A STUDY OF GENETIC INSTABILITY AND DNA REPAIR IN SELECTED MUTANTS OF *STREPTOMYCES CATTLEYA*

BY

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Abstract

Three mutants of Streptomyces cattleya NRRL 8057 with lesions in the DNA repair pathway were obtained by NTG mutagenesis. The mutants were selected on the basis of their resistance or sensitivity to Mitomycin C. Of the three mutants, two, R6 and R12, were selected as MMC resistant and one, S26, as MMC sensitive. They were also UV^r and UV^s, respectively. The mutants were subjected to UV irradiation in order to obtain UV kill curves. The curves generated for the two MMC^r mutants showed induction or derepression of a second repair system. In instability studies performed on them, the two MMC^r mutants showed increased instability compared to the wild type at low UV dosage. However, the instability dropped to below that of the wild type at high UV dosages. Both these mutants appeared to roughly mimic the plateau shown by the wild type as higher levels of UV irradiation were achieved. S26 in contrast showed a much higher initial incidence of instability, and no plateau was observed at high UV dosages. Single strand breaks were induced in the wild type and mutant strains by incorporation of P^{32} into their DNA. R6 showed a five-fold increase in instability after this treatment. R12 showed no significant change, and S26 showed a slight decrease, which may be due to the increased lethality of DNA damage sustained by this strain due to its relatively labile nature. The strains were

further analysed using polyacrylamide gel electrophoresis of total cellular proteins. S26 showed a startlingly different protein profile. R6 was almost identical to the wild type, while R12 was extremely similar, showing only the loss of one major band and a significant lowering of the amount of detectable protein in the region below this band.

UV repair in S.cattleya has been studied here using reactivation of UV-irradiated phage VC11 to overcome the potential interaction between UV irradiation and pleiotropic cellular effects. In an earlier study, a constitutive caffeine-inhibitable host mediated DNA repair system had been clearly identified in S.cattleya, and a second, arsenite-inhibitable, system was postulated to be present. Using the three mutants obtained in this study, the existence of the second system has been proved. These results allow for a more detailed analysis of the three mutants, and an assignation to each mutant of a phenotype based on the two DNA repair systems extant within it.

The effects of divalent metal ions on bacteria have been widely studied. Several studies have been performed in Aspergillus nidulans concerning the effects of manganese (Burr, Roper and Relton, 1982) and cobalt (Sexton and Roper, 1984; Daud et al, 1985) on the instability of that organism. Cobalt appeared to increase, and manganese, despite its well-documented mutagenic properties, appeared to decrease instability in A.nidulans. This led us to ask

the question as to how these ions would affect the genetic instability of S.cattleya wild type as well as that of the three mutants under study. It was shown that both Mn^{2+} and Co^{2+} slightly induced instability in S.cattleya wild type. A similar effect was seen in S26. R6 remained essentially unchanged, while there was a major increase in the stability level of R12 in the presence of manganese.

The possible induction of proteins concerned with DNA repair was attempted by irradiation of the wild type and mutant strains of S.cattleya with low levels of UV, followed by growth and testing at intervals for the presence of any new proteins arising through the growth period. This was done by SDS-polyacrylamide gel electrophoresis. The gels so obtained were silver stained and protein profiles obtained by scanning the gels. Unfortunately, only the wild type S.cattleya gave clear results. Observation of any UV induced proteins in the mutant strains was unsuccessful, due to technical reasons.

Due to their changed natures with regard to the DNA repair systems as compared to the wild type organism, it could be expected that the mutants would show correspondingly different reactions to mutagenesis. This was tested using three different mutagens, namely ethylmethanesulfonate (EMS), methylmethanesulfonate (MMS) and

N-methyl-N'-nitro-N-nitrosoguanidine (NTG). The reactions of the three mutants and the wild type S.cattleya were tested against a range of concentrations of these mutagens. Two specific aspects were concentrated on: the survival rate of the strains with increasing concentration of the mutagen, and the mutation rate (or percentage of mutants among the survivors). S26 showed a low survival rate with all three mutagens tested. However, in all cases R6 and R12 showed a higher survival rate than the wild type although with MMS the differences are the least marked. NTG had by far the most powerful effect on all strains, both where survival and mutagenesis were concerned. The mutagenesis curves of R6 after treatment with all three mutagens remained very similar to one another.

Chapter 1

Introduction

1.1 DNA repair: an overview

Most of the current knowledge of DNA repair systems and the mechanisms by which they operate has been gained through study of such systems in Escherichia coli. This organism has served as a reference and a model for many studies involving DNA repair in both prokaryotes and eukaryotes. DNA is damaged by many agents in a multitude of different ways; such damage can cause the induction of mutagenic repair mechanisms. Mechanisms for replicating damaged DNA have been shown to be functionally related to cellular control of mutagenesis induced by UV light (Coyne, PhD thesis, 1985). UV irradiation generates several photoproducts in DNA, and, of those known, the one that has been shown to have the most lethal and mutagenic effect is the intrastrand dimer of adjacent pyrimidines. Usually, dimerisation occurs between two thymines although thymine-cytosine and cytosine-cytosine dimers are also known (Howard-Flanders and Boyce, 1966; Howard-Flanders, 1968).

In bacteria, three basic processes of DNA repair are known to occur, although each process may be divided into several separate biochemical pathways. The main processes are photoreactivation, excision repair and postreplication repair (Smith, 1978). Each shall be discussed here in turn.

Photoreactivation involves restoration of damaged parts of the DNA molecule to their functional state in situ, without removing or excising such damage from the molecule (Smith, 1978). This can be the result of a spontaneous "decay" of potentially mutagenic or even lethal damage to relatively innocuous form. Alternatively, the enzymatic mechanism of photoreactivation, the enzymatic splitting of cyclobutyl pyrimidine dimers may take place. This photoreactivation event is mediated by a specific enzyme, consisting of a single polypeptide chain, that is found in both eukaryotic and prokaryotic cells. The enzyme forms a specific enzyme-substrate complex with cyclobutyl pyrimidine dimers in UV damaged DNA in the dark. Visible light (wavelength between 320 - 410 nm) is required to activate this complex. The enzyme, so activated, converts pyrimidine dimers to monomeric pyrimidines and is then released. Up to 80% of damage induced in DNA by UV radiation at 254nm can be repaired by photoreactivation. This said, it must also be mentioned that UV irradiation does cause types of damage other than the pyrimidine dimer. Some mutations originate from excisable UV damage that is not split by the

photoreactivating enzyme. This means that either such mutations are due to excisable UV damage that is not in the form of a pyrimidine dimer or that there exist certain types of pyrimidine dimers that are protected against the action of the photoreactivating enzyme in some way (Witkin, 1966). In organisms other than E.coli, photoreactivation is one of the options of repair of damaged DNA open to yeast, amongst several other pathways that will be discussed later (Hunnable and Cox, 1971). Photoreactivation has also been shown to take place in Streptomyces griseus (Eker et al.,1986) but does not appear to be operational in Streptomyces cattleya, the organism on which the present study has been performed (Coyne et al., 1984; R.Kirby, personal communication).

To distinguish them from the photoreactivation DNA pathway which requires light in order to function, the other DNA repair mechanisms known have been grouped under the collective title "dark repair" - i.e. they take place in the dark and do not need light activation of the enzymes involved in these processes. Also, despite the distinction drawn earlier between major headings under which repair processes fall, if we rigorously define "repair" as removal of lesions from DNA, then all forms of dark repair are essentially forms of excision repair (Hanawalt et al., 1979). There is an obvious generality in such a process - as long as the damage is confined to one strand of the DNA, a

common set of steps is required to repair such damage. The genetic basis of such repair systems in E.coli, particularly those responding to UV damage, has been well documented;there is a good understanding of the genes involved and of the simple sequences of enzymatic steps in the generalised excision repair process. In extremely simplified form, excision repair involves recognition of damage, the introduction of a single strand nick in the DNA chain near the lesion (incision step), the removal of the lesion and several bases adjacent to it on either side (excision step) and resynthesis initiation via the action of a DNA polymerase using the undamaged strand of DNA as a template, which are carried out simultaneously, and, finally, the closure of the remaining single strand gap enzymaticaly by polynucleotide ligase, resulting in a repaired DNA molecule (Smith, 1978). While this is the general mechanism for the major pathway of excision repair, it is not the only known excision pathway in the cell.

Genes from E.coli K12 that are important to DNA repair have been well characterised, but there is a fair amount of nomenclature overlap (Hanawalt et al., 1979). Of these genes, the uvr group is important. The uvrA and uvrB genes code for an endonuclease which initiates pyrimidine dimer excision, and the uvrC gene product is known to form a complex with the UvrAB protein in the incision step of excision repair and act in conjunction with this protein.

The gene products of these genes have been purified, and their enzymatic properties elucidated (Yeung et al., 1983). These workers showed that UvrABC proteins bind tightly to damaged sites in the cellular DNA in the presence of ATP. Initial attachment of UvrA is necessary before productive binding of UvrB and UvrC proteins takes place. The endonuclease complex generates two breaks on either side of a dimer site, one 7 nucleotides 5' to a pyrimidine dimer and the other 3 - 4 nucleotides 3' from it. No similar breaks are formed in the complementary, undamaged strand of DNA. The action spectrum of the UvrABC complex is wide, and it can incise DNA damaged by a variety of other agents as well as UV induced damage, which in itself can encompass more than simple pyrimidine dimer formation (Yeung et al., 1983). Mutations in the uvr genetic loci appear to have no effect on physiological functions of the cell other than through the modification of effects engendered by UV or other damage. This would appear to confirm the specific involvement of the uvr loci with DNA repair (Howard-Flanders, 1968).

After the initial incision event, which is apparently a common step, excision repair (as distinct from postreplication repair) can be divided into two specific pathways. In one of these, repair can occur in buffer as opposed to nutrient medium, and requires the presence of DNA polymerase I, the product of the polA gene. It may be

replaced by DNA polymerase III (polC) and/or DNA polymerase II (polB), which are, however, only 25% as effective. This pathway is effectively nonfunctional in polA$^-$ strains. There is evidence, however, that DNA polymerases I and III actually do most of the repair synthesis in vivo. This process, the mechanism of action of which is effectively summarised by the general protocol for excision repair of DNA given earlier,is the major pathway of excision repair (Smith, 1978). Because this system produces short patches of repair (up to 30 nucleotides), it has become known as "short patch excision repair". It is independent of nutrients and can take place in the presence of chloramphenicol (Coyne, PhD thesis, 1985). An endonuclease, (Endo III) which can recognise UV-induced lesions but which is not the uvrA/uvrB gene product has been isolated in E.coli. This, in conjunction with other available data, leads to the conclusion that there may be a second polA$^+$-dependent system in E.coli which acts on UV photoproducts other than cyclobutyl pyrimidine dimers (Smith, 1978).

The second pathway of excision repair requires a complete growth medium and functional recA, recB, lexA, uvrD, polC and lig genes. This pathway is able to produce long patches of repair replication (in the region of 1500 nucleotides) and is therefore termed "long patch excision repair" to distinguish it from the first observed pathway. recA$^-$recB$^-$ mutants did not show this pathway, but it was enhanced in a

polA$^-$ E.coli strain, which is blocked in the major pathway of excision repair. Long patch repair is irreversibly inhibited by chloramphenicol (Smith, 1978; Coyne, PhD thesis, 1985) and is blocked by impurities in the growth medium.

A phenomenon occurring in certain strains of E.coli, known as Liquid Holding Recovery (LHR), involves increased survival of UV irradiated cells if they are held in buffer for a number of hours before plating on nutrient medium as opposed to plating them immediately after irradiation (Smith, 1978). Genetic analysis of this occurrence showed that it is blocked by mutations in uvrA, uvrB or uvrC genes, suggesting that the basis of LHR is excision repair - specifically, since LHR occurs in buffer, the medium-independent, polA$^+$-dependent ("short patch") excision repair pathway. Where other genes are concerned, uvrD mutations also block LHR; lexA and recB mutations, however, in combination with recA mutations, greatly enhance LHR over levels observed with recA mutations alone. Several interpretations of this data are possible. One is that some of the lesions or excision gaps normally processed by the long patch excision repair pathway may be modified in such a manner that they are repaired by the polA$^+$-dependent repair pathway. In growth medium, should the cells have been plated directly, these excision gaps would be converted to lethal lesions; the holding time in buffer may give them a chance

to be shunted to the *polA*$^+$ repair pathway and be properly repaired, resulting in enhanced cell viability. This process is not, as far as is known, active in *Streptomyces cattleya*, the organism on which the current study has been done (Coyne *et al.*, 1984) However, it is observed to take place in yeast (Hunnable and Cox, 1971).

Both excision repair pathways discussed above could be described as nucleotide excision processes, as the entire region of DNA which has been damaged is excised. Another type of excision repair has been reported, involving base excision rather than nucleotide excision (Friedberg *et al.*, 1977;quoted by Smith, 1978). In this process, an altered base is recognised and severed from the sugar-phosphate DNA backbone by an N-glycosidase, and several specific enzymes of this type have been isolated for alkylated bases. This action leaves an apurinic or apyrimidinic site which is then susceptible to attack by a specific endonuclease. Subsequent steps presumably resemble those of the other excision repair processes described above. However, a great deal more needs to be known about this specific mode of excision repair before it may be fully understood.

Unrepaired pyrimidine dimers occasionally remain afer completion of DNA repair in the cell, and the resulting DNA is replicated. Such lesions produce gaps of up to 1000 nucleotides long in the daughter strands of replicated DNA

which correspond to the position and number of unexcised pyrimidine dimers in the parent strand, as they inhibit base pairing. This type of damage is repaired by what is termed a postreplication repair pathway. The mechanism of such repair entails the filling of the gaps in daughter strand DNA with undamaged material from the complementary parent strand via a recombination process, while the gaps formed in this way in the parental strand are filled in by repair replication. This mechanism has been observed in cells capable of performing excision repair, which would imply that some pyrimidine dimers do escape that repair mode and are encountered by a replication fork (Coyne, PhD thesis, 1985). Several rounds of replication and postreplicative repair may be required to produce a viable strand of DNA with the lesions "diluted out" (Smith, 1978).

This repair process has many genes in common with the processes controlling bacterial recombination; many of them, also, are similar to those functioning in the long patch excision repair pathway (Howard-Flanders and Boyce, 1966). They include recA, recB, lexA, uvrD, polA, polC, and lig, coding for polynucleotide ligase which is, as in the excision processes, essential for completion of repair. Here, as in the case of excision repair, several distinct biochemical pathways have been identified (Smith,1978). There appear to be at least five, all recA-dependent but otherwise distinct in their genetic requirements. Efficient

postreplication repair can occur in either polA$^-$ or polC$^-$ strains but is blocked in strains which lack both enzymes. It is not known which DNA polymerase (i.e. I or III) is preferred in postreplication repair occurring in wild type E.coli. A postreplication pathway is also amongst those seen to occur in yeast (Hunnable and Cox, 1971). In this organism, it is a plating- and medium-dependent system, depending on replication of damaged DNA which results in intragenic recombination.

The postreplication mechanism may be able to act on lesions other than unexcised pyrimidine dimers, in order to aid cell survival against mutagenic agents. Wild type E.coli cells are more resistant to the action of X-rays, alkylating agents and other mutagens than are rec$^-$ cells, suggesting that recombination and perhaps sister strand exchange are important in cell recovery from these treatments (Howard-Flanders, 1968). The rec$^-$ cells may in some cases be killed if they replicate a single pyrimidine dimer. This may occur when a gap is formed opposite a lesion in replicated DNA. Attempted recombination with the sister duplex may then abort in a rec$^-$ mutant, which could initiate degradation of the sister duplex. This would mean that even the intact strand would then be damaged opposite the dimer, which would have a detrimental effect on cell viability (Howard-Flanders, 1968).

Data have been presented in the literature by means of which it has been shown that *uvrA*$^-$ and *uvrB*$^-$ mutants are intrinsically more mutable than wild type strains while mutations in the *recA* and *lexA* loci essentially abolish UV induced mutations (Smith, 1978; Coyne, PhD thesis, 1985). It is interesting to recall that *recA*$^-$ and *lexA*$^-$ strains also appear to be deficient in the long patch excision repair pathway which has been implicated in UV induced mutagenesis. From such data, it could be suggested that the *polA*$^+$-dependent (short patch excision repair) system is largely error free (Witkin, 1976; Smith,1978). The second, media-dependent, *recA*$^+$-dependent excision repair pathway apparently produces low frequencies of UV induced mutations. At least one of the postreplication repair pathways is seen as mutagenic, however. It appears that post-irradiation protein synthesis is required for expression of UV induced mutations, since such mutagenesis is effectively prevented from occurring by treatment of cells with the protein synthesis inhibitor chloramphenicol (Smith, 1978). The *recA* and *lexA* genes would also appear to be implicated in this system. However, error prone repair does not appear to be a function of the constitutive DNA repair systems existing in *E.coli*. Rather, it is induced by the action of DNA damaging agents on the cellular genetic material. This inducible error prone DNA repair system is known as SOS repair, and it is discussed further in the following Section.

