Eco* RV-digested chromosomal DNA (lane 8, *B. pumilus* C1; lane 9, *E. coli* LK111; lane 10, *B. subtilis* 1A297); lanes 11 - 13, *Pst* I/*Eco* RI-digested chromosomal DNA (lane 11, *B. pumilus* C1; lane 12, *E. coli* LK111; lane 13, *B. subtilis* 1A297).

## 4.5 DISCUSSION

Several attempts to clone the *B. pumilus* C1 cyanide dihydratase in *E. coli* were unsuccessful. Although it is possible that the coding sequence for cyanide dihydratase was not represented in either of the *B. pumilus* C1 genomic libraries, there may be other reasons why the gene, or genes, could not be cloned. For example, the enzyme proteins may not have been expressed in *E. coli*, or, if expressed they may not have associated to form the active enzyme in this host. Another possibility is that, if there are indeed three genes (corresponding to the three peptides seen on SDS PAGE), the genes may not be closely linked on the *B. pumilus* C1 chromosome, making cloning all the genes on short DNA fragments extremely unlikely.

It may be noted that during the initial screening of the pEcoR251 genomic library, a fragment of *B. pumilus* C1 DNA was cloned which conferred on several *E. coli* clones the ability to degrade carboxymethyl cellulose (CMC) (Glaun, 1990). The isolation of cellulolytic *E. coli* clones demonstrated that *B. pumilus* C1 genes could be expressed in *E. coli*, and implied a measure of confidence in this library.

The requirement for manganoous ions for the production of cyanide dihydratase in *B. pumilus* C1, and the appearance of this activity only after mid-exponential phase, strongly suggests that there may be factors involved in the process of cyanide-dihydratase synthesis which are not provided in an *E. coli* host. For example, it could be that *E. coli* does not synthesize a necessary sigma factor for recognition of the promoter for the cyanide-dihydratase coding sequence. However, if there is more than one gene the possibility that the cloning problem is simply one of gene linkage cannot be ruled out.

The isolation of an *E. coli* clone (from the pEB1 genomic library), which was unable to degrade cyanide, but which exhibited a distinctly red phenotype on cyanide-TTC indicator medium, suggested that the cloned DNA encoded a *B. pumilus* C1 oxido-reductase which enabled *E. coli* to reduce TTC in the presence of cyanide. These indicator plates thus led to the isolation of a false-positive clone. However, genomic libraries of other cyanide-degrading organisms might be screened by this method in *E. coli* with greater success.

The reverse-genetics approach to the isolation of the gene, or genes, for cyanide dihydratase represents perhaps the best method available given the

demonstrated failure of the more direct techniques. However, the available NH<sub>2</sub>-terminal amino-acid sequence for cyanide dihydratase contains a number of amino acids which have a high codon redundancy. The synthetic oligomeric DNA probes corresponding to this amino-acid sequence also contained few guanosine and cytosine nucleotides and did not hybridize to chromosomal DNA of *B. pumilus* C1 with sufficient specificity to make screening the genomic libraries by colony-Southern-hybridization feasible.

## CHAPTER 5

### GENERAL DISCUSSION

These investigations into cyanide-degradation by *B. pumilus* C1 have revealed some interesting features of both the requirements for the production of cyanide dihydratase as well as the properties of this enzyme.

The enormous physical size of the enzyme is perhaps remarkable in view of the fact that it degrades a molecule as small as HCN. However, a high molecular weight is a common feature of most of the other cyanide-degrading enzymes which have been described (Fry and Millar, 1972; Ingvorsen *et al.*, 1991; Wang *et al.*, 1992), and has also been reported for some organic nitrilases and nitrile hydratases (for example, Harper, 1977; Nagasawa *et al.*, 1988; Goldlust and Bohak, 1989; Kobayashi *et al.*, 1990a). The elucidation of a catalytic mechanism for the cyanide dihydratase of *B. pumilus* C1 would be of interest for the comparison of this enzyme with nitrilases that catalyze the hydrolysis of organic nitriles, and may also provide an explanation for the influence that trivalent-metal cations and certain anions (such as azide) have on enzymatic activity.

The failure to clone the gene, or genes, for cyanide dihydratase in *E. coli* suggests that an entirely new cloning strategy may be necessary. An obvious alternative strategy would be to use *B. subtilis*, or a cyanide non-degrading strain of *B. pumilus*, as the cloning host. However, this approach presents certain problems as *Bacillus* cannot be transformed as efficiently as *E. coli* and there is also the problem of how to screen a genomic library in this host. The use of cyanide-sloppy agar overlays is a very crude method for screening cyanide-degrading clones and does not provide a reliable means of selecting only positive clones. An indicator medium, to distinguish between those transformants which can degrade cyanide and those which cannot, would be perhaps the best direct method for selecting positive clones. However, the cyanide-TTC indicator medium cannot be used with either *B. subtilis* or *B. pumilus* as both are capable of reducing TTC in the presence of cyanide.

A colony-Southern-hybridization procedure in *E. coli* using an oligomeric DNA probe, corresponding to a section of the amino-acid sequence of the cyanide dihydratase, remains one of the soundest approaches under the circumstances

and would indicate the presence of the DNA coding sequence of interest. If such a positive clone did not express the active cyanide dihydratase the DNA could be isolated and introduced into a host such as *B. subtilis* which is perhaps more likely to express *B. pumilus* genes. As a DNA probe to the NH<sub>2</sub>-terminal amino acids did not hybridize specifically to *B. pumilus* C1 chromosomal DNA, a section of the internal amino-acid sequence of cyanide dihydratase would have to be determined to permit the synthesis of a new oligomeric DNA probe.

An alternative approach, using transposons to generate a cyanide-dihydratase-minus mutant of *B. pumilus* C1, might also be employed to isolate the cyanide-dihydratase genes. However, the apparent complexity of the control mechanism of cyanide-dihydratase synthesis does raise the possibility that the isolation of such a mutant may not lead directly to the isolation of the coding sequence for the enzyme, but rather to the cloning of a sequence for an element involved in the events preceding the appearance of cyanide dihydratase.

It might be possible to isolate the genes for cyanide dihydratase by exploiting the fact that very low levels of cyanide dihydratase are produced when the manganous-ion concentration of the growth medium is low. If mRNA was isolated from a low-Mn<sup>2+</sup> culture of *B. pumilus* C1 (exhibiting low cyanide-degrading activity) and hybridized to a cDNA library prepared from a cyanide-degrading culture of the same organism, the cDNA which is not represented as mRNA in the culture exhibiting low cyanide-degrading activity, would remain unhybridized. The cyanide-dihydratase coding sequence could thus be isolated. This approach assumes that there is no difference between the high- and low-Mn<sup>2+</sup> cultures (apart from expression of cyanide dihydratase). However, the difference in cell yield between cultures grown with and without added Mn<sup>2+</sup> is alone reason to consider this assumption spurious. The apparent complexity of the system for cyanide degradation in *B. pumilus* C1 may confound efforts to isolate the genes for cyanide dihydratase by this method simply because the mRNA from the one culture, and the cDNA from the other, may not correspond to each other with respect to more than one gene, or set of genes. The result would be clones containing a number of coding sequences, amongst which would be that of cyanide dihydratase. However, this technique would be helpful in narrowing down the search for the cyanide-dihydratase coding sequence and would therefore concentrate the cloning effort in a defined area.

## APPENDIX A

### MEDIA, BUFFERS AND SOLUTIONS

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## APPENDIX A

### MEDIA, BUFFERS AND SOLUTIONS

#### A.1 MEDIA

##### A.1.1 CETRIMIDE AGAR

|                         |                                            |
|-------------------------|--------------------------------------------|
| 20.5 g                  | peptone                                    |
| 1.4 g                   | MgCl <sub>2</sub>                          |
| 10.0 g                  | K <sub>2</sub> SO <sub>4</sub>             |
| 0.5 g                   | cetrimide (cetyltrimethylammonium bromide) |
| 10.0 ml                 | glycerol                                   |
| 13.6 g                  | agar                                       |
| H <sub>2</sub> O to 1 l |                                            |

NOTE: adjust pH to 7.2 before adding the agar.

##### A.1.2 CYANIDE-TTC INDICATOR-MEDIUM

This medium distinguishes between *E. coli* clones which can reduce 2,3,5-triphenyltetrazolium chloride (TTC) in the presence of cyanide, and those that cannot.

To 250 ml of molten Luria agar (containing 2 % agar) add:

|          |                           |
|----------|---------------------------|
| 1.375 ml | filter-sterilized 1 % TTC |
| 0.510 ml | 50 mg Ap/ml               |
| 2.8 ml   | 367.5 mM KCN solution     |

This gives Luria agar containing 0.0054 % TTC, 100 µg Ap/ml, and 4 mM KCN.

##### A.1.3 0.2 % GLUCOSE LIQUID MINIMAL MEDIUM (Based on Spizizen, 1958)

To make 1 l of medium:

|                  |        |
|------------------|--------|
| D(+)-glucose     | 2 g    |
| H <sub>2</sub> O | 778 ml |

Autoclave. Then add:

|        |                                                                |
|--------|----------------------------------------------------------------|
| 200 ml | sterile SMM* salts                                             |
| 1 ml   | sterile 10 <sup>-4</sup> M MnSO <sub>4</sub> (autoclaved)      |
| 20 ml  | sterile 1 % MgSO <sub>4</sub> (autoclaved)                     |
| 1 ml   | filter-sterilized 0.5 % biotin (for <i>B. pumilus</i> strains) |

|              |                         |                                                 |
|--------------|-------------------------|-------------------------------------------------|
| * SMM salts: | 10 g                    | (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> |
|              | 70 g                    | K <sub>2</sub> HPO <sub>4</sub>                 |
|              | 30 g                    | KH <sub>2</sub> PO <sub>4</sub>                 |
|              | H <sub>2</sub> O to 1 l |                                                 |

Autoclave.

#### A.1.4 LURIA-BERTANI BROTH

|                         |               |
|-------------------------|---------------|
| 10 g                    | tryptone      |
| 5 g                     | yeast extract |
| 5 g                     | NaCl          |
| H <sub>2</sub> O to 1 l |               |

Sterilize by autoclaving.

For Luria agar medium add agar to 1.5 % before sterilizing.

#### A.1.5 NITROGEN-DEFICIENT GLUCOSE MINIMAL AGAR

For testing bacterial ability to use cyanide as a nitrogen source.

|        |                                                                     |
|--------|---------------------------------------------------------------------|
| 100 ml | sterile 10 × M9 salts (without NH <sub>4</sub> Cl)                  |
| 100 ml | sterile 100 mM glucose                                              |
| 1 ml   | sterile trace-metals solution B                                     |
| * 1 ml | filter-sterilized 0.5 % biotin                                      |
| 15 g   | sterile Oxoid purified agar in 798 ml distilled or de-ionized water |

This gives 1 l 10 mM glucose/ M9 salts/ trace metals/1.5 % agar.

\* only required in experiments involving strains of *B. pumilus*

**NOTE:** The 10 × M9 salts solution, 100 mM glucose solution, and the agar are autoclaved separately. All components of the medium are mixed while still quite hot and plates are poured.

#### A.1.6 SPORULATION AGAR

For *B. subtilis*, *B. pumilus* and *B. licheniformis* (Schaeffer *et al.*, 1965).

|                         |                                      |
|-------------------------|--------------------------------------|
| 8.0 g                   | Nutrient broth                       |
| 0.25 g                  | MgSO <sub>4</sub> ·7H <sub>2</sub> O |
| 1.0 g                   | KCl                                  |
| 15.0 g                  | Agar                                 |
| H <sub>2</sub> O to 1 l |                                      |

Autoclave, cool, and then add:

|       |                                                        |
|-------|--------------------------------------------------------|
| 10 ml | sterile 0.1 M $\text{CaCl}_2$                          |
| 2 ml  | sterile 5 mM $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ |

Pour plates.

Streak cells onto plates. Incubate at 37 °C overnight and then keep plates at room temperature for 3 - 4 days. Pipette 5 - 7 ml of sterile distilled water onto each plate and scrape off the cells and spores with a sterile spreader. Pour suspension into a sterile bottle. This constitutes the spore stock and should be stored at 4 °C.

### A.1.7 THORNLEY'S MEDIUM

To test for arginine-dihydrolase activity in *Pseudomonas* species.

|                             |                          |
|-----------------------------|--------------------------|
| 1.0 g                       | Peptone                  |
| 5.0 g                       | NaCl                     |
| 0.3 g                       | $\text{K}_2\text{HPO}_4$ |
| 0.01 g                      | Phenol red               |
| 10.0 g                      | L-arginine-HCl           |
| $\text{H}_2\text{O}$ to 1 l |                          |

Adjust pH to 7.2. Add 3 g agar and heat until solution is homogenous. Dispense in 3-ml aliquots into 5-ml screw-cap bottles. Autoclave.

Inoculate by stabbing to the bottom of the bottle. Seal by pouring liquid paraffin onto surface of agar. Incubate at 30 °C. Anaerobic formation of  $\text{NH}_3$  from arginine can be detected by a change in colour of the indicator to bright pink within 3 days.