Most UV damage studied to date has been the result of irradiation of bacteria with far-UV light of wavelength between 200nm - 300nm. Pyrimidine dimers have been shown to be the major component of such damage. However, similar phenomena occur with near-UV radiation (300nm - 400nm) (Hartman, 1981). Near-UV irradiation has an effect not only on cellular DNA but also on DNA repair systems (Webb, 1977; Hartman, 1981). A theory put forward by Hartman (1981) is that at these wavelengths repair proteins become the chromophores responsible for repair disruption; destruction of repair is not observed with shorter wavelengths as direct damage to DNA in this case rapidly inactivates cells before significant repair destruction can occur. Near-UV mutagenesis is strongly oxygen dependent (Webb, 1977; Hartman, 1981). Near-UV radiation strongly damages excision and postreplication repair systems, which enhances the lethal effects of DNA photoproducts produced itself and by far-UV irradiation. The mutational lesions incurred by DNA when it is irradiated by UV light at 365nm are subject to almost complete photoreactivation, which clearly implicates pyrimidine dimers (Webb, 1977).

While the models for excision and postreplication repair presented above are discussed as established fact, there is actualy a lot left to learn about many of the pathways mentioned and the genes involved therein. Furthermore, all the studies leading to the conclusions reached above have

been the result of work done on E.coli. It is important to remember that it is quite possible that repair systems in an organism such as S.cattleya, which is the subject of the present study, could function in a very different manner as it is intrinsicaly different from E.coli on many levels.

1.2 SOS response in E.coli

The SOS response has been the subject of several comprehensive reviews (Witkin, 1976; Walker, 1984; Walker, 1985; Hanawalt et al., 1979 [in part]). Unless otherwise referenced, the material discussed in this section has been drawn from these. In addition, good general simplified and historical overviews by Howard-Flanders (1979) and Sedgwick and Yarranton (1982) have been consulted.

The SOS response is induced in cells as a result of UV irradiation and other mutagenic treatments. It results in the induction of a number of manifestations, including prophage induction, chromosomal mutagenesis, Weigle mutagenesis, cellular filamentation and possibly several pathways of mutagenic, i.e. error prone, DNA repair. The long patch excision repair pathway is thought to be a manifestation of SOS repair in the excision repair process; however, it appears to show a relatively low incidence of mutagenesis. The SOS response is dependent on the recA and

lexA gene products, but more than seventeen other different genes are thought to be involved in the process. These have frequently been referred to as *din* (*d*amage *in*ducible) genes. The *recA* and *lexA* gene products appear to act as regulators of expression of these genetic loci. The RecA protein is a 40000 dalton molecule, previously known as Protein X, that is known to have at least two different roles in the cell. It is absolutely required for homologous recombination, in addition to the proteolytic activity necessary for its action as regulator of the SOS response. In this mode, it requires single stranded DNA and a nucleotide triphosphate for its activation, which is fully reversible (Phizicky and Roberts, 1981). LexA is a 22700 dalton protein which functions as a direct repressor of every known SOS gene, including *recA* (Walker *et al.*, 1982).

The simplest model for the SOS response involves a constitutive low level production of RecA protein by the cell (McPartland *et al.*, 1980). Under uninduced conditions, expression of the SOS response is prevented by the binding of the *lexA* gene product, functioning as a repressor, to the operator sequences of genes associated with the SOS response. These sequences have become known as "SOS boxes". When damage to DNA induces the SOS response, RecA is converted into a proteolytic enzyme which cleaves an -ala-gly- bond in LexA protein monomers (Walker *et al.*, 1986). In this form, LexA is unable to to carry out its repressor

functions any longer, being inactivated by the cleavage it has undergone. This results in increased expression of the various SOS genes in the cell, and facets of the SOS response mediated by these genes are observed. The specific proteolytic property of RecA on the LexA protein have recently been shown not to depend on RecA alone - i.e. specific cleavage of LexA can occur in the absence of RecA. This has prompted speculation that RecA may not itself be the direct catalytic agent in LexA cleavage but could rather interact with LexA, in its activated form, and change the conformation of the repressor molecule in such a way that the susceptibility of a specifically labile bond to simple hydrolysis by an exogenous water molecule is increased. Also, genetic evidence indicates that RecA is likely to play a further role in SOS processing apart from LexA cleavage. Two possibilities are that RecA is required for the cleavage of a protein which mediates an actual biochemical process of mutagenesis and/or that it participates mechanistically in SOS processing.

There is no proof that only one type of error-prone repair is induced in E.coli - in fact, there are at least two that have already been mentioned here, one concerned with a recombination or postreplication repair pathway and, to a point, the long patch excision repair pathway. It has been established that some mutagenic treatments will induce an error prone DNA polymerising activity capable of promoting

bacterial DNA replication past unexcised pyrimidine dimers. Error prone repair in many cases seems to be mediated by DNA polymerase III rather than DNA polymerase I, and the ability of the former to affect error prone repair may depend on the acquiring of an inducible factor. The hypothesis exists that SOS mutagenesis may involve the inducible inhibition of the 3'→5' proofreading exonuclease of DNA polymerases; it is known that this activity can be inhibited by RecA protein in both DNA polymerase I and polymerase III. The special "error prone" type of repair may be responsible for the increased survival associated with mutagenesis in irradiated wild type bacteria via the action of a special DNA polymerase which is more than ordinarily tolerant of abnormalities in template DNA. An error prone polymerase might thus increase the level of survival at the expense of increased frequency of mutations (Howard-Flanders, 1979).

To date, seven genes playing a role in homologous recombination have been identified - namely, recA, recB, recC, recF, recJ, recN and ruv. Of these, at least three - recA, recN and ruv - are induced by DNA damage and are controlled by the SOS regulatory circuit. Some of the products of these genes act in an error prone pathway of postreplication repair, as discussed earlier; a second process in which they are involved is double strand break repair in DNA. In E.coli, the latter has been shown to to be inducible and unequivocally governed by the $recA^+$ $lexA^+$

regulatory circuit. It was also shown that the process required synthesis of protein de novo after DNA replication, since it could be blocked by rifampicin or chloramphenicol. This type of repair requires a functional recA gene and a functional recN gene. There is, once again, a second pathway involved in the same type of repair that is dependent on recB instead of recN. The relationship of these two pathways to one another is not clear.

A number of factors determine which specific manifestations of the SOS response will be expressed. These include the strength and persistence of the inducing signal, which is a function of the dose of inducing treatment, and which will influence the amount of RecA protein which is activated and the time during which it remains so; the rate at which RecA protease activity cleaves repressors of a particular SOS gene - which may not all necessarily be LexA; the level of expression of each SOS gene required for its gene product to effect a physiological change; and, finally, the affinity of the LexA repressor for the operator of a particular gene. This last phenomenon means that the SOS system may in fact exist in a number of intermediate states of induction. Different genes in the SOS genetic loci bind LexA with different strength - e.g. LexA will bind strongly to recA, dinD, and the umuC and umuD operators but less strongly to those of lexA, dinA, dinB and others. This implies that an SOS signal of limited intensity would not result in the

derepression of the recA gene, and therefore a full SOS response would not occur. Because of the strength of LexA binding to the operators of uvrA and uvrB genes, a slightly stronger signal could result in the expression of the polA-dependent error free excision system or possibly the long patch excision repair system, both of which share the initial incision step mediated by the UvrABC enzyme complex. It would take a large and persistent SOS inducing signal, therefore, to induce the error-prone SOS DNA repair.

UV mutagenesis in E.coli is linked to SOS repair. The sequence changes of such mutations have been extensively analysed, and it has been concluded that the bulk of mutagenesis arising from SOS processing of damaged DNA templates is targeted. Targeted mutagenesis may consist of locally targeted mutations, where the presence of a lesion leads to the introduction of mutations at its actual site, and regionally targeted mutagenesis, where the lesion leads to the induction of mutations in its immediate vicinity but not directly at its site. The majority of mutations arising from SOS repair appear to be locally targeted, with the nature of the premutagenic lesion influencing the sequence change. However, untargeted mutagenesis has also been shown by various inducers of SOS functions in E.coli (Witkin and Wermundsen, 1972; Echols, 1982) although its incidence is not very high and it is definitely a minor component of UV mutagenesis in E.coli.

The role of the RecA protein in induced mutagenesis has been investigated by Blanco et al. (1982). The role of this protein in the mutagenic process could be attributed to the formation of a RecA-dependent protease complex through the proteolytic modification of a protein in the replication mechanism. Alternatively, the RecA activating sites on the DNA could be mutagenic in themselves in that the same RecA - DNA interaction required for the formation of the protease complex could be involved in the mutagenic event. The RecA protein requires the longer regions of single stranded DNA, furnished by the lesions in which mutagenic events are targeted, for its activation and the formation of the protease complex.

The SOS response in wild type E.coli has a definite duration - within four hours of the removal of the inducing signal, such cells return to equilibrium. RecA protease activity is reversed by the return of the protein to the inactive state, and the amount of the protein produced again approaches uninduced levels. LexA self regulation may play a role here - as RecA to cleavage of LexA diminishes, the uncleaved LexA repressor protein becomes available in larger quantities to repress the SOS response genes, starting with those with the highest affinity for the LexA protein. The recA gene, with its very high affinity for the repressor, is probably among the first genes to bind the protein (Gottesman, 1981). This

allows the cell to rapidly return to the uninduced state. Reestablishment of repression occurs, therefore, after the DNA damage in the cell has been dealt with; it is not, however, known whether this is accompanied by a concomitant inactivation or degradation of the functions of SOS response induced earlier.

It is clear that bacterial cells are willing to pay a high energy price to ensure the integrity of the genome and the survival of the bacterium under adverse mutagenic conditions (Hanawalt, 1982). This is obvious even if the sheer proliferation of repair mechanisms in E.coli alone is considered. Under normal circumstances, cellular DNA is repaired by constitutively expressed and predominantly error free pathways, but extreme environmental stress can induce emergency repair, such as the mutagenic SOS response. Despite the fact that the SOS response is highly mutagenic it is still advantageous to the cell as the increased survival due to DNA repair outweighs the disadvantages of increased mutation rates. Several of the genes involved in DNA repair in E.coli have been cloned and sequenced, the functions of their products elucidated, and several models of DNA repair proposed - and still, knowledge about the process of DNA repair, as essential to the cell as replication or transcription, is constantly increasing. A novel process of DNA repair, inducible like the SOS response but quite distinct from it, has recently come to light. It

has been termed the adaptive response, and this is the subject discussed in the following Section.

1.3 Non-SOS repair in E.coli: the adaptive response

A novel set of responses by bacteria to alkylation and oxidation damage has been elucidated in recent years, and termed the adaptive response (reviewed by Demple, 1987). The range of activities concerned with these inducible defences is comprehensive, from direct repair of specific damage to the induction of scavenging processes which inactivate harmful agents before they have the chance to act.

Much has been learned about the molecular basis of the adaptive response to DNA alkylating agents in the relatively few years since it has been discovered. Continuous exposure of E.coli to low levels of of NTG during early growth induces resistance to alkylation mutagenesis and confers increased resistance to killing by this agent (Demple, 1987; Cairns et al., 1981; Walker et al., 1986). The bacteria are, however, as sensitive as before to mutagens of other classes. The DNA repair enzymes induced during the adaptive response in E.coli have been discovered to possess unusual mechanisms (Demple, 1987). The AlkA protein encodes a broad specificity DNA glycosylase that removes N3-alkylpurines and

O^2-alkylpurines from duplex DNA. It functions in a polA-dependent pathway for repair of certain potentially lethal lesions (Cairns et al., 1981). A constitutively expressed glycosylase in E.coli is coded for by the tag gene, but this appears to act only on some of the lesions, leaving those it is unable to repair to the induced glycosylase.

Adapted bacteria are able to repair O^6methylguanine (O^6MeG), one of the major products of alkylation of the DNA molecule by alkylating agents, in a specific and unusual manner, involving a single event by a "suicide" enzyme that is apparently able to act only once (Demple and Karran, 1983; Walker et al., 1986; Demple, 1987). This enzyme, termed O^6MeG-DNA methyltransferase, employs a unique cysteine residue in the protein as the sole site for irreversible O^6-alkyltransfer. The enzyme in E.coli (19000 daltons) has been shown to be a part of the 39000-dalton product of the ada gene, which also has the distinction of being the only known regulatory locus of the adaptive response. In addition to its repair and direct regulatory roles, the gene also codes for a separate suicide methyltransferase for removal of methylphosphotriesters (MeP) arising from alkylation (methylation) of the DNA backbone. The Ada protein as a whole acts as a positive rather than negative regulator. A diagrammatic representation of the functions and action of the Ada protein in the cell is shown in Fig. 1. Alkylation of the Ada protein at the MeP repair site appears to be

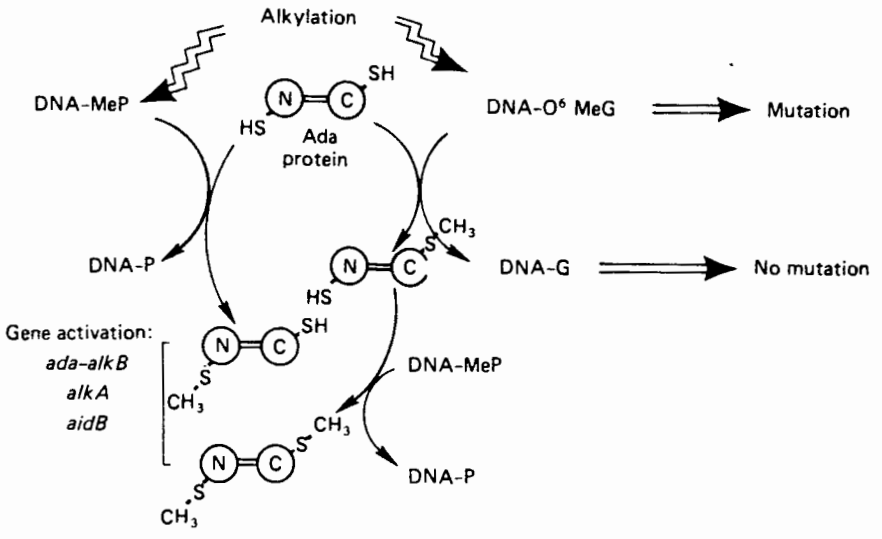


Figure 1

Suicide repair and gene regulation by Ada protein (Demple, 1987)

essential for the regulatory activity of the protein (Demple, 1987). The O^6MeG repair domain does not appear to bind directly to *ada* DNA; it could be involved in mediating the formation of Ada dimers or in transcriptional activation by interacting with RNA polymerase. Activation of the Ada protein involves a covalent protein modification and is therefore irreversible. The nature of the covalent trigger ensures that the system is extremely sensitive to low levels of the inducing signal but is only indirectly dependent on the concentration of the inducing molecules.

It has been shown that this system is most powerfully induced by methylating agents of the DNA. Ethylating agents like EMS do not act as inducers to any great extent, if at all, but *E.coli* becomes protected from their action by exposure to methylating agents (Cairns *et al.*, 1981). The system is extremely efficient, lowering the mutation rate by about 6000-fold (by comparison, the figure obtained with the error free constitutive *uvr* pathway of repair of UV induced damage reduces the mutation rate only by about 10-fold). The adaptive response enzyme O^6MeG-DNA methyltransferase acts very fast, with each O^6MeG lesion in the DNA being restored to its normal coding properties within half a second of its creation (Cairns *et al.*, 1981).

Inducible repair of oxidative damage to *E.coli* DNA has also

been reported (Demple and Halbrook, 1981; Demple, 1987). Resistance to the lethal and mutagenic action of hydrogen peroxide has been induced in E.coli in a manner similar to that observed with NTG - i.e. by pre-exposing the bacterium to low, non-lethal levels of the substance. Induced resistance to hydrogen peroxide toxicity is not part of any previously described inducible resistance to DNA damage. It must be noted that these two responses, to NTG and hydrogen peroxide, are distinct from one another as neither conferred any induced resistance to the inducing agent of the other. Both systems are also quite specifically distinct from the SOS response in E.coli, the only common factor being that all three are inducible responses to damage incurred by cellular DNA (Demple and Halbrook, 1981; see also Section 1.2). in E.coli, the adaptive response to oxidative damage, as induced by hydrogen peroxide, also confers elevated resistance to gamma rays and to near-UV light, which are capable of inducing similar damage to DNA (Demple, 1987). The low levels of H_2O_2 used to induce the adaptive response in E.coli have been shown to trigger the production of a large number of proteins in the cell, about 30 in all (Demple, 1987). A similar response has been observed in Salmonella typhimurium (Christman et al., 1985). In the latter organism, induction of 12 "early" and 18 "late" (produced in the longer term) polypeptides has been shown to occur. Of the "early" polypeptides, 9 (some scavenging agents and 2 heat shock proteins) are expressed

constitutively in OxyR1 mutants of S.typhimurium. The mutant OxyR1 protein appears to represent a 'gratuitous' activator of expression of such early proteins, and does not appear to require an inducing signal; the mutation is apparently dominant over the wild type gene (Demple, 1987). In E.coli, the situation appears to be extremely complex and is as yet not completely elucidated. The OxyR1 mutation occurs in this bacterium as well. While two of the OxyR1 regulated proteins are also under heat shock control, the mechanisms of activation of several "early" and all the "late" H_2O_2-inducible proteins, as well as their functions, are unknown. In addition, it is becoming apparent that at least one other regulatory locus in E.coli is also involved in the inducible response for oxidative damage (Demple, 1987).