## A.2 BUFFERS

### A.2.1 10 × LIGATION BUFFER

|         |                                                       |
|---------|-------------------------------------------------------|
| 660 µl  | sterile 1 M Tris-HCl (pH 7.5)                         |
| 66 µl   | sterile 1 M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ |
| 15.4 mg | dithiothreitol                                        |
| 174 µl  | sterile water                                         |
| 900 µl  | 10 × ligation buffer (without ATP)                    |

To make 1 ml of ready-to-use 10 × ligation buffer add 100 µl 100 mM ATP (pH 7.0) to 900 µl of the above buffer.

Notes: ATP is dissolved in water, filter-sterilized, and stored at -20 °C in small volumes (less than 100 µl). Each ATP sample should only be thawed once. The 10 × ligation buffer (without ATP) is also stored at -20 °C.

### A.2.2 10 × T4-POLYNUCLEOTIDE-KINASE BUFFER

|         |                                                                                      |
|---------|--------------------------------------------------------------------------------------|
| 1000 µl | 1 M Tris-HCl (pH 7.6)                                                                |
| 200 µl  | 1 M MgCl <sub>2</sub>                                                                |
| 200 µl  | 500 mM dithiothreitol (DTT)                                                          |
| 200 µl  | 10 mM spermidine                                                                     |
| 4 µl    | 500 mM EDTA (pH 8.0)                                                                 |
| 396 µl  | water                                                                                |
| 2000 µl | 500 mM Tris-HCl 100 mM MgCl <sub>2</sub> 50 mM DTT 1 mM spermidine 1 mM EDTA, pH 7.6 |

### A.2.3 TRIS-BORATE-EDTA DNA-ELECTROPHORESIS BUFFER

To make 2 l of a 10 × stock solution:

|                         |                      |
|-------------------------|----------------------|
| 216 g                   | Tris base            |
| 110 g                   | boric acid           |
| 80 ml                   | 500 mM EDTA (pH 8.0) |
| H <sub>2</sub> O to 2 l |                      |

### A.2.4 TE BUFFER (pH 8.0)

|         |                                  |
|---------|----------------------------------|
| 100 µl  | sterile 1 M Tris-HCl (pH 8.0)    |
| 20 µl   | sterile 500 mM EDTA (pH 8.0)     |
| 9.88 ml | sterile water                    |
| 10 ml   | 10 mM Tris-HCl 1 mM EDTA, pH 8.0 |

To prepare TE buffer at pH 7.6 use 1 M Tris-HCl (pH 7.6) and 500 mM EDTA (pH 7.6).

### A.2.5 10 mM Tris-HCl 50 mM NaCl, pH 8.0

|        |                      |
|--------|----------------------|
| 100 ml | 200 mM Tris solution |
| 5.85 g | NaCl                 |

Add water to approximately 1.9 l and adjust the pH to 8.0 using a concentrated solution of HCl (32 %). Adjust the volume to 2 l with water. Store at 4 °C.

### A.2.6 10 mM Tris-HCl 250 mM NaCl, pH 8.0

|         |                      |
|---------|----------------------|
| 100 ml  | 200 mM Tris solution |
| 29.22 g | NaCl                 |

Add water to approximately 1.9 l and adjust the pH to 8.0 using a concentrated solution of HCl (32 %). Adjust the volume to 2 l with water. Store at 4 °C.

**A.2.7 10 mM Tris-HCl 360 mM NaCl, pH 8.0**

|         |                      |
|---------|----------------------|
| 100 ml  | 200 mM Tris solution |
| 42.08 g | NaCl                 |

Add water to approximately 1.9 l and adjust the pH to 8.0 using a concentrated solution of HCl (32 %). Adjust the volume to 2 l with water. Store at 4 °C.

**A.2.8 10 mM Tris-HCl 50 mM NaCl 12 % sucrose, pH 8.0**

For protein purification by sucrose-gradient centrifugation.

|         |                                       |
|---------|---------------------------------------|
| 12.5 ml | 200 mM Tris solution                  |
| 0.73 g  | NaCl                                  |
| 30 g    | analytical-grade sucrose (e.g. Merck) |

Add water to approximately 200 ml, stir to dissolve the sucrose, and adjust the pH to 8.0. Adjust the volume to 250 ml with water and store at -20 °C.

**A.2.9 10 mM Tris-HCl 50 mM NaCl 39 % sucrose, pH 8.0**

For protein purification by sucrose-gradient centrifugation.

|         |                                       |
|---------|---------------------------------------|
| 12.5 ml | 200 mM Tris solution                  |
| 0.73 g  | NaCl                                  |
| 97.5 g  | analytical-grade sucrose (e.g. Merck) |

Add water to approximately 200 ml, stir to dissolve the sucrose, and adjust the pH to 8.0. Adjust the volume to 250 ml with water and store at -20 °C.

**A.3 SOLUTIONS****A.3.1 ANTIBIOTIC SOLUTIONS**

*Ampicillin stock solution (50 mg Ap/ml):*

Dissolve 0.5 g ampicillin in water and make the volume up to 10 ml. Dispense in 1-ml aliquots in eppendorf tubes and store at -20 °C. Ampicillin is assumed to be self-sterilizing.

*Chloramphenicol stock solution (10 mg/ml):*

Dissolve 0.1 g chloramphenicol in 10 ml of 50 % ethanol. Store in a sterile standard container at -20 °C. Chloramphenicol is assumed to be self-sterilizing.

*Rifampicin stock solution (10 mg/ml):*

Dissolve 10 mg rifampicin in 1 ml of methanol. Store in a sterile eppendorf tube at -20 °C. Rifampicin is assumed to be self-sterilizing.

A.3.2 EDTA STOCK SOLUTION

Mix 186.1 g disodium ethylenediaminetetra-acetate.2H<sub>2</sub>O with 800 ml water and 18 g NaOH. Stir vigorously to dissolve and adjust the pH to 8.0. Adjust the volume to 1 l to give 500 mM EDTA.

A.3.3 10 × M9 SALTS SOLUTION

|         |                                              |
|---------|----------------------------------------------|
| 12.00 g | Na <sub>2</sub> HPO <sub>4</sub> (anhydrous) |
| 6.00 g  | KH <sub>2</sub> PO <sub>4</sub> (anhydrous)  |
| 1.00 g  | NaCl                                         |
| 1.07 g  | NH <sub>4</sub> Cl                           |

H<sub>2</sub>O to a final volume of 200 ml.

(Sambrook *et al.*, 1989).

For nitrogen-deficient minimal media the NH<sub>4</sub>Cl is omitted.

A.3.4 PICRIC ACID REAGENT

|       |                                       |
|-------|---------------------------------------|
| 5 ml  | 1.2 % picric acid solution GR (Merck) |
| 6 ml  | 0.5 M Na <sub>2</sub> CO <sub>3</sub> |
| 1 ml  | H <sub>2</sub> O                      |
| 12 ml |                                       |

A.3.5 RNase STOCK SOLUTION

Dissolve RNase A in 10 mM Tris-HCl 15 mM NaCl (pH 7.5) at a concentration of 10 mg RNase/ml. Heat at 100 °C for 15 min, allow to cool slowly to room temperature, aliquot into microfuge tubes, and store at -20 °C.