Yet another inducible repair system in E.coli, this time dealing with repair of damage caused by near-UV irradiation, has been reported. This system is also distinct from the SOS response (Peters and Jagger, 1981). These authors suggest that this type of repair may be an adaptation to environments exposed to near-UV solar radiation. Demple (1987) puts forward such a response as a possible means by which adaptive responses to oxidative and alkylating damage to DNA may have been selected for in successive generations of organisms living in such environments.

The different responses to damage discussed here are all

distinct from one another, yet there do appear to be similarities between them. The adaptive responses to alkylation and oxidation share with the SOS and heat shock responses the presence of positive regulators acting on the systems (Demple, 1987). Multiple functions are seen in at least two of the proteins acting as such regulators, and, while such functions have not yet been elucidated in the other cases, they may yet be proved to exist there too. During the short time that the inducible adaptive responses have been known, a lot has been learned about them; however, much still remains to be done to bring our knowledge of these fascinating systems on a par with the vast reservoir of information that exists about the types of DNA repair processes elucidated earlier in both eukaryotic and prokaryotic organisms. Similar systems, as well as quite different ones, exist in other organisms also, and have been studied as well. These systems are the subject of the next Section.

1.4 DNA repair in other organisms

SOS-like DNA repair responses have been studied in S.typhimurium (Guerrero and Barbe, 1982; Barbe et al., 1983). The SOS responses in E.coli include, amongst other things, inhibition of division, cessation of respiration, induction of prophages if they are present in the cell, and

rexA lexA dependent inducible error prone repair of damaged DNA. The so-called Protein X, associated with the induction of SOS responses, and the product of the recA gene have been shown to be identical (McEntee, 1977).

A similar system of of SOS responses has been reported in S.typhimurium (Guerrero and Barbé, 1982). In this study, the authors suggest that there may be possible discrimination in the pathways and conditions of expression of the various SOS functions in this bacterium. They showed that certain injurious treatments, for example thymine deprivation, will trigger induction of prophage but not cessation of respiration; other agents, e.g. UV irradiation, were shown to induce both. This work was performed in a $recA^{+}_{-}$ thy^{-} mutant strain of S.typhimurium. The same treatments have little effect on the respiration of a $recA^{-}$ mutant of S.typhimurium, since the cutoff of respiration is an SOS function and requires, as a consequence, the RecA protein for its expression. Inhibition of respiration, as an SOS function, is therefore present in S.typhimurium and is, as in E.coli, controlled by the recA gene (Guerrero and Barbé, 1982). The authors also show that the class of injury sustained by the DNA (e.g. alkylation, crosslinks) may be important for the expression of one or another SOS function. This discrimination amongst SOS response functions could be due to the fact that, for induction, each type of response needs a different range of DNA degradation, and/or a

different type of damage in the DNA (Guerrero and Barbé,1982).

Barbé et al. (1983) have reported indirect induction of SOS functions in S.typhimurium. This study supports the hypothesis that a differentiation of expression of various SOS functions exists, as postulated by Guerrero and Barbe (1982). According to the data presented in the later study, degradation of DNA could be an important factor in the mechanism of expression of the SOS system. D'Ari and Huisman (1982) have suggested the existence of two branches of SOS response in E.coli - one being related with DNA replication arrest without damage while the other would be with damage and resulting DNA arrest. For RecA protein activation, the former would require additional degradation, which may be what was observed by Barbé et al. (1983). Differential expression of SOS functions has been observed in many organisms, but the molecular basis of this behaviour is still not completely clear.

DNA repair mechanisms have also been studied in Bacillus subtilis. Mita et al. (1983) have shown that two DNA repair processes, independent of one another, are functional in competent cells of this bacterium. Competent cells form the basis of this study for several reasons. They are non-dividing and appear to be metabolically less active, which means that DNA damage might be repaired by an excision

repair process but not by a postreplication repair process. However, due to other data, these workers suggest that a repair system of the latter type is also active in these cells. Furthermore, in this organism competent cells seem to be derived from sporulating cells and DNA repair in these specific cells may have some unique feature related to cellular differentiation (Mita et al., 1983).

The SOS-like system in B.subtilis has been characterised by Love and Yasbin (1984). The bacteria display a set of responses very similar to those observed in E.coli and S.typhimurium. The development and maintenance of competence is apparently also involved in the SOS system of B.subtilis. Various strains of this organism with different mutations in the DNA repair pathways were used to investigate the effect of such mutations on the expression of inducible phenomena (Love and Yasbin, 1984). The results of this study indicated that the SOS-like system in B.subtilis is regulated at two or more genetic loci. A model for the mechanism of this system in B.subtilis has been put forward. Certain genes in this organism, notably tsi and recE, clearly affect the expression of multiple cellular processes; their products could be involved in the regulation or the induction of the system. Other genes are more likely to serve functional rather that regulatory roles. According to the model proposed by Love and Yasbin (1984), the tsi gene product is the primary trigger in the induction of the SOS-like system.

The gene is stimulated in some manner by damage to cellular DNA or the replication thereof and initiates the expression, under these circumstances, of each of the SOS phenomena. This is achieved by the activation of the recE gene by the tsi gene product. The RecE protein then acts to induce or derepress genes associated with specific functions encompassed by the SOS response. The recA, recB, and recG genes also appear to have regulatory roles; it is proposed that one way in which they could exercise this function is the constitutive repression by the products of these genes, singly or in concert, of the synthesis of the recE gene product. These regulators may become inactivated in induced cells by tsi or recE gene products, allowing for amplification of RecE protein in the cell (Love and Yasbin, 1984).

It appears that the control of the SOS phenomenon in B.subtilis is more complex than in E.coli, as it has multiple genetic loci concerned with DNA repair that are also involved in the regulation of induction of the SOS response. The competence phenomenon is also apparently associated with the SOS system in B.subtilis, with the bacterium having the distinction of being naturally competent. No other naturally competent organism possesses an inducible system such as the SOS response (Yasbin et al., 1986). Error prone repair has been associated with the SOS system in E.coli; however, should B.subtilis prove to be

capable of error prone DNA repair, it would be the only naturally competent organism known to date to show this this type of repair mechanism. Such a capacity has, indeed, been established in B.subtilis. The error prone repair system in this organism can be characterised by its ability to promote mutagenesis by UV; it has also been shown that this system is is necessary for the generation of EMS-induced mutations in B.subtilis (Yasbin et al., 1986). In this characteristic, B.subtilis appears to be more similar to Saccharomyces cerevisiae, where an error prone repair mechanism is essential for EMS mutability (Prakash, 1974; Prakash and Higgins, 1982) than to E.coli, which is only partially dependent on error prone repair to exhibit EMS induced mutagenesis (Yasbin et al., 1986). B.subtilis has also been shown to possess the adaptive response to NTG (Hadden et al., 1983).

Another naturally competent organism is Neisseria gonorrhoeae (Yasbin et al., 1986; Campbell and Yasbin, 1984a). The organism has been shown to possess an excision repair system but not photoreactivation or error prone DNA repair (Campbell and Yasbin, 1984b). The sensitivity of N.gonorrhoeae to UV, EMS and MMS in comparison to the repair proficient strains of B.subtilis and E.coli was noted. It was suggested that such sensitivity may be due to the absence of a form of postreplication repair (Campbell and Yasbin, 1984b). An absence of an adaptive response to these

agents in N.gonorrhoeae was also postulated.

In Micrococcus radiodurans, much higher levels of resistance to damage by such agents as UV and mitomycin C than those seen in E.coli have been observed (Moseley and Copland, 1978). In both organisms, it has been suggested that repair of damage induced by these two agents has common steps. In the above study, however, it was shown that a repair pathway which is involved in the removal of DNA crosslinks but not in repair of UV induced damage exists in M.radiodurans, thus refuting that claim at least partially although the existence of a separate repair pathway concerned with both types of damage has also been postulated (Suzuki and Nakazawa, 1976). A study on Micrococcus luteus has shown that two main systems of DNA dark repair exist in that organism, namely, an excision repair pathway and an inducible postreplication repair pathway (Zherebtsov and Tomlin, 1982). However, constitutive pathways of postreplication repair have been shown to act when the inducible pathway is suppressed by chloramphenicol. There is a close similarity of the effects of chloramphenicol treatment on survival, DNA replication and postreplication repair in UV irradiated M.luteus and E.coli. This implies that the repair physiologies of the two organisms must be very similar in many respects. However, some repair pathways in M.luteus do show a higher effectiveness compared to E.coli (Zherebtsov and Tomlin, 1982).

In Streptomyces, the first concrete evidence for the existence of DNA repair mechanisms was provided by the isolation of UV sensitive mutants (uvs mutants) of Streptomyces coelicolor by Harold and Hopwood (1970a, 1970b). Six genetic loci, termed uvs A - F, were genetically analysed in these mutants. They were shown to exist in two diametrically opposed clusters on the chromosome (uvsA, uvsC and uvsD in one cluster, and uvsB and uvsE in the other) with one gene, uvsF, occurring separately, apart from either locus. The uvs A, B, C and D genes appear to be involved in the same DNA repair mechanism, and are thought to have a tentative correlation with the uvr A, B, C and D genes in E.coli, although this was not confirmed (Harold and Hopwood, 1970a). The uvrE gene is seen as being active in a different repair mechanism, probably one which is saturable at low UV doses. The uvsF gene is thought to be required for the operation of a system which is able to repair a portion of the UV-caused damage to DNA also repairable by the proposed uvs ABCD system (Harold and Hopwood, 1970a).

In Streptomyces fradiae, several DNA repair pathways have been shown to exist (Baltz, 1986). Error free excision repair is present, as is mutagenic error prone repair which has implications in EMS-induced mutagenesis in this organism (Baltz, 1986; Stonesifer and Baltz, 1985). The presence of a second error prone repair system dealing with the repair of

bulky lesions has also been postulated. Recently, the existence of an adaptive response to NTG by S.fradiae has been demonstrated (Baltz and Stonesifer, 1985), but appears to be significant only at relatively low doses of NTG. At higher doses, error prone repair is induced, and predominates (Baltz, 1986).

1.5 Genetic instability

Genetic instability is a phenomenon that occurs in many different ways in a multitude of organisms. Although the bacterial genome is usually very stable, there is a growing number of known microbial instabilities characterised by either transposition of short DNA sequences from one part of the genome to another, rearrangements involving sizeable parts of the bacterial DNA molecules, or the loss of genetic material such as plasmids and of linkage groups from plasmids, chromosome, or organelle genomes (Williamson, 1986). Gene amplification might also be responsible for instability in some organisms, including Streptomycetes (Cullum et al., 1986; Usdin et al., 1985; Dyson et al., 1986). Genetic instability also occurs in eukaryotes such as yeast (Williamson, 1986) and Aspergillus nidulans (Roper, 1986); the latter organism and aspects of its instability will be further discussed in Chapter 4.

In Streptomycetes, genetic instability is often displayed and this genus has often been studied with a view to elucidating this phenomenon. Gene amplification is known to occur in Streptomyces (Usdin et al., 1985; Dyson et al., 1986; Cullum et al., 1986; Schrempf, 1982). It has been postulated that repeated DNA sequences in Streptomyces may have some connection with genetic instability in some species which are known not to harbour plasmids (Usdin et al., 1985). By virtue of their sequence homology, the repeated sequences may act as "hot spots" for recombinational events, whether legitimate or not. Repeated DNA sequences, which are found less often in lower types of bacteria, may also play a role in the control of morphological and secondary metabolic changes that occur irreversibly in Streptomycetes in the course of their growth and development (Usdin et al., 1985). Instability arising from chromosomal alterations involving amplifications and rearrangements of specific nucleotide sequences has been reported in several species of Streptomyces (Schrempf, 1982). These sequences are present in low copy number in the wild type strains, and share extensive homology with one another. Parts of amplified sequences have been shown to exist in several copies in the chromosomal DNA of 20 different species of Streptomyces and are postulated to have arisen by the acquisition of insertion elements by the organisms (Schrempf, 1982). Three hypotheses have been put forward by means of which genetic instability in

Streptomyces could be explained (Kirby and Lewis, 1981). The first involves insertion or deletion of transposable element(s), with genes coding for antibiotic resistance, which are able to exert an effect on the promoters of other genes. The second hypothesis also relies on the insertion or deletion of a transposable element. In this case, however, the insertion sequence would produce pleiotropic effects on genes coding, for example, for aerial mycelium production by interfering with the operon which specifies antibiotic biosynthesis and resistance. In the third case, a controlling element which does not interfere directly with genes expressing antibiotic resistance is postulated; nor does it bear such genes itself. Rather, it exerts its influence by switching antibiotic resistance and other genes on or off by means of its orientation. Such a controlling element could either act in *cis* from a position on the chromosome close to the genes it affects or in *trans* from a plasmid.

Spontaneous genetic instability and DNA amplification in *Streptomyces lividans* has been biochemically and genetically analysed by Dyson *et al*. (1986). Two major pathways of instability have been discovered, both of which are characterised by the generation of DNA deletions and amplifications. A model has been put forward by means of which gene amplification could arise, based on the rolling circle replication model. The model must as yet remain

speculative, for the authors (Dyson et al., 1986) are not absolutely certain whether the Streptomyces amplified DNA they studied was chromosomal or extrachromosomal; the rolling circle replication model has so far been more commonly attributed to the replication of extrachromosomal DNA.

Unstable genes in several Streptomyces species have been studied by Cullum et al. (1986). From this study it appears that gene amplification is not ubiquitous in instability events, although its occurrence is frequent enough to warrant interest. There is more repetitive DNA in Streptomyces than in most other prokaryotes (Usdin et al., 1985) and this may be coupled to the fact that instability in Streptomyces is in many ways unique amongst genetic instabilities shown by bacteria (Cullum et al., 1986). In most cases, Streptomyces instability is reversible, implying that the observed phenotypic changes may not be the result of an irreversible loss of genetic material.

Streptomyces cattleya, the organism on which the present study has been performed, is an industrially important organism which produces thienamycin, a beta-lactam antibiotic (Kahan et al., 1978; Aoki and Okuhara, 1980) and a cyclopentenedione antibiotic known as G2201-C (Noble et al., 1977) as secondary metabolites. S.cattleya shows a high spontaneous genetic instability, characterised by the loss

of a large DNA fragment which engenders the simultaneous loss of the ability to form aerial mycelia and of arginosuccinate synthetase activity in the cell. This results in an Amy^- Arg^- phenotype of such variants; the spontaneous frequency of occurrence of these variants is in the region of 0.5%, and this frequency can be enhanced further by treatment with DNA damaging agents (Usdin et al., 1985; Coyne et al., 1984). The genetic basis of the instability in S.cattleya must be attributed to factors related to the chromosomal DNA, as no plasmid has been found to exist in this organism (Usdin et al., 1985). This coinheritance of the loss of the aerial mycelium and the arginine auxotrophy is important, as it shows that other genes apart from those coding for antibiotic production or resistance are affected by the instability phenomenon in Streptomyces (Coyne, PhD thesis, 1985).

1.6 Aim of this project

Genetic instability can affect antibiotic production in strains of Streptomyces that produce them. Instability may be reduced by altering environmental conditions, such as the temperature or the media, but such remedies are likely to have a harmful effect on the physiology of antibiotic production (Cullum et al., 1986). The ability to control instability in this industrially important class of organism

would be desirable, and more could be achieved in this field if the mechanisms of instability and the molecular basis of regulation of the phenomenon were better understood.

Inhibitors of DNA repair have been shown to affect the frequency of genetic instability in S.cattleya (Coyne et al., 1984). If DNA repair systems were involved in instability, study of such systems using mutants in DNA repair pathways would afford valuable insight into the manner in which genetic instability functions in S.cattleya. If a relationship between the two phenomena could be established, it is not impossible to contemplate a means of controlling the high rate of spontaneous genetic instability in this organism, by manipulation of its DNA repair pathways or production of mutants in such pathways that are genetically stable yet yield high quantities of industrially important secondary metabolites. This would have great implications in enhancing the commercial and economic potential of this valuable organism. This study was undertaken in order to investigate the possible role of DNA repair in genetic instability in S.cattleya and to investigate such a relationship, should any be shown, with a view to controlling instability via manipulation of repair pathways.