### A.3.6 SOLUTIONS FOR PROTEIN DETERMINATION

|                          |                                                                                 |
|--------------------------|---------------------------------------------------------------------------------|
| Reagent A:               | 2 % $\text{Na}_2\text{CO}_3$ in 0.10 M NaOH                                     |
| Reagent B <sub>1</sub> : | 1 % $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in water                          |
| Reagent B <sub>2</sub> : | 2 % sodium, or potassium, tartrate in water                                     |
| Reagent C:               | 50 ml Reagent A + 0.5 ml Reagent B <sub>1</sub> + 0.5 ml Reagent B <sub>2</sub> |
| Reagent E:               | 1 ml commercial Folin-Ciocalteu phenol reagent + 1.8 ml water                   |

(Adapted from Lowry *et al.*, 1951). See section 2.3.7 for the assay method.

### A.3.7 SOLUTIONS FOR DETERMINATION OF AMMONIA

See Fawcett and Scott (1960). The assay method is described in section 3.3.11

4 M NaOH:  
160 g NaOH/l

|                         |          |
|-------------------------|----------|
| Sodium phenate:         |          |
| 25 g                    | phenol   |
| 800 ml                  | water    |
| 78 ml                   | 4 M NaOH |
| H <sub>2</sub> O to 1 l |          |

Store at 4 °C in the dark. Allow the solution to warm up to room temperature before use.

0.02 M NaOCl:  
0.25 ml 15 % technical grade NaOCl (15 % w/v NaOCl  $\approx$  2.02 M)  
H<sub>2</sub>O to 25 ml

0.01 % Sodium nitroprusside:  
A 1 % solution of  $\text{Na}_2\text{Fe}(\text{CN})_5\text{NO} \cdot 5\text{H}_2\text{O}$  in water is diluted to 0.01 % in water.  
Store at room temperature in the dark.

### A.3.8 SOLUTIONS FOR DETERMINATION OF CYANATE

See Martin and McClelland (1951). The assay method is described in section 3.3.11

*Copper-pyridine reagent:*

|         |                                                   |
|---------|---------------------------------------------------|
| 2.95 ml | 0.276 M $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ |
| 1.95 ml | water                                             |
| 1.0 ml  | pyridine stock solution (12.39 M)                 |
| 5.9 ml  |                                                   |

Prepare a fresh solution of the reagent for each experiment.

*Cyanate stock solution (50 mM):*

Dissolve 0.1626 g NaOCN in 10 mM Tris-HCl 50 mM NaCl, pH 8.0 and adjust the volume to 50 ml with the same buffer. Store at room temperature (in the dark). A fresh stock solution should be prepared every few weeks as sodium cyanate is slowly hydrolysed to  $\text{Na}_2\text{CO}_3$  and urea.

A.3.9 SOLUTIONS FOR DETERMINATION OF FORMAMIDE

See Ingram (1960) and Fry & Millar (1972). The assay method is described in section 3.3.11

*3.5 M NaOH:*

7 g NaOH in 50 ml

*2.3 M Hydroxylamine hydrochloride:*

15.98 g  $\text{NH}_2\text{OH} \cdot \text{HCl}$  in 100 ml

Store at room temperature (in the dark).

*4 M HCl:*

9.822 ml concentrated HCl solution (10.18 M) made up to 25 ml with water.

*1.23 M  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  / 0.075 M HCl:*

Dissolve 8.31 g  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  in 20 ml water. Add 0.469 ml 4 M HCl and make volume up to 25 ml with water. Store at room temperature.

*Stock formamide solution (400 mM):*

Mix 0.1 ml of concentrated  $\text{HCONH}_2$  solution (25.05 M) and 6.17 ml 10 mM Tris-HCl 50 mM NaCl, pH 8.0 to give 6.27 ml 400 mM  $\text{HCONH}_2$  10 mM Tris-HCl 50 mM NaCl (pH  $\approx$  8). Store at room temperature in the dark.

### A.3.10 SOLUTIONS FOR DETERMINATION OF THIOCYANATE

See Baily (1957). The assay method is described in section 3.3.11

*Ferric ammonium sulphate stock solution (100 mM):*

Dissolve 4.82 g  $\text{NH}_4\text{Fe}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$  in water and make the volume up to 100 ml with water. Dilute to 15 mM for the thiocyanate assay.

*2 M HCl:*

Dilute concentrated HCl solution (10.18 M) 1-in-5 in water.

*Sodium thiocyanate stock solution (100 mM):*

Dissolve 0.2027 g NaSCN in water and make the volume up to 25 ml.

*Stock thiocyanate assay mixture:*

Mix together:

|         |                                 |
|---------|---------------------------------|
| 7.0 ml  | 15 mM ferric ammonium sulphate  |
| 4.55 ml | water                           |
| 0.7 ml  | 2 M HCl                         |
| 7.0 ml  | acetone                         |
| 14.0 ml | 2-butanone (methylethyl ketone) |

Dispense into eppendorf tubes in 950- $\mu\text{l}$  volumes for the thiocyanate assay. Prepare a fresh solution of the assay mixture for each experiment.

### A.3.11 SOLUTIONS FOR COLONY WESTERN-BLOTS

See Sambrook *et al.* (1989).

*Lysozyme stock solution (50 mg/ml):*

Prepare a 50 mg/ml solution of lysozyme in water. Filter-sterilize and store in 50- $\mu\text{l}$  aliquots at  $-20^\circ\text{C}$ .

*0.01 M HCl:*

Mix 0.1 ml concentrated HCl solution (10.18 M) with 101.7 ml water.

*DNase I stock solution:*

Prepare a 1 mg/ml DNase I stock solution in 0.01 M HCl.

*Lysis buffer:*

To prepare a 10 × strength stock solution:

|         |                                      |
|---------|--------------------------------------|
| 60.55 g | Tris                                 |
| 43.83 g | NaCl                                 |
| 5.16 g  | MgCl <sub>2</sub> ·6H <sub>2</sub> O |

Add water to approximately 450 ml and adjust the pH to 7.8 with concentrated HCl (32 %). Adjust the volume to 500 ml with water to give a 1 M Tris-HCl 1.5 M NaCl 50 mM MgCl<sub>2</sub>·6H<sub>2</sub>O solution. Autoclave and then store at room temperature.