Chapter 2

Generation, isolation and primary characterisation of Streptomyces cattleya mutants

Summary

Three mutants of Streptomyces cattleya NRRL 8057 with lesions in the DNA repair pathway were obtained by NTG mutagenesis. The mutants were selected on the basis of their resistance or sensitivity to Mitomycin C. Two MMC resistant mutants, R6 and R12, and one MMC sensitive mutant, S26, were isolated. S26 was also UV^S while the other two were UV^r. The mutants were subjected to UV irradiation in order to obtain UV kill curves. The curves generated for the two MMC^r mutants showed induction or derepression of a second repair system. These mutants initially showed higher instability than the wild type, but it dropped to below wild type levels at higher UV dosages. Both these mutants appeared to roughly mimic the plateau shown by the wild type as high levels of UV irradiation were achieved. S26 in contrast showed a much higher initial incidence of instability, and no plateau was observed at high UV dosages. Single strand breaks were induced in the wild type and mutant strains by incorporation of P^{32} into their DNA. R6 showed a five-fold

increase in instability after this treatment. R12 showed no significant change, and S26 showed a slight decrease, which may be due to the increased lethality of DNA damage to this strain because of its highly labile nature. The strains were further analysed by polyacrylamide gel electrophoresis of total cellular proteins. S26 showed a startlingly different protein profile. R6 was almost identical to the wild type, while R12 was extremely similar, showing only the loss of one major band and a significant lowering of the amount of detectable protein in the region below this band.

2.1 Introduction

Mitomycin C belongs to a class of antibiotics which inhibits DNA synthesis in bacteria, and DNA appears to be the primary and direct target of their lethal action. Kersten *et al.* (1964) have reported that mitomycin C causes DNA and RNA degradation in bacteria, although the increase in DNAase activity observed in *E.coli* after treatment with the antibiotic does not appear to be directly caused by an enzyme-inducing effect of mitomycin C. Mitomycin C forms complexes with DNA, most notably with guanine-rich regions, and induces crosslinks (Kersten *et al.*, 1964). It is possible that in their reduced form the mitomycins may function partly as bifunctional alkylating agents (Tomasz *et al.*, 1974). Mitomycin C is not only seen as selectively inhibiting cellular DNA synthesis, but also as having a plethora of other activities, such as inducing phage lambda development in *E.coli* K12 and affecting the cellular genetic mechanism by mutagenic action and stimulation of the recombination rate in *E.coli* (Ijima and Hagiwara, 1960).

Ijima and Hagiwara (1960) underline the similarity of effects in *E.coli* obtained by treatment with mitomycin C and treatment with UV radiation. Damage due to mitomycin C and UV light, DNA crosslinking and thymine dimer formation

respectively, is repairable by a similar molecular mechanism, possibly controlled by the same genetic loci (Suzuki and Nakazawa, 1976). A mutant, mcl, of the bacterium Bacillus subtilis which was sensitive to both mitomycin C and ultraviolet radiation has been isolated (Okubo and Romig, 1965). It was proved that only one mutational step was required for B.subtilis to acquire sensitivity to both these agents, and further, that this mutation has a debilitating effect on at least one reaction involved in recombination. It has been suggested that UV sensitivity and recombination may have a close relationship and that the processes of recombination and dark-repair could have common enzyme(s) and/or share common steps. One such step could be the repair of single stranded unpaired damaged sections of DNA. There is evidence that repair of DNA damage caused by alkylating agents may occur by a similar process. Okubo and Romig (1965) give as the simplest explanation for the results they had obtained the fact that UV and mitomycin C sensitivity, as well as the altered transformability of their mutant, probably have a common genetic basis. This conclusion is borne out by a later study in the same bacterial species (Mahler, 1966). The author sees repair of mitomycin C- and UV-caused damage to be essentially similar, namely, an endonucleolytic cut at the site where damage or distortion has occurred, with exonucleolytic enlargement; repair with nucleotides complementary to the unexcised strand would then follow this step. There is

evidence (Dolbeare, quoted by Mahler, 1966) that similar enzymatic events may initiate excision of damage caused by both agents, following the discovery that an enzyme (an enzymatic fraction) isolated from Micrococcus lysodeikticus will degrade DNA that has been treated and, presumably, damaged by either UV and mitomycin C but which has no effect on native transforming DNA. Mitomycin C has been used in the present study as an analogue to UV in terms of action against damage caused by either agent in the afflicted cell, as such a system is easier to control and to standardise.

Work done on Haemophilus influenzae (Small et al., 1976) indicated, however, that crosslinks in the cellular DNA may not be the only biologically important effect of mitomycin C and that damage other than crosslinking caused by this agent is repaired by an enzyme system that does not remove UV-induced pyrimidine dimers.Therefore, while similarities in the action of Mitomycin C and UV have been noted in many studies, in H.influenzae their differences are emphasised. For example, while UV irradiation causes a delay in DNA synthesis after which the process resumes fully, mitomycin C does not appear to cause this but rather a slowing or a stoppage of synthesis of DNA in the cell (Small et al., 1976).

The incidence of genetic instability in Streptomyces as distinguished by the loss of the organism's ability to

produce an aerial mycelium appears to be fairly widespread. The loss of this ability, resulting in Amy$^-$ (Aerial mycelium$^-$) clones, is usually linked to arginine auxotrophy; as a consequence, such cells very frequently show an Amy$^-$ Arg$^-$ phenotype. The enzyme that has been lost, with respect to the arginine auxotrophy, is arginosuccinate synthetase (Redshaw et al., 1979). In the three Streptomyces species studied in that work, the Amy$^-$ isolates were not seen to revert to either Amy$^+$ or Arg$^+$. This, together with the high frequency of curing observed, leads the authors to suggest that a deletion of genetic material, quite possibly an extrachromosomal element or a plasmid, has taken place. No plasmid has so far been discovered in S.cattleya; nevertheless, this organism is not exempt from the Amy$^-$ Arg$^-$ linkage. Amy$^-$ variants arise in S.cattleya spontaneously at high frequencies, and this can be enhanced by DNA damaging agents (Coyne et al., 1984; Usdin et al., 1984). It is this high incidence of Amy$^-$ variants, indicating a high level of spontaneous genetic instability in S.cattleya, that prompted this study.

2.2 Methods

2.2.1 Materials

The materials, media and reagents used are listed in Appendix B. Any abbreviations used are clarified in Appendix A.

2.2.2 Bacterial strains

All work was done on Streptomyces cattleya NRRL 8057, originally supplied by Glaxo Research Ltd. in 1977.

2.2.3 Maintenance of bacterial strains

The wild type S.cattleya and the three mutants obtained from it were grown for long term storage on Malt 3 Agar slants. All Streptomyces cultures were grown at $30^{o}C$. Strains were also maintained at $-20^{o}C$ as spore suspensions in 20% glycerol.

2.2.4 Preparation of spore suspensions

Spores from a single colony of S.cattleya, wild type and mutant, were streaked out on Malt 3 Agar slants. These were incubated at 30^{O}C until the cultures had sporulated completely. The spores were then scraped off into 20% glycerol. In this form the strains could be kept frozen at -20^{O}C for several months without losing viability. For NTG mutagenesis experiments, spore suspensions were prepared by scraping the spores from one slant into 10ml 0.05M Tris-malate buffer (pH 9.0).

2.2.5 Determination of the Minimum Inhibitory Concentration (MIC) of mitomycin C for S.cattleya wild type

A number of Malt 3 Agar plates were prepared, containing steadily increasing concentrations of mitomycin C in the range of 0.1ug/ml to 10ug/ml. 100ul of undiluted spore suspension was plated out on each plate, and the resulting colonies were scored after incubation at 30^{O}C for one week. The MIC value was taken as the concentration of MMC in the plates above which no growth was seen to occur.

2.2.6 NTG mutagenesis

A spore suspension of S.cattleya wild type was made in 10ml 0.05M Tris-malate buffer (pH 9.0).It was divided into two equal parts. One half was set aside as the control. The other was added to a known mass of NTG crystals, such that the final concentration was 1mg/ml, and allowed to shake gently at room temperature for one hour. After this time the suspension was centrifuged in corex tubes in a benchtop centrifuge for approximately twenty minutes or until a firm pellet was obtained. The supernatant was drained off and the pellet was resuspended in 20% glycerol.

2.2.7 Isolation of mitomycin C sensitive mutants (MMC^S)

The NTG-treated spores were plated out on Malt 3 Agar plates in such dilutions as to give countable colonies (1×10^{-3} dilution was shown to be the best).

The mitomycin C sensitive mutants were searched for by replica plating these plates to a series of plates with gradually increasing concentrations of MMC. Colonies which grew well on the control MMC-free plates and which were killed before the MMC reached the MIC value established for the wild type organism earlier were taken as putative MMC^S

mutants and subcultured onto fresh Malt 3 Agar plates. The accurate MIC of the putative mutants was determined by replica plating the colonies obtained on another series of plates containing MMC, the concentratons ranging from 0.05ug/ml to 1.0ug/ml, and testing their growth on these plates.

2.2.8 Isolation of mitomycin C resistant mutants (MMC^r)

This type of mutant was obtained by plating out 100ul of undiluted mutagenised spore suspension per plate on Malt 3 Agar plates containing 1.5ug/ml and 2.0ug/ml MMC. Colonies which showed growth at these high levels of MMC were replica plated onto a series of Malt 3 Agar plates containing a range of MMC concenrations ranging from 1.0ug/ml to 10ug/ml to determine the MIC of these mutants in the manner described above.

2.2.9 UV irradiation: generation of kill curve profiles of S.cattleya wild type and mutants

UV irradiation was carried out at 254nm using an English Electric G8T5 tube at an irradiation level of 5 J $m^{-2}s^{-1}$. All irradiation measurements were made with a UV meter model 225 (Ultraviolet Products, San Gabriel, California, USA).

The volume irradiated was 10ml in a glass Petri dish. UV kill curves of S.cattleya spores were carried out by irradiating a given volume of a spore suspension filtered using cotton wool to remove mycelial material and taking samples every 15 seconds, over irradiation levels ranging from 0 - 750 J m^{-2}. Spores so irradiated were plated out on Malt 3 Agar plates in a series of dilutions and incubated at $30^{o}C$ for four to five days, after which period the number of survivors at each irradiation level was scored and the results plotted in a graph.

2.2.10 Stability determination

Spore samples were irradiated as described above. Stability was determined by scoring the number of Bald (Amy^{-}) variants of S.cattleya present over an UV irradiation range of 0 - 750 J m^{-2}.

2.2.11 Induction of single strand breaks in DNA of S.cattleya spores

S.cattleya wild type and the three mutants were grown on Malt 3 Agar containing 0.1 mCi ml^{-1} of P^{32} orthophosphate. The cultures were incubated at $30^{o}C$ until sporulation was complete. The spores were harvested in 20% glycerol and

stored at -70^{0}C for 8 weeks. The effect of P^{32} induced single strand breaks on genetic instability was then determined as described above.

2.2.12 PAGE analysis of cellular proteins in S.cattleya wild type and mutants

2.2.12.1 Preparation of samples

Vegetative cells grown in Malt 3 Broth for 3 days were prepared by adding one third sample to two thirds sample buffer in a final volume of 15ml. The cells were then put through the French press in three consecutive runs at a pressure of 4 tons. Following this treatment small aliquots of each sample were taken and the requisite amount of Bromophenol blue indicator added (see Appendix B). Samples were then boiled for 5 minutes and run on polyacrylamide protein gels.

2.2.12.2 SDS polyacrylamide gel electrophoresis of proteins

Electrophoresis was as described by Laemmli and Faure (1973) and O'Farrel (1975). The procedure was performed using a Hoeffer gel apparatus. To ensure formation of an even interface between the resolving gel and the stacking gel, the resolving gel was overlaid with isopropanol after

it had been poured. This was poured off before the application of the stacking gel mixture, once the resolving gel had completely solidified. The amount of sample usually loaded was 100ul. Samples were mixed with disruption buffer containing 0.1% bromophenol blue indicator in the ratio 1:2 and this mixture was boiled for 5 minutes and allowed to cool before being loaded. The gel was subjected to electrophoresis at a constant voltage of 35V for 5 to 6 hours. Gels were stained in Coomassie blue stain overnight and then destained with gentle shaking and frequent changes of destain until protein bands became clearly visible.

2.3 Results

2.3.1 Determination of MIC of mitomycin C on S.cattleya wild type

It was necessary to determine the MIC of mitomycin C on this organism as putative mutants arising from the mutagenesis experiments would be selected on the basis of their resistance or sensitivity to mitomycin C.

The MIC of mitomycin C on the wild type organism was shown to be 0.8 - 0.9 ug/ml.

2.3.2 Mutagenesis and isolation of the mutants

Two mitomycin C resistant (MMC^r) mutants, designated R6 and R12, and one mitomycin C sensitive (MMC^S) mutant, S26, were obtained from the spores exposed to NTG mutagenesis. The MIC values of mitomycin C for these mutants were determined as described earlier. In the case of the two MMC^r mutants the MIC proved to be 8ug/ml of Mitomycin C, which is considerably higher than that of the wild type S.cattleya. The MMC^S mutant, S26, showed an MIC value of 0.1g/ml mitomycin C.

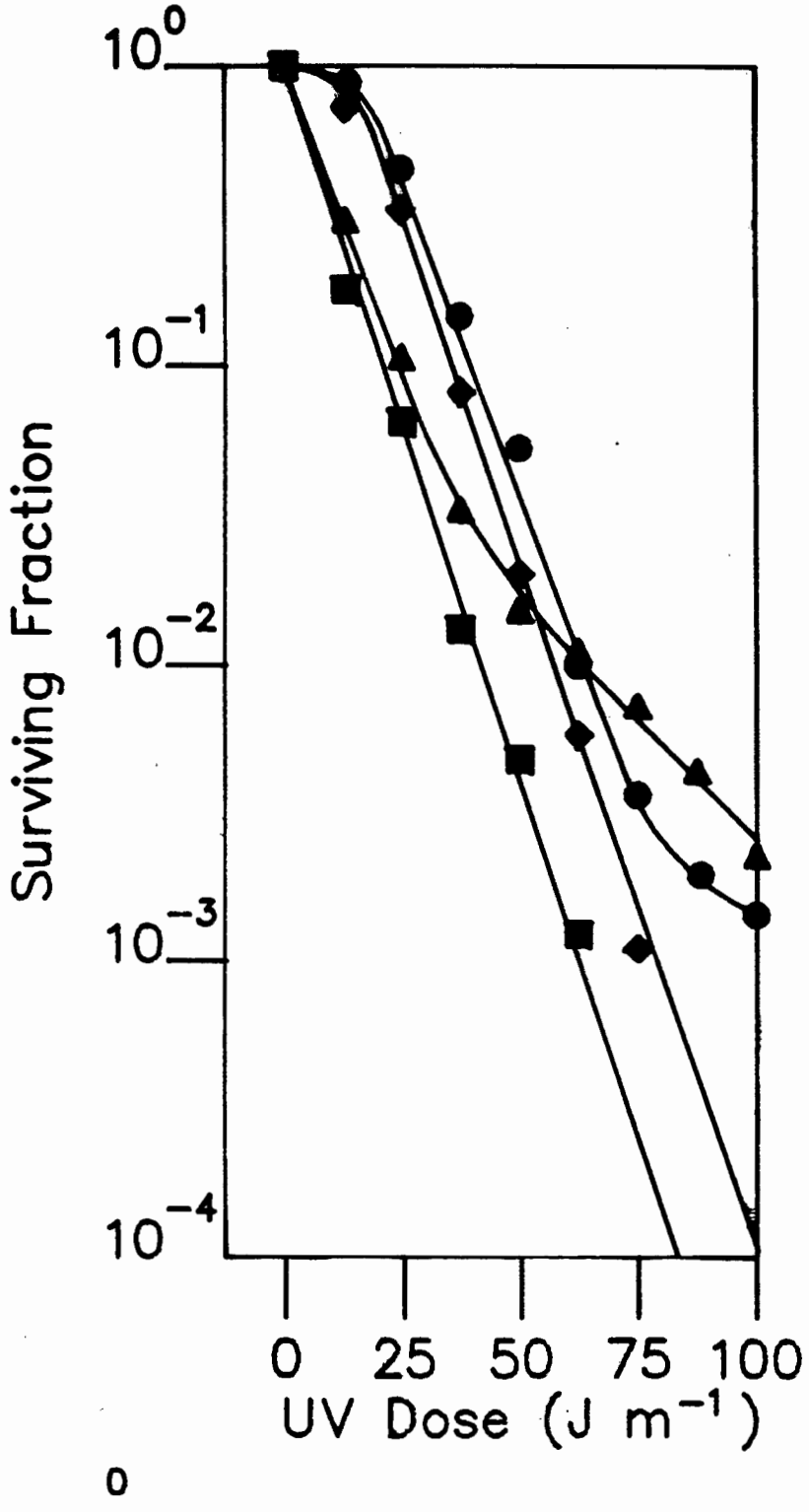
Physiologically, R6 behaved in a manner most resembling the wild type organism as far as growth and sporulation were concerned although there was a slightly higher incidence of Amy^- colonies. R12 was quite different - its growth was slower, and the frequency of Amy^- colonies higher. S26 proved to be the most labile of the mutants obtained; its growth was frequently erratic, definitely slower than that of either of the other two mutants and of the wild type organism, and the Amy^- phenotype occurred much more frequently than in any of the other three strains.

2.3.3 UV kill curves

All mutants showed UV survival curves which were quite distinct from the wild type organism and from each other (Fig. 2). Both the MMC^r mutants show a distinct second shoulder at higher UV dosages compared to the wild type profile, and in the case of R6 the primary shoulder is enhanced. However, the primary shoulder is completely absent in R12. S26 is linear over the UV dose range tested, lacking both the shoulders observed in the other mutants and in the wild type organism. The enlargement of the primary shoulder in R6 suggests that the mutation in this mutant resulted in an increase in activity of the repair system causing this shoulder. The secondary shoulders occurring in the kill

Figure 2

Spore survival curves of Streptomyces cattleya wild type (◆), S26 MMC^S mutant (■), R6 MMC^r mutant (●) and R12 MMC^r mutant (▲).



curves of R6 and R12 are possibly the result of the induction by the mutation of a second, inducible DNA repair system in S.cattleya.

2.3.4 Stability determination

The results of this experiment are shown in Fig. 3. R6 and R12 both initially show greater instability than that of the wild type but both are slightly less unstable than the wild type at high UV dosages, with R12 duplicating the levelling off of the wild type curve and R6 showing a slight decease in its instability as the UV dose increases. S26 showed a much higher initial level of instability than any of the other strains, and this increases sharply with the rise in UV dosage. There is no evidence of a plateau similar to those seen in the wild type and the other two mutants occurring at high levels of UV irradiation.

Figure 3

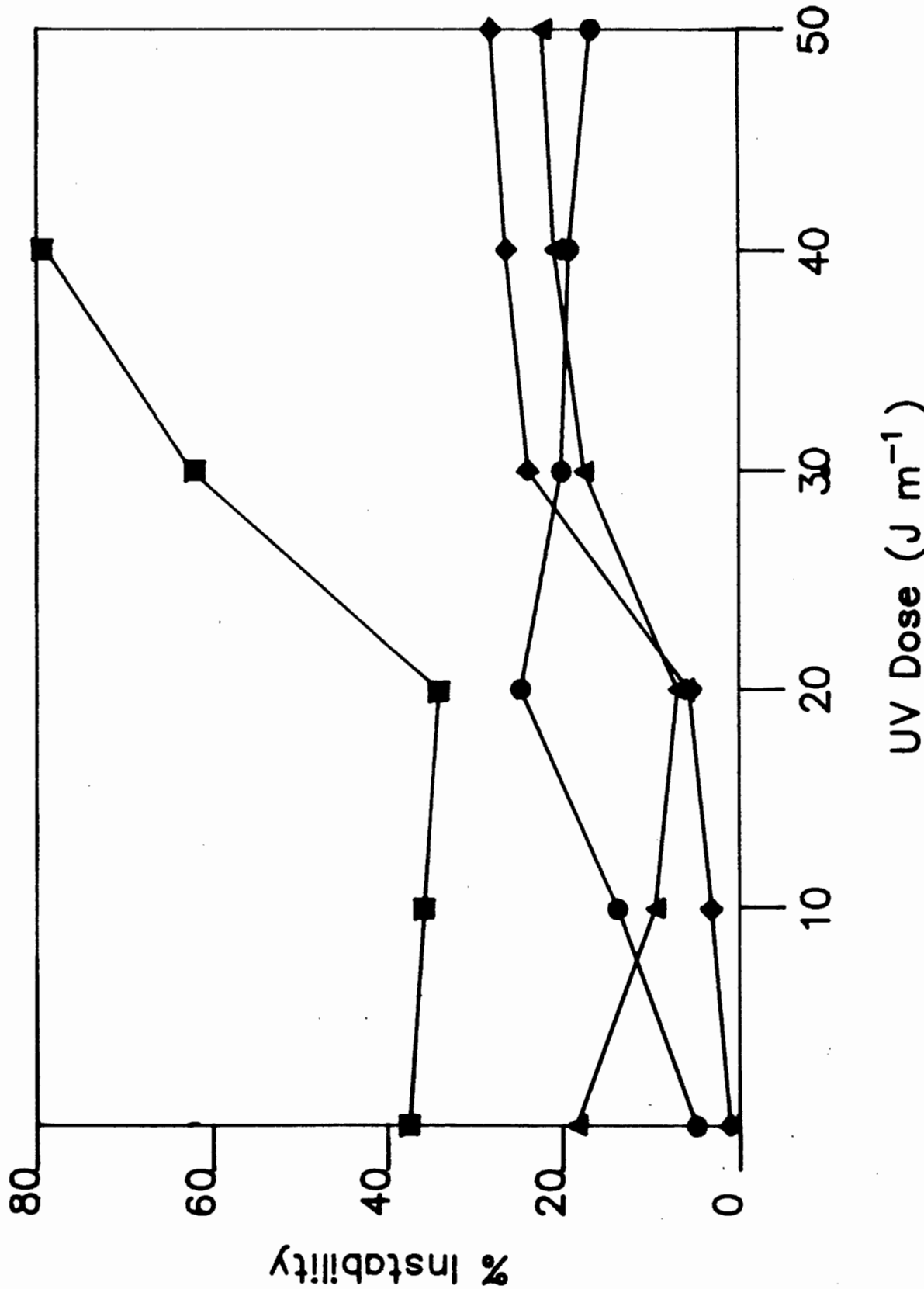
Genetic instability of the Streptomyces cattleya wild type and three mutant strains.

◆- wild type

■- MMC^S S26

●- MMC^r R6

▲- MMC^r R12



2.3.5 Induction of single strand breaks in DNA of S.cattleya spores (wild type and mutants)

The results of this experiment are set out in Table 1 below.

Table 1: Effect of single strand DNA breaks induced by P^{32} on genetic instability in S.cattleya wld type and DNA repair mutants

presence of P^{32} / strain	- P^{32}	+ P^{32}
wild type	0.5%	8.4%
R6	6.0%	31.0%
R12	18.0%	19.0%
S26	42.0%	26.0%

2.3.6 SDS-PAGE analysis of cellular proteins in S.cattleya wild type and mutants

The protein gel obtained from this experiment is shown in Fig. 4. The difference in the protein profiles between S26 and the wild type S.cattleya is immediately obvious. Where R6 and R12 are concerned, they are in essence similar to the wild type and quite similar to one another. R12, however, shows a loss of a major protein band indicated on the gel, and much lower amounts of proteins whose molecular weights fall in the region below this band are observed. The latter phenomenon is unfortunately not easily visible on the gel photograph.

Figure 4

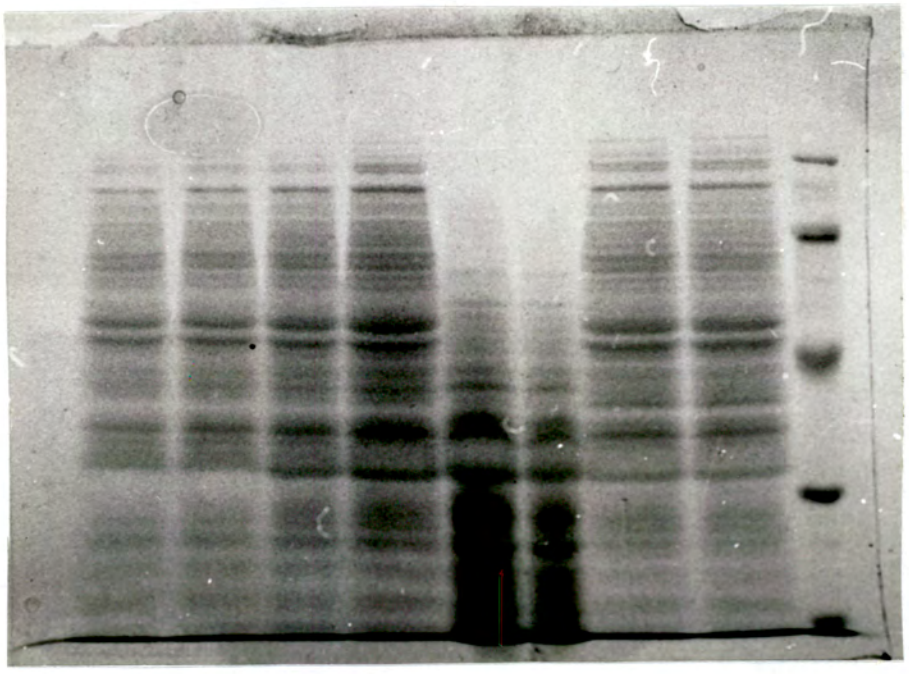
Polyacrylamide gel analysis of total cellular protein of S.cattleya wild type and mutants.

Lane A: R12

Lane B: R6

Lane C: S26

Lane D: wild type organism



A B C D

2.4 Discussion

Mitomycin C has been used in this experimental system to select for DNA repair mutants as it is easy to work with and the system is easier to standardise than conventional methods of selecting UV resistant/sensitive mutants of Streptomyces. The use of mitomycin C in this context is justified by reports that the effects of this antibiotic are very similar to those of ultraviolet radiation in E.coli.

The spore survival curves of mutants R6 and R12 showthe presence of a second shoulder at higher UV dosages - i.e. these mutants are only more resistant than wild type S.cattleya at these levels of radiation. This would suggest that the MMC^r mutations have derepressed or changed the level of expression of a second repair system induced at these dosages. R12 shows a similar degree of sensitivity to low levels of UV irradiation as S26. This, coupled with the loss of the primary shoulder in the survival curves, would seem to indicate that the same constitutive repair system has been lost in both mutants.

The very high instability level of S26 shows that the mutation has had an effect on the mechanism responsible for this phenomenon. The MMC^r mutations, on the other hand,

appear to have produced a level of instability that is considerably lower than that of the wild type at high UV dosages. The instability, therefore, seems to be consistent with the type of mutation concerned with mitomycin C that the mutants display. Since this enhancement of instability, after irradiation with UV light, has been observed before in this organism, it is possible that instability in S.cattleya is related to DNA repair in this organism. It would be interesting to know the precise nature of the mutation in a mutant like S26, and the mechanism of its action and its effect on the cell.

It is possible that part of the answer lies in the obviously very different proteins that S26 appears to be producing as compared to the wild type, as can be seen in Fig. 4. Alternatively, the accumulation of the low molecular weight proteins in S26 may be due to a protease or another enzyme with a similar action which was induced in the mutated strain. It is possible that this is in fact the case, since the production of serine proteases in late stages of growth of a Streptomyces species has been reported (Ginther, 1970). This possibility is attractive because it could explain the poor growth and the other negative physiological characteristics acquired by S26. But such a detailed protein analysis has not yet been done.

Chapter 3

Effect of sodium arsenite and caffeine on DNA repair systems of S.cattleya

Summary

UV repair in S.cattleya has been studied here using reactivation of UV-irradiated phage VC11 to overcome potential interaction between UV irradiation and pleiotropic cellular effects. In an earlier study, a constitutive caffeine-inhibitable host mediated DNA repair system had been clearly identified in S.cattleya, and a second, arsenite-inhibitable, system was postulated to be present. Using the three mutants obtained in this study, the existence of the second system has been proved. These results allow for a more detailed analysis of the three mutants, and an assignation to each mutant of a phenotype based on the two DNA repair systems extant within it.

3.1 Introduction

Caffeine is a substance that man has been exposed to for a very long time. A controversy exists as to whether caffeine actually has any harmful effects on humans, such as mutagenic or carcinogenic properties, although it certainly appears that it is addictive to a degree. However, caffeine has marked effects on the lower forms of life. It has been shown to induce chromosomal abnormalities in eukaryotic cell culture lines, but it is in the prokaryotes that its effects become most marked. Several DNA repair processes sensitive to caffeine have been described, and it clearly has potent mutagenic effects in E.coli, both alone and in combination with other substances (Timson, 1977).

The biological effects of caffeine are diverse, and within a given system may be multiple and complex. It can be said with certainty that caffeine has mutagenic effects on microorganisms - to take an example, such activities have been known for a long time in E.coli (Timson, 1977). It has been found that the mutation rate engendered by caffeine was directly proportional to the growth rate of the subject bacteria (Kubitschek and Bendigkeit, 1964; quoted by Timson, 1977). Caffeine has been shown to be a frame-shift mutagen in some E.coli strains, and it was thought that it could

induce two different kinds of mutational response in E.coli which were directly dependent on the cell environment. Furthermore, the substance was also seen to act as an antimutagen in E.coli (Grigg and Stuckley, 1966; quoted by Timson, 1977). Caffeine was also shown to be mutagenic in Klebsiella pneumoniae over a range of concentrations (Voogd, Jacobs and van der Stel, 1972). An interesting parallel was that the range of mutants obtained with caffeine closely matches that produced by UV irradiation. The effect of caffeine on E.coli in conjunction with UV light is striking - in one study, the combination induced the formation of ten times as many mutants as observed with UV irradiation alone (Lieb, 1961; quoted by Timson, 1977). It was suggested that the effect was due to caffeine interference with a dark-repair enzyme system. In other studies, it has also been proposed that the caffeine-induced increase in mutation frequency was due specifically to the prevention by caffeine of the excision of radiation induced dimers in the DNA (Timson, 1977), especially since the synergism between caffeine and UV light did not seem to be duplicated by, for example, X-ray irradiation and caffeine. Other workers have shown that antagonistic effects of caffeine and UV in mutation induction in certain strains of E.coli can be explained by the postulate that caffeine inhibited an error-prone (postreplication) repair system in these strains (Witkin and Farquharson, 1969; quoted by Timson, 1977).

Caffeine has been shown to be able to act directly on DNA. Although it does not behave as a purine analogue or become incorporated into bacterial nucleic acids , there is evidence that caffeine may affect the rate of DNA synthesis. A temporary reduction in DNA synthesis, for example, was produced in UV-irradiated E.coli cells by caffeine (Lieb, 1961; quoted by Timson, 1977). It was suggested that caffeine binds preferentially to single stranded DNA, and in UV-irradiated DNA treated with non-lethal quantities of caffeine this binding occurs near regions of UV-induced conformational changes (Timson, 1977).

However, the major effect of caffeine, and the one that has drawn the most interest, is the effect it has on DNA repair mechanisms of many organisms. In host cell reactivating (hcr^+) strains of E.coli, caffeine will inhibit the repair of UV lesions in DNA. Because UV^r strains of E.coli did not seem to be affected by (non-lethal) levels of caffeine, it has been suggested that it selectively blocked excision repair while leaving recombination repair unaffected (Grigg, 1972). Caffeine would appear to act by interfering with the action of the excision enzyme of the dark repair system in a radiation-resistant strain of E.coli. The enzyme which is responsible for both host cell reactivation and dark reactivation is believed to be the same protein, normally present in the cell and induced neither by phage infection

nor by UV light; this enzyme is inhibited by caffeine (Metzger, 1964; quoted by Timson, 1977). It had earlier been suggested that it is the interference of caffeine with the dark repair system that is responsible for the enhancement of UV-induced mutation in E.coli in its presence. Caffeine has been shown in two separate studies to have prevented excision of UV-induced thymine dimers by binding to the excision enzyme (Timson, 1977). But however caffeine affects the repair mechanisms of E.coli, the process does not seem to be associated with the known effect of caffeine on cAMP (Chandra et al., 1974). Caffeine has been shown to inhibit the excision repair capacity of Salmonella typhimurium (Williams and Clarke, 1971). In a more recent study, caffeine has been shown to significantly repress Mitomycin C induced reversion of a S.typhimurium strain from his^- to his^+ (Kim and Levin, 1986). These workers attribute this repression of MMC-induced mutagenesis to interference by caffeine with an error-prone repair process resulting in destruction of cells with damaged DNA. In two strains of Micrococcus radiodurans resistant to radiation damage caffeine did not sensitise the cells to irradiation, which suggests that the very accurate repair mechanism existing in this organism is not caffeine-sensitive (Sweet and Moseley, 1974).

In two later studies on E.coli some further aspects of the effect of caffeine on DNA repair were investigated. uvrA

E.coli strains that lack excision repair capacity have been used in this study (Fong and Bockrath, 1979). These cells can overcome their repair deficiency by resorting to another repair pathway, namely the postreplication repair pathway . Caffeine, when added to wild type parent cells of the uvrA mutant strain before or immediately after UV irradiation, inhibited the formaton of of single strand breaks in the cell DNA; however, once formed, single strand breaks were not subject to repair inhibition by caffeine. Postreplication repair was not affected in the uvrA mutant strains. From the data presented, it was suggested that caffeine binds tightly to the irradiated DNA, particularly at regions affected by UV irradiation thereby competing with the dimer-specific endonuclease for binding sites at the dimers and thus preventing the formation of single strand breaks (Timson, 1977). In suport of this theory, Rothman (1980) has shown that caffeine inhibits the excision step in DNA repair. Excision repair is a tightly organised process; caffeine does not disturb this coordination. Inhibition of only one step could lead to a compensating adjustment in the rates at which the other steps are occurring. The connection between the specific effects of caffeine on DNA excision repair and its general effects on cell metabolism or its specific binding to irradiated DNA is still unclear, especially in the light of the controversy that seems to exist with respect to the binding sites of caffeine within the cell.

With regard to sodium arsenite, a study by Rossman *et al.* (1975) has shown that arsenite interferes with DNA repair by inhibiting a rec-dependent function. This study showed that the wild type and excision repair deficient strains of *E.coli* exhibited a decreased survival in the presence of arsenite after UV irradiation but $recA^-$ cells were not affected in this manner. Arsenite inhibition of DNA repair has not been as widely studied as caffeine has in this respect, and consequently its mechanism of action and other properties are less well known.