To prepare lysis buffer for use, mix the following together:

|                            |                                  |
|----------------------------|----------------------------------|
| 20 ml                      | 10 × lysis buffer stock solution |
| 3 g                        | bovine serum albumin (BSA)       |
| 0.16 ml                    | 50 mg/ml lysozyme solution       |
| 0.2 ml                     | 1 mg/ml DNase solution           |
| H <sub>2</sub> O to 200 ml |                                  |

*TNT:*

To prepare a 10 × strength stock solution:

|         |      |
|---------|------|
| 3.03 g  | Tris |
| 21.95 g | NaCl |

Add water to approximately 200 ml and adjust the pH to 8.0 with concentrated HCl (32 %). Add 1.25 ml Tween 20 and adjust the volume to 250 ml with water to give a 100 mM Tris-HCl 1500 mM NaCl 0.5 % Tween 20 solution.

To prepare TNT for use:

|                            |          |
|----------------------------|----------|
| 50 ml                      | 10 × TNT |
| H <sub>2</sub> O to 500 ml |          |

*Blocking buffer:*

|                            |                                         |
|----------------------------|-----------------------------------------|
| 20 ml                      | 10 × TNT                                |
| 10 g                       | Commercially available skim-milk powder |
| H <sub>2</sub> O to 200 ml |                                         |

*TNT 0.1 % BSA:*

|                            |          |
|----------------------------|----------|
| 50 ml                      | 10 × TNT |
| 0.5 g                      | BSA      |
| H <sub>2</sub> O to 500 ml |          |

TNT 0.1 % BSA 0.1 % Nonidet P-40:

|                            |              |
|----------------------------|--------------|
| 25 ml                      | 10 × TNT     |
| 0.25 g                     | BSA          |
| 0.25 ml                    | Nonidet P-40 |
| H <sub>2</sub> O to 250 ml |              |

### A.3.12 SOLUTIONS FOR ISOLATION OF PLASMID DNA FROM *E. coli*

Procedures for both large- and small-scale isolation of plasmid DNA from *E. coli* are described in Appendix B.

#### *Solution I:*

|                    |                                  |
|--------------------|----------------------------------|
| 250 µl             | sterile 1 M glucose              |
| 125 µl             | sterile 1 M Tris-HCl (pH 8.0)    |
| 100 µl             | sterile 500 mM EDTA (pH 8.0)     |
| 4.525 ml           | sterile water                    |
| 5 ml 50 mM glucose | 25 mM Tris-HCl 10mM EDTA, pH 8.0 |

#### *Solution II:*

|                  |               |
|------------------|---------------|
| 200 µl           | 10 M NaOH     |
| 1 ml             | 10 % SDS      |
| 8.8 ml           | sterile water |
| 10 ml 0.2 M NaOH | 1 % SDS       |

#### *Solution III (5 M acetate):*

|         |                       |
|---------|-----------------------|
| 60 ml   | 5 M potassium acetate |
| 11.5 ml | glacial acetic acid   |
| 28.5 ml | water                 |

### A.3.13 SOLUTIONS FOR POLYACRYLAMIDE GEL ELECTROPHORESIS

15 % Resolving gel (20 × 20 cm plates):

|                           |                                      |
|---------------------------|--------------------------------------|
| 18.75 ml                  | 40 % acrylamide 0.2 % bis-acrylamide |
| 16.7 ml                   | 1.125 M Tris pH 8.8                  |
| 0.5 ml                    | 10 % ammonium persulphate            |
| 0.5 ml                    | 10 % SDS                             |
| H <sub>2</sub> O to 50 ml |                                      |

Mix, add 0.05 ml N,N,N',N'-tetramethyl-ethylenediamine (TEMED), mix again, and pour gel. Pipette water onto top surface of gel mixture to ensure that the gel sets with a straight surface. Pour off water before adding stacker gel.

**6.7 % Stacker gel:**

|                           |                           |                      |
|---------------------------|---------------------------|----------------------|
| 2 ml                      | 40 % acrylamide           | 0.2 % bis-acrylamide |
| 4 ml                      | 0.375 M Tris              | pH 6.8               |
| 0.3 ml                    | 10 % ammonium persulphate |                      |
| 0.125 ml                  | 10 % SDS                  |                      |
| H <sub>2</sub> O to 12 ml |                           |                      |

Mix, add 0.02 ml TEMED, mix again, and pour onto stacker gel.

**Sample application buffer:**

|                           |                              |
|---------------------------|------------------------------|
| 2 ml                      | 0.375 M Tris-HCl, pH 6.8     |
| 2 ml                      | 10 % SDS                     |
| 2 ml                      | glycerol                     |
| 0.5 ml                    | 2-mercaptoethanol (optional) |
| 1 mg                      | bromophenol blue             |
| H <sub>2</sub> O to 10 ml |                              |

**Tank buffer:**

|                         |         |
|-------------------------|---------|
| 30 g                    | glycine |
| 6 g                     | Tris    |
| 2 g                     | SDS     |
| H <sub>2</sub> O to 2 l |         |

**Page Blue G90 Stain (0.1 %):**

Dissolve 1 g Page Blue G90 in 900 ml water by stirring on a magnetic stirrer (this takes some time). Add 30 ml concentrated phosphoric acid (85 %) and adjust the volume to 1 l with water to give 0.1 % Page Blue G90 3 % phosphoric acid.

**Coomassie Brilliant Blue R250 Stain:**

Dissolve 1 g of Coomassie Brilliant Blue in 225 ml water. Add 225 ml methanol (technical grade) and then 50 ml of concentrated acetic acid (final volume = 500 ml).

**A.3.14 SOLUTIONS FOR PREPARATION OF COMPETENT *E. coli* CELLS**

The procedure for preparing competent cells of *E. coli* is described in Appendix B.

**Transformation and Storage Buffer (TSB):**

|         |                                                  |
|---------|--------------------------------------------------|
| 10 ml   | Luria broth containing 10 % PEG 4000             |
| 100 µl  | sterile 1 M MgCl <sub>2</sub> ·6H <sub>2</sub> O |
| 100 µl  | sterile 1 M MgSO <sub>4</sub> ·7H <sub>2</sub> O |
| 500 µl  | dimethyl sulphoxide (DMSO)                       |
| 10.7 ml |                                                  |

Notes: Luria broth/10 % PEG 4000 is prepared as a bulk solution, dispensed into standard containers in 10-ml aliquots, and sterilized by autoclaving. DMSO is assumed to be self-sterilizing.

*TSB 20 mM glucose:*

|                |                        |
|----------------|------------------------|
| 120 $\mu$ l    | sterile 500 mM glucose |
| <u>2.88 ml</u> | TSB                    |
| 3 ml           |                        |

### A.3.15 SOLUTIONS FOR SOUTHERN HYBRIDIZATION

Modified from Church and Gilbert (1984).

*1 M Sodium phosphate buffer (pH 7.2):*

Dissolve 179.07 g  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  in approximately 900 ml of water and add 4 ml concentrated phosphoric acid (85 %  $\text{H}_3\text{PO}_4$ ). Measure the pH and adjust to 7.2 if necessary. Autoclave.

*25 % SDS stock solution:*

Add water to 250 g of SDS to a volume of approximately 900 ml. Heat gently with stirring to dissolve. Allow to cool to room temperature and adjust the volume to 1 l with water.

*Church hybridization buffer ([Na<sup>+</sup>] = 1.9 M):*

|                |                                     |
|----------------|-------------------------------------|
| 0.5 g          | Commercial skim-milk powder         |
| 50.0 ml        | 1 M sodium phosphate buffer, pH 7.2 |
| 1.0 ml         | 100 mM EDTA                         |
| 28.0 ml        | 25 % SDS                            |
| <u>20.5 ml</u> | water                               |
| 100 ml         |                                     |

Note: Dissolve the skim-milk powder in the water and then add the other solutions (the SDS should be added last). This gives a 500 mM phosphate 0.5 % milk powder 1 mM EDTA 7 % SDS solution.

*Wash buffer A ([Na<sup>+</sup>] = 0.25 M):*

|                                |                                     |
|--------------------------------|-------------------------------------|
| 100 ml                         | 25 % SDS                            |
| 20 ml                          | 1 M sodium phosphate buffer, pH 7.2 |
| 5 ml                           | 100 mM EDTA                         |
| $\text{H}_2\text{O}$ to 500 ml |                                     |

This gives a 40 mM phosphate 5 % SDS 1 mM EDTA solution.

*Wash buffer B* ( $[Na^+] = 0.11\text{ M}$ ):

|                         |                                     |
|-------------------------|-------------------------------------|
| 40 ml                   | 25 % SDS                            |
| 40 ml                   | 1 M sodium phosphate buffer, pH 7.2 |
| 10 ml                   | 100 mM EDTA                         |
| H <sub>2</sub> O to 1 l |                                     |

This gives a 40 mM phosphate 1 % SDS 1 mM EDTA solution.

### A.3.16 SOLUTIONS FOR WESTERN BLOTS

*Electroblotting buffer:*

For a 10 × strength stock solution:

|                         |         |
|-------------------------|---------|
| 30.3 g                  | Tris    |
| 144 g                   | glycine |
| H <sub>2</sub> O to 1 l |         |

This gives a 250 mM Tris-HCl 1.92 M glycine stock solution which can be autoclaved and stored at room temperature.

To prepare the buffer for protein transfer:

|                         |                                                     |
|-------------------------|-----------------------------------------------------|
| 500 ml                  | 10 × strength electroblotting buffer stock solution |
| 1000 ml                 | technical-grade methanol                            |
| H <sub>2</sub> O to 5 l |                                                     |

This gives normal-strength electroblotting buffer containing 20 % methanol. Store at 4 °C. The solution can be used three times before being discarded.

*Phosphate-buffered saline (PBS):*

To prepare a 10 × stock solution:

|                         |                                                                       |
|-------------------------|-----------------------------------------------------------------------|
| 19 ml                   | Solution A (1 M NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O)   |
| 81 ml                   | Solution B (1 M Na <sub>2</sub> HPO <sub>4</sub> ·12H <sub>2</sub> O) |
| 85 g                    | NaCl                                                                  |
| H <sub>2</sub> O to 1 l |                                                                       |

This gives 10 × PBS, pH 7.4. Dilute 1-in-10 before use to get normal-strength PBS (10 mM sodium phosphate 0.145 M NaCl, pH 7.4).

*Blocking buffer* (PBS/0.05 % Tween 20/2 % skim-milk powder):

|                         |                             |
|-------------------------|-----------------------------|
| 100 ml                  | 10 × PBS                    |
| 0.5 ml                  | Tween 20                    |
| 20 g                    | Commercial skim-milk powder |
| H <sub>2</sub> O to 1 l |                             |

*Washing buffer:*

|                         |          |
|-------------------------|----------|
| 100 ml                  | 10 × PBS |
| 0.5 ml                  | Tween 20 |
| H <sub>2</sub> O to 1 l |          |

*Alkaline-phosphatase substrate buffer:*

|        |                                      |
|--------|--------------------------------------|
| 6.06 g | Tris                                 |
| 2.92 g | NaCl                                 |
| 5.08 g | MgCl <sub>2</sub> ·6H <sub>2</sub> O |

Dissolve in approximately 450 ml of water and adjust the pH to 9.5. Adjust the volume to 500 ml with water to give a 100 mM Tris-HCl 100 mM NaCl 50 mM MgCl<sub>2</sub>·6H<sub>2</sub>O solution.

*Alkaline-phosphatase substrate solution:*

## Stock solutions:

## 1. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP):

Dissolve 50 mg of BCIP (Sigma Chemical Co., B-8503) in 1 ml of N,N'-dimethylformamide. Store in the dark at -20 °C.

## 2. Nitro-blue tetrazolium (NBT):

Dissolve 75 mg of NBT (Sigma Chemical Co., N-6876) in 700 µl of 100 % N,N'-dimethylformamide and add 300 µl of water. This gives a solution of 75 mg NBT/ml in 70 % N,N'-dimethylformamide. Store in the dark at -20 °C.

Mix 150 µl of BCIP with 100 ml of alkaline-phosphatase substrate buffer and then add 150 µl NBT.

A.3.17 SUCROSE SOLUTIONS FOR FRACTIONATION OF DNA FRAGMENTS

## 10 % sucrose

|                            |                       |
|----------------------------|-----------------------|
| 20 g                       | sucrose               |
| 11.68 g                    | NaCl                  |
| 4 ml                       | 1 M Tris-HCl (pH 8.0) |
| 2 ml                       | 500 mM EDTA (pH 8.0)  |
| H <sub>2</sub> O to 200 ml |                       |

This gives 200 ml of 20 mM Tris-HCl 1 M NaCl 5 mM EDTA 10 % sucrose. Autoclave.

40 % sucrose

Dissolve 80 g sucrose in 150 ml water. Autoclave.

Add:

|       |                               |
|-------|-------------------------------|
| 40 ml | sterile 5 M NaCl              |
| 2 ml  | sterile 500 mM EDTA (pH 8.0)  |
| 4 ml  | sterile 1 M Tris-HCl (pH 8.0) |
| 4 ml  | sterile water                 |

This gives 200 ml of 20 mM Tris-HCl 1 M NaCl 5 mM EDTA 40 % sucrose.

A.3.18 TRACE-METALS SOLUTION A

Used in continuous selection system at the University of Cape Town for selection of cyanide-utilizing pseudomonads from activated sewage sludge (section 2.3.2).

|        |                                                      |
|--------|------------------------------------------------------|
| 0.30 g | FeSO <sub>4</sub> ·7H <sub>2</sub> O                 |
| 0.22 g | MgCl <sub>2</sub> ·6H <sub>2</sub> O                 |
| 0.13 g | Co(NO <sub>3</sub> ) <sub>2</sub> ·6H <sub>2</sub> O |
| 0.04 g | CaCl <sub>2</sub> ·2H <sub>2</sub> O                 |
| 0.04 g | ZnSO <sub>4</sub> ·7H <sub>2</sub> O                 |

Make up to 1 l with de-ionized water. Autoclave.  
(Adapted from White *et al.*, 1988).