The finding of an "error-free", caffeine inhibitable DNA repair system and an "error-prone", arsenite inhibitable system in *E.coli* had prompted members of the University of Cape Town *Streptomyces* research group to begin a study on the effect of these inhibitors on repair systems found in *Streptomyces*, specifically *Streptomyces cattleya*, and the connection of such repair systems with the genetic instability of this organism (Coyne *et al.*, 1984). The study was conducted using the reactivation of UV-irradiated phage VC11 (Coyne and Kirby, 1986) in order to avoid pleiotropic cellular effects and to ascertain whether the chemicals were in fact acting at the level of DNA repair. Working on the wild type organism, these workers proved the existence of a caffeine inhibitable host mediated repair system in

S.cattleya. The presence of an arsenite inhibitable repair system was postulated, but not confirmed.

In the present work, the three mutants of S.cattleya were subjected to to the same experimental protocol as the wild type organism in the above study . The changes in the DNA repair mechanisms in these mutants have enabled the unequivocal confirmation of the presence of a secondary repair system in S.cattleya which is inhibited by sodium arsenite and can thus be postulated to have some similarity to the "error-prone" system found in E.coli.

3.2 Methods

3.2.1 Materials and media

All materials and media used are described in Appendix B.

3.2.2 Production of high titre VC11 phage lysate

300ul of a wild type S.cattleya spore suspension was added to each of several test tubes containing 3ml of melted soft Nutrient Broth Agar at 42^{o}C. Following this, 4ul of VC11 phage was added per container. Once these manipulations were complete the soft NBA was immediately poured over Nutrient Broth Agar plates containing 0.5% (w/v) glucose and 8mM calcium nitrate. The plates were allowed to set and and were then incubated at 30^{o}C for 24 hours. When the plaques had become visible, the slurry from the top layer of the plates was scraped into 20ml total volume of Nutrient Broth containing 0.5% (w/v) glucose and 8mM calcium nitrate. The slurry present in this suspension was removed by centrifugation at 15000rpm for 10 minutes in a SS34 rotor. The supernatant was sterilised by filtering it through a 0.22u Millipore filter to prevent mycelial contamination. The resulting high titre phage lysate was stored at 4^{o}C.

3.2.3 UV irradiation

This was performed as described in Section 2.2. , using the phage instead of a bacterial spore suspension.

3.2.4 Determination of the effect of caffeine on DNA repair in S.cattleya wild type and mutants

The MIC of caffeine on S.cattleya was determined in an earlier study to be 3mg/ml (data not shown). Plates of Nutrient Broth Agar were prepared containing caffeine at the concentration of 1mg/ml and supplemented by 0.5% (w/v) glucose and 8mM calcium nitrate. 500ul of VC11 phage which had been subjected to UV irradiation (see previous section) was suspended in 5ml nutrient broth and tenfold serial dilutions of the phage were prepared at each irradiation level by transfering 200ul of the phage to 2ml soft Nutrient Broth Agar at $42^{O}C$. S.cattleya spores (125ul per container) were added to each phage dilution. Each phage sample was then plated on Nutrient Broth Agar containing 0.5% (w/v) glucose and 8mM calcium nitrate. Similar experiments were performed with the wild type organism and the mutants, in the absence of caffeine, to act as controls. The plates were

assayed for plaques following incubation at 30°C for 24 hours.

3.2.5 Determination of the effect of sodium arsenite on DNA repair in S.cattleya wild type and mutants

In a separate study, the MIC of sodium arsenite was determined to be 0.45mg/ml. The experiment in this study was performed using a sodium arsenite concentration of 0.15mg/ml in NBA plates supplemented with 0.5% (w//v) glucose and 8mM calcium nitrate. The experimental protocol was otherwise identical to that described above in Section 3.2.4.

3.3 Results

3.3.1 Effect of caffeine and sodium arsenite on wild type S.cattleya

Addition of caffeine induced increased killing by UV light. These results confirmed the results obtained by Coyne et al. (1984) and further support the theory of the existence of a caffeine inhibitable repair system in this organism. Addition of sodium arsenite did not appear to affect the wild type bacterium, as had also been observed in the prior study (Fig 5a).

3.3.2 Effect of caffeine and sodium arsenite on the MMC^S mutant S26

A 25% drop in UV flux required for 10% VC11 survival was observed using S26 as bacteriophage host compared to the wild type (Fig. 5b). This matches the caffeine-inhibited level of the wild type. Addition of caffeine to S26 does not further reduce repair. Addition of arsenite, however, reduces the UV flux required for 10% VC11 survival to 50% of the wild type. Such inhibition of UV repair by sodium arsenite was not observed in the wild type S.cattleya (Fig

5a).

3.3.3 Effect of caffeine and sodium arsenite on MMC^r mutant R6

R6 shows a very unusual result for a bacteriophage UV survival curve (Fig.5c), namely, the presence of a shoulder in the bacteriophage UV survival curve which is usually linear . Caffeine drops the R6 shoulder in that it arises only after a sharp drop in survival; arsenite eliminates the shoulder completely.

3.3.4 Effect of caffeine and sodium arsenite on MMC^r mutant R12

In both absence and presence of caffeine R12 matches the survival curves shown by S26 (Fig. 5d). However, addition of arsenite increases bacteriophage survival.

All the results described here were shown to be reproducible by repeating the experiment on at least three separate occasions. The graphs were plotted with data averaged from the multiple experiments performed.

Figure 5

(a) Bacteriophage UV survival curve of wild type S.cattleya

(b) Bacteriophage UV survival curve of MMC^S mutant S26

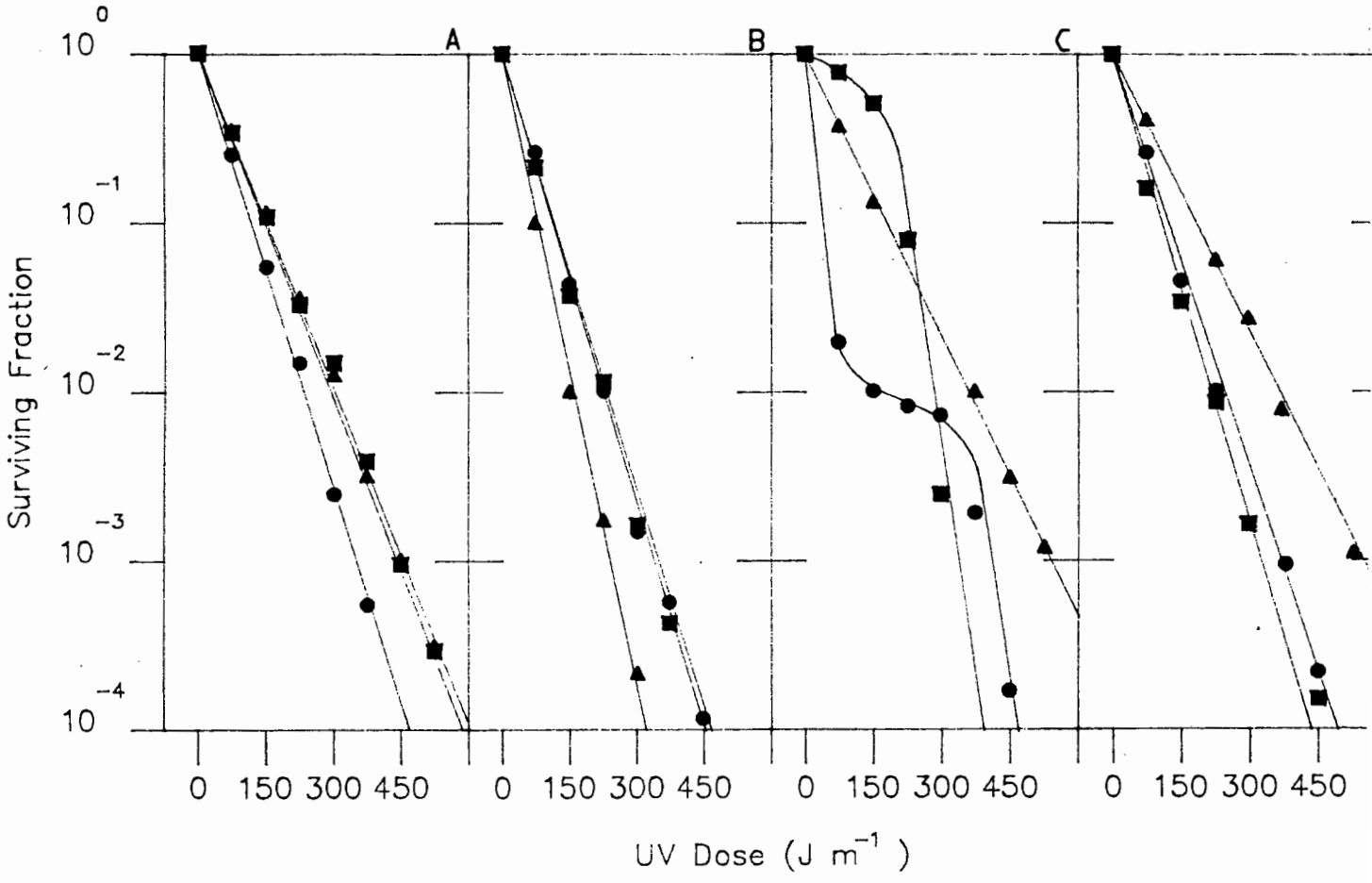
(c) Bacteriophage UV survival curve of MMC^r mutant R6

(d) Bacteriophage UV survival curve of MMC^r mutant R12

■- No additives

●- + caffeine

▲- + sodium arsenite



3.4 Discussion

The profiles obtained from all four strains were compared. The reduced level of UV-irradiated bacteriophage reactivation in S26 is similar to the level of repair found in caffeine-inhibited wild type *S.cattleya*. This may imply that that S26 possesses a mutation in the constitutive caffeine inhibitable host mediated DNA repair system described by Coyne *et al.* (1984). The arsenite inhibition of phage survival in S26 suggests two possibilities. Either the mutation in S26 has uncovered a previously undetected constitutive DNA repair system which is normally overwhelmed by the caffeine inhibitable system, or it has caused derepression of an inducible repair system. Coyne *et al.* (1984) proposed the existence of an inducible arsenite inhibitable system which was not detectable at the bacteriophage level as the half-life of such a system (20 minutes in *E.coli*) was short compared to the 4 - 6 hours required for the spore germination in *Streptomyces* before bacteriophage attachment occurs. The failure to detect such a system in the wild type would support the derepression hypothesis. The very high level of genetic instability in S26 (Fig. 3) shows that the mutation here has had an effect on the mechanism responsible for this phenomenon. To carry the above two alternatives a step further, it would seem that this very high instability is due to either the

essential role the constitutive caffeine inhibitable system in S.cattleya plays in the suppression of instability both in the presence and absence of UV, which system this mutant appears to have lost, or the derepressed arsenite-inhibitable second repair system is responsible for the greater instability.

Coyne et al (1984) have shown that the caffeine inhibitable DNA repair system, termed uvrl, was not directly involved in genetic instability, as the effect of single strand breaks induced by P^{32} on instability was not inhibited by caffeine. As the uvrl system is a primary source of single strand breaks and their subsequent repair in the normal cell, it would seem to be "error-free" as in E.coli. This would imply that both the mutants which could be classed $uvrl^-$, S26 and R12, should have decreased genetic instability, which is not the case. However, it must be remembered that both mutants are also affected in the second, induced repair system, uvr2. P^{32}-induced single strand breaks do not increase instability in R12 and there is a slight dcrease in the instability of S26, although the latter could easily be due to increased lethality of DNA damage in this poorly growing strain. Under similar circumstances, a five-fold increase in genetic instability is seen in R6. This mutant has an enhanced uvrl repair system, and the additive effect of this to the P^{32}-induced single strand breaks together with the enhanced uvr2 system could be causing the increased genetic

instability in this organism. The uvr2 system would therefore appear to be central to the instability phenomena. The effect of a $uvr2^-$ mutant on genetic instability would be a critical factor in elucidating that phenomenon. It would also be helpful if the $uvr1^-$ genotype could be studied in a $uvr2^+$ background, something that would be much easier to achieve if S.cattleya had a direct system for genetic manipulation. Unfortunately such a system has not been developed to date.

The VC11 survival curve of R6 has a shoulder. Induction of a repair system at higher levels of UV damage to the phage DNA could explain this. This would require the rapid detection of such damage and induction of the repair enzymes. Both inhibitors suppress this effect, suggesting that they might be interfering with either or both of these two processes. The presence of such a shoulder in the usually linear phage survival curve is unusual enough to warrant comment and invite speculation. A wealth of interpretations is possible of this interesting result, but there is at present little concrete data to either confirm or deny any of them. It is therefore wise to guard against overinterpretation. However, several suggestions can be discussed. These results do match the spore survival curve obtained for R6 earlier (Fig.2) and suggest that a second UV repair system is being enhanced by the MMC^r mutation although the mechanism is unclear. The system may show enhancement due to its

acquisition of multiple hit kinetics in what is essentially a single hit kinetics process. The elimination of the shoulder by arsenite can argue for the presence of a arsenite inhibitable second repair system. There is, however, potential for a third repair system in S.cattleya that is actually enhanced rather than inhibited by arsenite, as exibited by the very similar phage reactivation curves shown by the two MMC^r mutants, R6 and R12, in the presence of arsenite (Fig. 5c, 5d). This putative third system has a basis in the literature, as Baltz (1986) has reported a third DNA repair system in Streptomyces fradiae. Where uvr1 is postulated to be error free and uvr2 error prone, it is possible that such a system, which may be named uvr3, could also be error free, or at least more so than uvr2 appears to be.

The close simlarity between the S26 and R12 phage reactivation curves in the presence or absence of caffeine suggests that R12 is also defective in the constitutive caffeine inhibitable repair system. This means that it is likely that the mutation has eliminated this system. The increase in bacteriophage survival during growth on arsenite can be explained if control of the arsenite inhibitable system has been affected as well. The spore UV survival curve (Fig. 2) supports the idea that a second inducible repair system which has been affected by the mutation is present and that of the loss of the constitutive system.

These results confirm the presence of a caffeine inhibitable "error-free"-like host mediated repair system (uvr1) in S.cattleya. This system is responsible for the repair of single strand DNA breaks and as these events induce genetic instability in this organism, loss of the system results in increased genetic instability. In S26, the loss of the uvr1 system also results in a derepression of an arsenite inhibitable "error-prone"-like host mediated inducible DNA repair system (uvr2). This system also seems to be the one affected in R6, where the unusual shoulder is found, and in R12 where arsenite induction of DNA repair is detected, although the idea of a third system to account for this must not be dismissed out of hand.

Thus, from the available data, S26 could be described as a $uvr1^{-}$ $uvr2^{d}$ (derepressed) mutant; R6 could be described as a $uvr1^{e}$ (enhanced) $uvr2^{e}$ (enhanced) mutant; and R12 could be described as a $uvr1^{-}$ $uvr2^{c}$ mutant.

The pleiotropic nature of the mutants of the two DNA repair systems could be due to either a closely interlinked system of control of DNA repair or to the genes of these repair systems being physically close on the S.cattleya chromosome, and comutation by NTG, which is common in Streptomyces, has occurred. Of course, both these effects could be occurring together.

The legitimate recombination system in Streptomyces does not appear to be integrated with the DNA repair system as in E.coli, since no UV sensitive mutants have been isolated which are recombination negative (Harold and Hopwood, 1971). Furthermore, a S. lividans rec^- mutant has been isolated which was found not to be UV sensitive (R.Kirby, personal communication). It is thus possible that the legitimate recombination system is at least partially responsible for genetic instability in Streptomyces, especially as most species contain a high proportion of repeated DNA sequences (Usdin et al., 1985). The interrelationship between the uvr1, uvr2 and rec systems in Streptomyces may be essential to the full understanding of genetic instability of these organisms. The independence of the rec function from the SOS response normally associated with it may be due to a unique control system for DNA repair. The induction of genetic instability by UV does not suport the complete independence of this phenomenon from DNA repair; therefore some degree of interaction is still required, if the rec system is implicated.

Unfortunately, the lack of a genetic recombination and cloning system in S.cattleya means that direct genetic analysis of these mutants and the identification of the gene or genes involved in the two systems is not possible. However, it should be possible to clone the MMC^r genotype

into another species of Streptomyces where such a system does exist, which would allow a more detailed analysis.

Chapter 4

Effect of manganese and cobalt ions on the genetic instability of S.cattleya wild type and mutant strains

Summary

The effects of divalent metal ions on bacteria have been widely studied. Several studies have been performed in Aspergillus nidulans concerning the effects of manganese (Mn^{2+}) (Burr, Roper and Relton, 1982) and cobalt (Co^{2+}) (Sexton and Roper, 1984; Daud et al., 1985) on the instability of that organism. Cobalt appeared to increase, and manganese, despite its well-documented mutagenic properties, appeared to decrease instability in A.nidulans. This led us to ask the question as to how these ions would affect the genetic instability of S.cattleya wild type as well as that of the three mutants under study. It was shown that both Mn^{2+} and Co^{2+} slightly induced instability in S.cattleya wild type. A similar effect is seen in S26. R6 remained essentially unchanged, while there was a major increase in the stability level of R12 in the presence of manganese.

4.1 Introduction

An extensive and fairly detailed study has been made on the mutagenic action of manganous chloride on E.coli under various conditions (Demerec and Hanson, 1951). These workers saw manganous compounds as potent mutagens which induced large numbers of mutations under conditions producing little or no killing. $MnCl_2$, the compound studied in this work, was unusual in that the effectiveness of this chemical as a mutagen could show large variations depending on the physiological state of the organism. For example, the effectiveness of the chemical in inducing mutations was very much affected by materials used in the pre-treatment washing of the bacteria - the mutagenic effect was considerably less if the bacteria were washed in water as compared to when they were washed in saline or not at all. When different salts were used in the washing of the bacteria, it was found that the mutagenic effect of $MnCl_2$ increased with the increase in concentration of the salt used. Survival, however, decreased under the same conditions (Demerec and Hanson, 1951). It was suggested that washing the cells in water "leaches out" a certain proportion of whatever bacterial component is necessary for $MnCl_2$ action and thus the cells are better able to resist this action. When further experiments were performed in order to determine to

what extent the physiology of the bacteria influenced the mutagenic effect, $MnCl_2$ induction of mutation was compared in resting bacteria and in actively growing bacteria, as well as in resting bacteria grown in aerated and non-aerated broth. Such conditions appeared to have a fair amount of influence on the effect of $MnCl_2$ on the bacteria, in that it was found that the induction of mutants was much higher in resting cells as compared to growing cells. Furthermore, fewer mutants were produced in bacteria raised in non-aerated cultures than in those raised under conditions of aeration. In tests to determine the effect of other ions on the mutagenic effect of manganese, it was found that cobalt reduced this by a factor of one hundred, second only to Mg and Zn. In another experiment, the effect of pH on conditions of mutagenesis was tested. It was seen that within the range that is tolerable by bacteria, the pH does not seem to affect the observed mutagenic effect of $MnCl_2$. Mutagenesis appeared to be proportional to the concentration of the mutagen only up to a certain level, after which it remained unaffected by further rises in concentration. A similar observation was made with respect to time. It was found that the mutagenic effectiveness of $MnCl_2$ increased with time up to a period of 30 minutes and was then not increased further.

Demerec and Hanson concluded that the results may be explained by differences in permeability and uptake ability

of bacteria, given the effects that bacterial morphology and differences in bacterial response when washed in different chemicals have on mutagenesis. Situations favouring high uptake of $MnCl_2$ are also situations of high mutability; it can be suggested that manganese probably binds to specific sites on the proteins or nucleic acids of the cell in a weak complex. Also, seeing that the mutability of Mn^{2+} is modified in the presence of certain metal and other ions, it could be that the induction of mutagenesis by $MnCl_2$ is initiated by some reaction involving a certain enzyme or a group thereof. There are several lines of evidence indicating that the action of manganese is indirect. Firstly, its effect is not specific but rather general, increasing mutability in many loci of a genetic system. A second line of evidence is that treatment with $MnCl_2$, UV light and X-rays produces similar patterns of genetic efects. There is usually a delay in the expression of mutants in all these cases, indicating that the pattern of expression of induced mutation is characteristic of the mutation locus and does not depend on the treatment by which mutations are induced. Thirdly, and perhaps most convincingly, the effect of treatment with both UV and $MnCl_2$ is not additive. At present the genetic effect of $MnCl_2$ can be assigned to changes induced outside the genetic system in either the cytoplasm or the nucleus of the bacterial cell. These changes are assumed to affect the metabolic activity of the cell and its repair systems, thus causing genetic

instability and an increase in the mutation rate (Demerec and Hanson, 1951).

Orgel and Orgel (1965) have reported an induction of mutation in phage T4 by the manganous ion. These mutants, as well as other base analog mutants, are also fully reversible by Mn^{2+} in a kind of antimutagen capacity. Mg^{2+} is seen as protecting E.coli against the mutagenic effects of Mn^{2+}, probably by competing with it for entrance into and maintenance within the bacterial cell. An interesting phenomenon is the fact that the DNA polymerase of E.coli will incorporate ribonucleotides in place of deoxyribonucleotides if incubated with Mn^{2+} instead of Mg^{2+}(Orgel and Orgel, 1965). This may be due to the fact that almost all Mn^{2+} complexes with oxygen- and nitrogen-containing ligands are isomorphous with the corresponding Mg^{2+} complexes, a relationship apparently unique to these two ions (Orgel and Orgel, 1965). The ionic radius of Mn^{2+} is greater than that of the magnesium ion by approximately 0.1Å; it is therefore to be expected that the stereochemistry of complexes containing these two ions would often be qualitatively extremely similar, but that certain groups would be displaced from their normal positions by distances of the order of 0.1Å. In some cases, if Mg^{2+} is present at the active site, such displacements may have a hand in upsetting the speciicity of enzyme action without affecting the basic process; this might be the basis for the

understanding of the aberrant action of the manganese-substituted DNA polymerase.

The effect of Mg^{2+} on manganese mutagenesis, as described above, is repeated in a study of that process in yeast (Putrament et al., 1975). These workers assumed that the manganese cation would decrease the fidelity of DNA polymerase(s) and thus induce point mutations in the organism (Demerec and Hanson, 1951; Orgel and Orgel, 1965). The data produced in this particular study lead the authors to conclude that manganese acts as an error-producing factor only on replicating mitochondrial DNA (Putrament et al., 1975). Mn^{2+} may induce mutations by interaction with template DNA rather than DNA polymerase, but the authors suggest that the more likely hypothesis would be that the cation interacts directly with the enzyme. It may completely inhibit the activity of the manganese-sensitive mitDNA polymerase and cause the remaining enzymes involved in mitDNA replication become error-prone.

In stark contrast to all the reported instances of Mn^{2+} causing mutagenesis and therefore increased instability, Burr, Roper and Relton (1982) report it has a stabilising influence on Aspergillus nidulans. The stabilising effect of manganous chloride was also demonstrated on mutagen-treated Penicllium chrysogenum (Sermonti and Morpurgo, 1959; quoted by Burr, Roper and Relton, 1982). In the A.nidulans study,

it was shown that Mn^{2+} either substantially decreased instability in certain strains studied, or had no effect at all in other strains. In no case did it exhibit its usual and very well documented highly mutagenic and instability-increasing action. Aside from the observations of Sermonti and Morpurgo (1959) and the Aspergillus study, only three further reports with possible relevance to a stabilising action of the manganese ion have been made. Burr, Roper and Relton (1982) quote Whiting et al. (1979) as having shown that unscheduled DNA synthesis in hydrazine-treated human cell cultures was stimulated by Mn^{2+}; Litman (1971), quoted by the same authors, reported that both the rate and fidelity of replication of GC rich regions of DNA by a Micrococcus luteus polymerase was enhanced by Mn^{2+} as compared to Mg^{2+}. Finally, Eichhorn (1971) showed that Mn^{2+} stabilised the DNA helix by preventing unwinding and/or facilitating reannealing.

Two studies have been done involving the presence and action of cobalt ions on Aspergillus nidulans (Sexton and Roper, 1984; Daud et al., 1985). These studies involved spontaneous duplications and transpositions of a large chromosomal segment in this organism. Strains of A.nidulans in which the segment was duplicated were shown to be unstable at mitosis. Some of these duplication strains showed marked stabilisation by cobalt, which, by extrapolation, implies that cobalt ions stabilised their instability and

consequently probably induced a rise in the general level of instability shown by the organism.

The A-factor (autoregulatory factor) and similar derivatives in Streptomyces griseus are pleiotropic endogenous effector molecules, mediating a restoration of aerial mycelium, spore and antracycline formation by surface cultures of blocked, non-sporulating and non-antibiotic producing ($Amy^{-}Ant^{-}$) strains of S.griseus. The A-factor has been suggested to play a role either in the transport of trace elements from the medium into the cells or as a coeffector of a metal-containing enzyme (Grafe et al., 1985). Cobalt was the only metal ion out of several tested which showed synergistic interference with the A-factor in its inducing effect on cytodifferentiation of the blocked mutant strain S.griseus 86. The presence of cobalt was found to improve aerial mycelium formation and other positive effects on the strain growth and differentiation were observed. It was postulated that the metal could perhaps play a role in mediating the A-factor induced cytodifferentiation of the blocked S.griseus mutants. Possibly the A-factor may exert its effect by chelating trace elements in the medium and enabling them to be transported through the lipophilic membrane (Gräfe et al., 1985). The occurrence of just such a cobalt-chelating molecule has been recently established in S.cattleya (Bushell and Fryday, 1983).

The reports of the action of Mn^{2+} and Co^{2+} have thus been numerous and occasionally conflicting. Since these substances appear to be instrumental in several ways to the stability, or lack of it, of many different organisms, this was deemed to be a good reason to investigate their effect on the instability in S.cattleya , both in the wild type and in the three mutants obtained in this study.

4.2 Methods

4.2.1 Materials and media

All materials and media used are described in Apendix B.

4.2.2 Determination of the effect of manganese ions (Mn^{2+}) on S.cattleya wild type and mutants

The source of the manganese ions for this experiment was the salt Manganese (II) chloride.

Five different concentrations of $MnCl_2$ were prepared in five 200ml aliquots of Malt 3 Agar (0.005M, 0.01M, 0.02M, 0.03M, 0.05M). Spore suspensions of S.cattleya wild type or the mutants, diluted so as to give countable colonies (1×10^{-3}), were plated on these plates. These were then incubated at $30^{O}C$ for 4 to 6 days. The appearance of the bacteria and any changes in their size or viability were noted. To avoid pleiotropic effects due to the presence of manganese in the growth media, approximately 1000 colonies per strain were transferred to gridded Malt 3 Agar plates free of any additives and allowed to reach full sporulation by being incubated at $30^{O}C$ for at least a week.

4.2.3 Determination of the effect of cobalt ions (Co^{2+}) on S.cattleya wild type and mutants

The source of cobalt ions for this experiment was the salt cobalt (II) chloride.

Five different concentrations of $CoCl_2$ were prepared in five 200ml aliquots of Malt 3 Agar, as in the previous experiment. The concentrations used here were 0.001M, 0.002M, 0.005M, 0.01M and 0.05M. The remainder of the experimental protocol was identical to that described in Section 4.2.2.

4.2.4 Scoring of results

The manganese/cobalt treatment was expected to affect in some manner the instability of S.cattleya. Instability was therefore used as a measure of the results of this experiment. Instability was taken here, as previously, to mean the loss of the aerial mycelium and therefore of the ability to sporulate. It has thus been expressed here as the percentage frequency of Bald (Amy^-) colonies produced in the wild type S.cattleya and the three mutants by the treatment.

4.2.5 Determination of mutagenic properties of cobalt and manganese ions on S.cattleya wild type and mutant strains

This was tested directly by plating undiluted spore suspensions of the wild type or mutant strains onto Malt 3 Agar containing 10ug/ml rifampicin in addition to 0.001M or 0.002M Co^{2+}, and 0.005M or 0.02M Mn^{2+}. The plates were incubated at $30^{O}C$ until such time as colonies appeared, a time period of about four to five days, and the numbers of mutant colonies arising from the treatment were scored at this time.

4.3 Results

4.3.1 Effect of manganese and cobalt ions on genetic instability in S.cattleya wild type and mutant strains

4.3.1.1 Qualitative results

4.3.1.1.1 S.cattleya wild type

The entire range of concentrations of Mn^{2+} tested engendered 100% Amy^- phenotype in S.cattleya wild type, with two distinct morphological types appearing. Both were small and smooth in appearance, unlike the usual ridged appearance of Bald colonies; however, one type was a light beige and the other a darker brown in colour. As far as the cobalt is concerned, no growth at all was observed above the lowest concentration tested, i.e. 0.001M. All the colonies observed had the Amy^- phenotype, as with manganese. The morphology of the colonies was more similar to the usual appearance shown by Amy^- colonies.

4.3.1.1.2 R6

A large number of very small colonies were observed at 0.001M Co^{2+}. Unlike the wild type, R6 showed almost 70% sporulation while on the cobalt-containing media; it also grew at a higher concentration, the cutoff point being 0.002M Co^{2+}. Again, large numbers of very small colonies were observed, with a pinkish tinge to them not found in the wild type. No growth was observed at higher cobalt concentrations. With manganese, this mutant showed strong growth at all the concentrations tested. The sporulation levels on the manganese-containing plates were high (60% - 70%), again unlike the wild type organism.

4.3.1.1.3 R12

The results obtained with cobalt using this mutant were slightly anomalous. Small numbers of colonies (on average, about 7 per plate) which showed a low incidence of sporulation were observed on the plates containing 0.001M cobalt. No growth was observed on the media with 0.002M or 0.005M Co^{2+}; but several large and bald (Amy^-) colonies were observed at the concentration of 0.01M. These colonies had a distinct pink tinge to them, and appeared to behave as though an agarase-like enzyme had been induced in them in that they had spread down into and through the media until

they were brought up by the bottom of the Petri dish. Growth was shown at all concentrations of manganese, but only small numbers of colonies were observed throughout. The colonies were generally larger than those observed with R6. At all concentrations but one, all the colonies showed the Amy^- phenotype whilst still on the manganese-containing media; at 0.05M, however, the bulk of the colonies had begun showing signs of incipient sporulation on the manganese plates.

4.3.1.1.4 S26

This mutant showed growth, albeit very poor, at all the tested concentrations of manganese. Very small colonies were observed in the presence of both cobalt and manganese in the media. A few pinprick colonies grew at 0.005M cobalt but no growth was observed at concentratons higher than this.

4.3.1.2 Quantitative results

4.3.1.2.1 S.cattleya wild type

Colonies from plates containing the highest concentrations of Mn^{2+} and Co^{2+} at which growth was seen to occur were transferred onto fresh Malt 3 Agar plates with no additives. After an incubation period of 18 days of the colonies taken from the 0.001M cobalt plates, only 20% had retained their Amy^- character. After a further subculturing step to test the incidence of revertants, it was concluded that only 10% of the tested colonies could be said to have been permanently and stably altered by the cobalt treatment and retained the Amy^- phenotype. This is a slight increase on the percentage instability frequency for untreated wild type S.cattleya (see Table 2).

The two different phenotypes occurring in the manganese plates were taken into account when colonies were transferred onto fresh Malt 3 Agar plates, and representatives of both types were tested; however, there appeared to be no differences in their subsequent behaviour. The incidence of Bald (Amy^-) colonies, after an incubation period of 19 days, was 18%. However, in this case too there were some revertants, effectively halving this percentage to

a final frequency of about 9%.

4.3.1.2.2 R6 and R12

As distinct from the wild type organism, both these mutants, to a greater or lesser extent, showed sporulation while still growing on media contaning manganese. Only 5% of colonies transferred to fresh Malt 3 Agar plates remained Amy^-, in both cases.

Where cobalt is concerned, R6 showed, again, sporulation on cobalt-containing media whereas the wild type did not. The limiting concentration of cobalt in this mutant was slightly higher than that of the wild type S.cattleya (0.002M) and the colonies that grew at this concentration were all Amy^- whilst still on the cobalt containing media. However, only about 5% of the colonies stayed Amy^- once transferred to cobalt-free Malt 3 Agar. R12 showed some sporulation on cobalt-containing media, but much less than R6. Colonies transferred onto fresh Malt 3 Agar stayed true to type as manifested on the cobalt media. 20% of the colonies transferred to fresh, additive-free media remained Amy^-.

4.3.1.2.3 S26

All the colonies growing on media containing manganese or cobalt were Amy$^-$. Transferred onto additive-free media, 51% of the colonies taken from the cobalt plates remained Amy$^-$ while 41% of the colonies from the manganese-containing plates did so.

All the above results have been summarised in Table 2.

Table 2: Effect of manganese and cobalt ions on instability in S.cattleya wild type and mutant strains

Strain / Media: Malt 3 Agar	WT*	R6	R12	S26
No additives	2%	6%	17%	38%
+ Co^{2+}	10%	5%	20%	51%
+ Mn^{2+}	9%	5%	5%	41%

4.3.2 Mutagenesis by cobalt and manganese ions in S.cattleya wild type and mutant strains

The results obtained in this experiment are summarised in Table 3. There was a distinct decrease in the number of mutations induced in R12 by manganese as the concentration of manganese was increased. In R6, the mutagenesis level remained relatively unchanged after a small rise at low levels of Mn^{2+}. By contrast, there was a large induction in the wild type organism. S26 results are misleading, as there is a high level of lethality masked by the low numbers of mutants obtained.

The cobalt induced mutagenesis values cannot be meaningfully explained because of the very high lethality the treatment engenders. It does, however, appear that at 0.001M Co^{2+} in the medium a fairly high percentage of R6 survivors are in fact mutagenised by the treatment; in R12 a similar phenomenon is observed but to a much lesser extent. This data is not shown, as it cannot be substantiated; the conclusions tentatively discussed above are based on subjective observation of the growth of these mutants on cobalt-containing media.