A.3.19 TRACE-METALS SOLUTION B

|          |                                      |
|----------|--------------------------------------|
| 134.51 g | MgSO <sub>4</sub> ·7H <sub>2</sub> O |
| 2.00 g   | CaCO <sub>3</sub>                    |
| 4.50 g   | FeSO <sub>4</sub> ·7H <sub>2</sub> O |
| 1.44 g   | ZnSO <sub>4</sub> ·7H <sub>2</sub> O |
| 0.64 g   | MnSO <sub>4</sub> ·H <sub>2</sub> O  |
| 0.25 g   | CuSO <sub>4</sub> ·5H <sub>2</sub> O |
| 0.24 g   | CoCl <sub>2</sub> ·6H <sub>2</sub> O |
| 0.06 g   | H <sub>3</sub> BO <sub>3</sub>       |
| 51.3 ml  | Concentrated HCl (32 %)              |

Make up to 1 l with de-ionized water. Autoclave.  
(Adapted from Bauchop and Elsdén, 1960).

**A.3.20 TRIS-BUFFERED PHENOL**

|        |                               |
|--------|-------------------------------|
| 500 g  | commercial crystalline phenol |
| 0.6 g  | 8-hydroxyquinoline            |
| 7.5 ml | 2 M NaOH                      |
| 6.0 ml | 1 M Tris-HCl (pH 7.6)         |
| 130 ml | water                         |

Leave overnight at room temperature to dissolve. Add an equal volume of 500 mM Tris-HCl (pH 8.0) and mix by inversion (or stirring) for 15 min. Remove the upper aqueous phase and extract the phenolic phase with an equal volume of 100 mM Tris-HCl (pH 8.0) as above. Repeat the 100 mM Tris-HCl (pH 8.0) extractions until the pH of the phenolic phase is above 7.8 (measured with pH paper). Store the equilibrated phenol under  $1/_{10}$  of a volume of 100 mM Tris-HCl (pH 8.0) at 4 °C for up to 1 month (or at -20 °C for long-term storage).

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## APPENDIX B

### STANDARD METHODS

#### B.1 PROCEDURE FOR SMALL-SCALE ISOLATION OF PLASMID DNA FROM *E. coli*

Plasmid DNA was isolated from a 5-ml overnight culture of *E. coli* (Luria broth + 100 µg Ap/ml, 37 °C) by the alkaline-lysis method of Ish-Horowicz and Burke (1981). The cells from 1.5 ml of the culture were harvested by microfuging for 1 min at room temperature. The cell pellet was resuspended in 200 µl of room-temperature Solution I (Appendix A) and mixed with 400 µl of freshly prepared, room-temperature Solution II (Appendix A). The solutions were mixed by inversion, incubated on ice for 7 min, and then mixed with 300 µl of ice-cold Solution III (Appendix A). The solution was incubated on ice for 7 min, microfuged for 5 min at 4 °C, and then transferred to ice. A 730-µl volume of the supernatant was pipetted into a clean microfuge tube containing 730 µl of room-temperature isopropanol and mixed by inversion. The DNA was recovered by microfuging for 12 min at room temperature (or 4 °C). The DNA pellet was resuspended in 450 µl of TE buffer (pH 8.0), mixed with 50 µl 3 M sodium acetate (pH 5.2) and 1 ml of room-temperature absolute ethanol, and incubated at room temperature for 5 min. The reprecipitated DNA was recovered by microfuging for 12 min at 4 °C. The DNA pellet was washed with 1 ml of cold 70 % ethanol, air dried, and finally resuspended in 50 µl TE buffer (pH 8.0). Volumes of 5 µl of this DNA preparation were used per restriction-endonuclease digestion, and 1 - 5 µl were used for *E. coli* transformations.

#### B.2 PROCEDURE FOR LARGE-SCALE ISOLATION OF PLASMID DNA FROM *E. coli*

A 200-ml Luria-broth culture of *E. coli* (containing 100 µg Ap/ml) was grown overnight at 37 °C with vigorous shaking. The cells were harvested by centrifugation at 3000 × g for 10 min, resuspended in 4.5 ml of Solution I (Appendix A), incubated at room temperature for 5 min, and then placed on ice for 2 min. The solution was mixed with 9 ml of cool Solution II (Appendix A),

incubated on ice for 5 min, mixed with 6.8 ml of ice-cold Solution III (Appendix A), and incubated on ice for a further 10 min. The entire volume (c. 25 ml) was transferred to a sterile SS34 centrifuge tube and centrifuged at  $12\,000 \times g$  for 15 min to remove the cellular debris. The supernatant was transferred to two clean, sterile centrifuge tubes. Two volumes of absolute (100 %) ethanol were added to each tube, the solutions were mixed well, and incubated for 10 min, or longer, on ice. The precipitated DNA was recovered by centrifugation for 15 min at  $27\,000 \times g$  and the pellet was drained. Each DNA pellet was resuspended in 2.1 ml of TE buffer (pH 8.0) and the two solutions were combined. Cesium chloride (5.1 g) was dissolved in the DNA solution. The solution was mixed with 100  $\mu$ l 10 mg/ml ethidium bromide, and then centrifuged at  $27\,000 \times g$  for 15 min. The solution was removed from the debris and the refractive index was adjusted to 1.394 - 1.396. The solution was sealed in a Beckman Quickseal™ ultracentrifuge tube and centrifuged at 55 000 rpm in a Beckman VTi65 rotor for 12 - 16 h.

The centrifuge tube was irradiated with ultraviolet light (350 nm), the fluorescent band of plasmid DNA was removed in a small volume (approximately 500  $\mu$ l), and the ethidium bromide was removed by three extractions with NaCl-saturated isopropanol. The DNA was dispensed into microfuge tubes in 150- $\mu$ l volumes, mixed with 2 volumes of sterile water, and 2 volumes of absolute ethanol (or 1 volume of isopropanol). The tubes were incubated on ice for 10 min and then microfuged for 10 min at 4 °C. The DNA pellets were resuspended in TE buffer (pH 8.0) so that the total volume of the combined solutions was 200 - 500  $\mu$ l.

The DNA was further purified by mixing 166- $\mu$ l volumes of DNA with 333  $\mu$ l of sterile water, precipitating with 1 ml absolute ethanol, and resuspending in 450  $\mu$ l TE buffer (pH 8.0). A final precipitation step was carried out by adding 50  $\mu$ l 3 M sodium acetate (pH 5.2), 1 ml absolute ethanol, and incubating on ice for 15 min or longer. The DNA was recovered by microfuging for 12 - 15 min at 4 °C (or room temperature). The DNA was washed with 1 ml of cold 70 % ethanol, air dried, and resuspended in 200  $\mu$ l TE buffer (pH 8.0).

### B.3 ISOLATION OF *B. pumilus* C1 CHROMOSOMAL DNA

Sterile Oxoid nutrient broth (200 ml in a 1-l conical flask) was supplemented with manganous ions to 0.01 mg  $Mn^{2+}$ /l, inoculated with a single colony of *B. pumilus* C1, and grown at 30 °C with shaking for 15 h. The culture was harvested by centrifugation at  $16\,000 \times g$  for 10 min at 4 °C. The cell pellet was resuspended in 20 ml of Solution I (Appendix A), incubated at room temperature for 15 min, and then mixed with filter-sterilized lysozyme (20 mg/ml) at a final concentration of 0.5 mg lysozyme/ml. The solution was incubated at 37 °C for 30 min with gentle agitation and then mixed with 2.28 ml of sterile 500 mM EDTA, pH 8.0 (final EDTA concentration 50 mM). An equal volume (22.8 ml) of Tris-buffered phenol (Appendix A) was added and the solution was swirled gently for 5 min. The aqueous and organic phases were separated by centrifugation at  $16\,000 \times g$  for 10 min at 4 °C. The upper DNA-containing phase was transferred to a Sorvall SS34 centrifuge tube. The lower phenol phase was mixed with an equal volume of TE buffer, pH 8.0 (Appendix A) and the solution was swirled gently for 5 min to extract DNA which had been partitioned into the organic phase during the initial phenol-treatment step. The phases were separated by centrifugation as before and the upper DNA-containing phase was transferred to a clean SS34 tube as before.

The phenol phase was discarded and the two aqueous phases were each mixed with an equal volume (22.8 ml) of a 24:1 mixture of chloroform and isoamyl alcohol. The solutions were mixed gently by inversion for 5 min and the phases were allowed to separate. The upper aqueous phase in each case was transferred to a clean tube. An equal volume (22.8 ml) of n-butanol was added to each of the two aqueous phases and the solutions were mixed gently by inversion for 5 min. n-Butanol withdraws water from the aqueous phase thereby concentrating the DNA. The phases were separated by centrifugation at  $16\,000 \times g$  for 10 min at 4 °C. The milky, DNA-containing lower phase in each case was transferred to a clean SS34 tube. The chloroform/isoamyl alcohol and n-butanol steps were repeated as above (smaller volumes were used as the volume of the aqueous phases had been reduced).

The DNA-containing solutions were dispensed into lengths of dialysis tubing (32 mm diameter), allowing for an increase in volume of the solutions, and dialysed once against 5 l of TE buffer (pH 8.0) at 4 °C for 17 h. The dialysed solutions were mixed with RNase (Appendix A) at a final concentration of 0.1 mg RNase/ml

and incubated at 37 °C for 15 min. RNase was removed by extraction with Tris-buffered phenol, followed by two extractions with chloroform/isoamyl alcohol and n-butanol as before (with the exception that the phenol phase was not extracted with TE buffer).

The DNA-containing solutions were dialysed once against 5 l of TE buffer (pH 8.0) as described before. The two DNA-containing solutions (of approximately 20 ml each) were stored in sterile standard containers at 4 °C. The purity of the isolated chromosomal DNA was assessed spectrophotometrically and by agarose-gel electrophoresis.

#### B.4 PREPARATION OF COMPETENT *E. coli* CELLS

The dimethyl sulphoxide (DMSO) method for preparation of competent *E. coli* cells was described by Chung and Miller (1988).

*E. coli* was grown overnight at 37 °C in 5 ml of Luria broth in a standard container. A 200- $\mu$ l volume of this stationary-phase culture was pipetted into 20 ml of fresh Luria broth in a 200-ml conical flask and grown at 37 °C with vigorous shaking until the optical density at 600 nm was in the range 0.3 - 0.6 (c. 2 h to reach  $OD_{600nm} = 0.4$ ). The cells were harvested by centrifugation at  $3000 \times g$  for 5 min at 4 °C (rotor not pre-cooled), resuspended in 2 ml of Transformation and Storage Buffer (TSB; Appendix A) in the centrifuge tube, and incubated on ice for 15 min to 6 h. Competent cells (100  $\mu$ l) were mixed with 10 - 20  $\mu$ l of the DNA to be transformed, placed on ice for 10 min, and then mixed with 900  $\mu$ l of expression broth (TSB containing 20 mM glucose). The mixture was incubated at 37 °C in a waterbath for 30 - 60 min to allow the antibiotic-resistance marker genes on the transformed plasmids to be expressed. The expression mixture was plated in 100- $\mu$ l volumes on Luria agar medium containing 100  $\mu$ g Ap/ml, and incubated at 37 °C overnight.

## B.5 RECYCLING DE52 ANION-EXCHANGE MATRIX

Used DE52 anion-exchange matrix was flushed with 2 - 3 bed volumes of 1 M NaCl directly after use, and was then stored at 4 °C as a slurry in water or buffer (containing 0.02 % sodium azide) before recycling the matrix.

The supernatant from the used slurry was decanted and the slurry was placed in a large beaker (2-l or bigger). De-ionized water was added to 1.9 l followed by 100 ml of concentrated (32 %) HCl. The slurry was stirred with a glass rod for 5 - 10 min, and filtered on a Buchner funnel with 2 discs of Whatman No.1 filter paper. The slurry cake was washed with 10 - 15 l of de-ionized water (or until the pH of the filtrate was between pH 4 and 5). The slurry was filtered dry and then transferred into a large beaker again. The Whatman filter discs were removed and de-ionized water was added to 1.9 l, followed by 100 ml 10 M NaOH. The slurry was stirred with a glass rod for 5 - 10 min and then filtered on a Buchner funnel again with 2 discs of Whatman No.1 filter paper. The slurry cake was washed with 10 - 15 l of de-ionized water (or until the pH of the filtrate was between pH 8 and 9), filtered dry, and then transferred into a large beaker again. The Whatman filter discs were removed and 1.5 l of 10 mM Tris-HCl 50 mM NaCl (pH 8.0) buffer was added. The slurry was stirred gently on a magnetic stirrer and the pH was adjusted back to pH 8.0). The slurry was filtered on a Buchner funnel with 2 discs of Whatman No.1 filter paper, washed with 1 l of 10 mM Tris-HCl 50 mM NaCl (pH 8.0) buffer, and filtered dry. The regenerated anion-exchange matrix was stored at 4 °C in 10 mM Tris-HCl 50 mM NaCl (pH 8.0) buffer containing 0.02 % sodium azide (the volume of the slurry was about 150 % of the volume of the dry slurrycake).



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