Table 3: Mutagenicity by manganese in S.cattleya wild type and mutant strains

	Average number of mutants/plate		
	No Mn^{2+}	Mn^{2+}	
		0.005M	0.02M
wild type	61.75	400.00	-
R6	150.00	280.00	324.00
R12	275.00	300.00	114.10
S26	288.00	100.80	-

4.4 Discussion

There appears to be a mild induction of instability by both cobalt and manganese in S.cattleya wild type, with both ions inducing the instability to roughly the same extent. A similar induction occurs in S26, with cobalt causing the higher degree of induction (an increase of 13%). R6 does not change, except for a minor 1% drop in instability caused by both ions tested. In R12 there is a major increase in stability in the presence of manganese, while cobalt has the opposite effect, causing a small increase of the instability level of this strain.

The phenomenon occurring in R12 is difficult to explain. There seems to be a parallel drop in instability in R6, but it is much smaller; and in the presence of cobalt the reactions of the two strains are opposite to one another. S26, with which R12 shares a similarly affected uvrl DNA repair system, shows a parallel instability induction in the case of cobalt, which then implies that the action of cobalt may be in some way connected to the uvrl system or its enzymes - especially in the light of the fact that R6 shows no such effect. But R12 is unique amongst the strains in its reaction to manganese.

There are two theories that could explain this phenomenon. The first one is that perhaps there is a difference in permeability or active manganese uptake between R12 and the other strains. It is impossible on the strength of the available data to differentiate between a possibly increased or decreased uptake capability; in the first case, the effect of the manganese ion could be similar to that in A.nidulans (Burr,Roper and Relton, 1982) and the strain would thus be stabilised, while in the second the R12 strain would not take up as much manganese as the other strains and would thus be protected from any negative mutagenic or instability-inducing effects. In the presence of manganese, the instability level of R12 actually approaches that of the untreated wild type organism. The increased instability in the wild type and S26 in the presence of manganese could be taken as evidence in support of the second hypothesis, but in this case it is R6 which now requires an adequate explanation. However, Streptomyces being a highly comlex organism that is almost intermediate between prokaryotic and eukaryotic nature,it is possible that the first hypothesis is correct and that Streptomyces joins some of the fungi in showing a stabilising effect of manganese.

The second theory involves an enzyme or an enzyme system in R12 that is stabilised by the presence of manganese. This is a likely hypothesis in the sense that R12 is unique in the mutation it possesses, and it is conceivable that an enzyme

or enzymes altered by this mutation is/are actively stabilised by the manganese ion. It is possible that such an enzyme may have remained unaltered in S26, as its phenotype and therefore its mutation is different from that of R12. A slight similarity of action of manganese on R6 suggests that the same enzyme or enzymes have been affected by the metal ion in a similar manner to that observed in R12. While the actual identity of the enzyme(s) affected in this way remains difficult if not impossible to pinpoint on the given data, a likely candidate would be a DNA polymerase, which has been shown to be affected by the action of manganese in earlier studies. An interesting speculation is invited by the observation of Orgel and Orgel (1965) of the altered action of DNA polymerase when cells are grown in the presence of manganese instead of magnesium. The changed enzyme loses the specificity of action and sequesters ribonucleotides instead of deoxyribonucleotides in DNA polymerisation. Such an effect would in itself be mutagenic and repairable, since the ribonucleotide bases would have to be excised and replaced by the correct deoxyribonucleotide bases for the DNA to function correctly. In a mutant where a repair system is already active such mistakes could be readily repaired, which might account for the lowering of instability in R6 and R12. A higher level of polymerase expression might be taken as a possible explanation, or a part thereof, for the high incidence of sporulation of R6 and R12 while still on the cobalt- or manganese-containing

media. S26, mimicking as it does the wild type organism with a similar response at its own much higher instability level, may have kept the enzyme concerned unaltered, or possesses a mutation in its own repair systems such that the repair of this particular kind of damage is impaired. It must be mentioned that S26 is by its very nature an extremely labile and unstable strain; the variation in instability that it shows, for all its face value similarity to that shown by wild type S.cattleya in the presence of cobalt and manganese, may be no more than a reminder of this fact.

In as much as the mutagenesis results for manganese are concerned, it is interesting that R12, the mutant which showed the greatest stabilisaton by manganese, now shows a decline in the rate of manganese mutagenesis. The two phenomena are doubtless connected at the level of the mutation in R12. It could be surmised, since this mutant appears to have lost the error free repair system of the wild type organism, that the second repair system discovered in S.cattleya, uvr2, is what is now responsible for the decreased mutagenesis by manganese as well as for the increase in stability in the strain. However, this is an intriguing prospect, since uvr2 has been tentatively characterised as an error prone system and as such should not be capable of such stabilising action. It may be possible that there are in fact two inducible repair systems in S.cattleya, one repressed and one induced by arsenite

(induction of repair by arsenite is actually seen in R12; see Fig.5) and that the latter is an error free repair system which is responsible for the control of the above discussed phenomena in the R12 mutant.

Chapter 5

Effect of UV irradiation on proteins in S.cattleya wild type and mutant strains

Summary

The possible induction of proteins concerned with DNA repair was attempted by irradiation of the wild type and mutant strains of S.cattleya with low levels of UV, followed by growth and testing at intervals for the presence of any new proteins arising through the growth period. This was done by SDS polyacrylamide gel electrophoresis. The gels so obtained were silver stained and protein profiles obtained by scanning the gels at 450nm in a Beckman DU-8 spectrophotometer. Unfortunately, only the wild type S.cattleya gave clear results. Observation of any UV induced proteins in the mutant strains was unsuccessful, due to technical reasons.

5.1 Introduction

All facets of DNA repair in the organisms in which such processes have been studied are governed by the action of enzymes, which are either produced constitutively or induced by the action of DNA damaging agents on cellular DNA.

Different enzymes have been postulated for different processes - in E.coli, for example, the error free short patch excision repair pathway is dependent on the presence of DNA polymerase I (Smith, 1978) while there is evidence that other, more error prone pathways rely more on DNA polymerase III or DNA polymerase II (Howard-Flanders, 1968; Grossman et al., 1975). In contrast, some enzymes, probably produced constitutively by the cell, are required for all the repair processes because their action restores the integrity of the genome - polynucleotide ligase, which is responsible for joining DNA molecules together, is such an enzyme (Howard-Flanders, 1968). It has been established that some enzymes exist which show multiple functions. These can be exemplified by the RecA protein, which is induced in the cell during the SOS response to damage in the DNA molecule. Enzymes concerned with DNA repair, and there are many whose functions and mechanisms of action are well understood, have been more fully discussed in Chapter 1.

The work presented in this chapter is concerned with identifying possible proteins arising from treatment of S.cattleya wild type and mutant strains with low doses of UV light. UV irradiation is known to produce damage at the DNA level, and any proteins seen to appear in irradiated cells may well be concerned with the repair of such damage within the cell. It was thus necessary to compare the differing responses of the wild type and mutant strains to such treatment. It was thought that such an approach would give a valuable insight into the different ways in which the mutant strains cope with damage repair, compared to the wild type and to one another.

5.2 Methods

5.2.1 Preparation of samples

Cultures of the wild type and mutant strains were prepared as follows: 100ml of Malt 3 Broth was inoculated with a loopful of fully sporulated bacteria and allowed to grow for 24 hours at $30^{o}C$ with gentle aeration. After this period the cultures were harvested by centrifugation at 19000 rpm for 10 minutes in a JA20 rotor (Beckman). The pellets were resuspended in 10ml Streptomyces Minimal Medium (Appendix B). Half of this cell suspension of each strain was subjected to irradiation with low doses of UV, with the wild type strain and mutants R6 and R12 each receiving a dose of 60 J m^{-2} (12 seconds at 5 J m^{-2}) and mutant S26, which showed too high a level of killing at this dosage, receiving 10 J m^{-2} (2 seconds at 5 J m^{-2}). The other half of each of the cell suspensions was left unirradiated to serve as a control. Following the irradiation, all of the suspensions were centrifuged once more under identical conditions as desribed above and the pellets were thoroughly resuspended in 5ml Streptomyces Minimal Medium. Once adequate suspension was achieved, each suspension was divided into nine 500ul aliquots and these were placed at $30^{o}C$, with gentle shaking. At predetermined intervals growth was stopped by adding

200ul of the sample to 400ul of sample buffer (Appendix B), containing 0.01% bromophenol blue. Samples thus obtained were boiled for 10 minutes and then cooled. To free any protein still possibly bound the samples were sonicated in 10-second bursts with 10 second intervals for one minute. Following this the samples were run on an SDS polyacrylamide gel, as described previously. After completion of electrophoresis, the gels were stained.

5.2.2 Silver staining

This method of staining is highly sensitive and can detect as little as 15ng of protein per band. The method, adapted from the procedure of Oakley et al. (1980), was obtained from J. Burger, Department of Microbiology, University of Cape Town. All the solutions are described in Appendix B. The Fixer, Developer, $KMnO_4$ and $AgNO_3$ solutions, must be freshly prepared before use. The other solutions can be prepared as stocks and used as such.

The gel was placed in a clean Tupperware container after electrophoresis, and stained was allowed to proceed at room temperature with gentle shaking. It is important to handle the gel with gloves at all times.

The solutions used in the staining procedure were added

sequentially to the same container after the previous solution had been carefully poured or aspirated off. A tabulated version of the method, containing the order in which solutions were added and the length of time they require to act on the gel, is given below. The quantity specified refers to the quantity of reagent necessary per gel.

Solutions (in sequential order)	Vol/gel	Soaking time
(1) Fixer	(100ml)	45 minutes
(2) Solution A	(100ml)	15 minutes
(3) 0.01M $KMnO_4$	(100ml)	15 minutes
(4) Solution A	(100ml)	15 minutes
(5) 10% ethanol	(100ml)	15 minutes
(6) distilled H_2O	(100ml)	15 minutes
(7) 0.1% $AgNO_3$	(100ml)	15 minutes
(8) H_2O dip	(100ml)	30 seconds
(9) 10% K_2CO_3	(100ml)	90 seconds
(10) Developer	(100ml)	until discrete bands appear; but must not allow background to get too dark.
(11) Solution A	(100ml)	5 minutes

5.2.3 Analysis of gels

Silver stained gels do not dry well - they shrink and darken, and much of the resolution is lost. To overcome this, gels were scanned electronically using a Beckman DU-8 spectrophotometer at 450nm. The protein profiles obtained in this manner were directly comparable.

5.3 Results

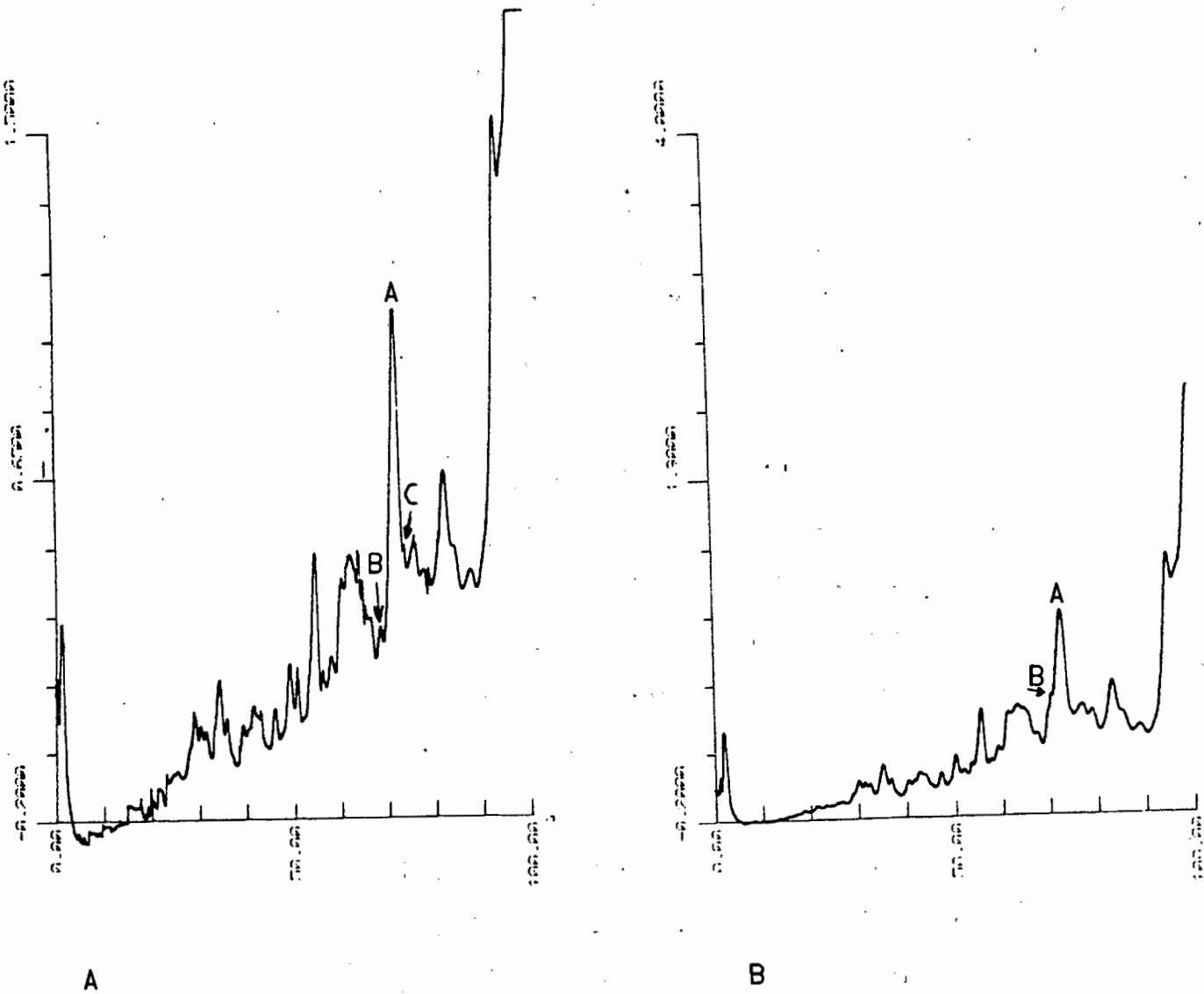
The comparative profiles obtained for the UV irradiated and non UV irradiated cells of wild type S.cattleya obtained by scanning the silver stained gels are shown in Fig.6. The peaks that may be of interest have been marked A, B and C. If the two peaks designated as B in Fig 6a (unirradiated cells) and Fig.6b (irradiated cells) are in fact equivalent, then there would appear to be a slight induction of production of this protein after UV irradiation of the cells. In contrast the protein designated as A decreases after UV irradiation. It is possible that the protein signified by peak A has been significantly reduced as protein B has been induced. A possible explanation is that a constitutive enzyme is being replaced by an enzyme that is induced - perhaps an error prone polymerase in an SOS-like system. An alternative and more complex view is that the peaks designated as A and B do not in fact correspond. Rather, it could be argued that peak C in Fig.6a is equivalent to peak A in Fig 6b, and that peak A in Fig.6a is in fact equalled by peak B in Fig 6b. In this case, the protein signified by peak C would have been significantly induced by treatment with UV, with a concomitant drop in the level of expression of the other protein. It could be argued that the induced protein is perhaps acting as a repressor of some kind to the expression of the other. If the distances

of each peak to the point of origin are measured in order to give a clearer picture as to the actual identity of each peak, the second hypothesis is strongly suggested as the correct one. However, it is difficult to give any weight to either hypothesis at this time, as the data is ambiguous and due to vagaries of scale it is not easy to accurately correlate the equivalent peaks in the two images.

Unfortunately, severe difficulties were experienced with the three mutant strains. After several attempts, it proved impossible to obtain a gel with banding clear enough for scanning purposes. Those bands that did appear were too faint to be useful, and there appeared also to be a large amount of protein degradation which resulted in very high background levels as the gels were stained. Within the time limit of this project it was not possible to investigate and rectify the reasons for these occurrences. This investigation is therefore unfortunately not as complete as it may have been.

Figure 6

Protein profiles of wild type S.cattleya after attempted induction of proteins by UV irradiation (a) Non-UV irradiated cells (control); (b) UV irradiated cells.



5.4 Discussion

In the light of the differences in total cellular protein seen in Fig.4, the results of this experiment could have been of great interest. Unfortunately, the levels of protein expression or the extent of their degradation in the cells of the mutant strains has prevented a complete analysis.

A possible explanation for the failure to obtain clear protein banding in protein gels of the three mutants could be that the mutations have also affected the expression of other unrelated proteins in the cell. Oblique confirmation of this could perhaps be taken from the sudden appearance of an agarase-like enzyme produced by mutant R12 during treatment with divalent metal ions, as reported in Chapter 4. If such new proteins have been produced, it is possible that they could include intracellular proteases which degrade cellular proteins. Such proteases have already been observed by Ginther (1970) in another Streptomyces species, and so it is not inconceivable that they may arise in S.cattleya. The production of such enzymes could have been stimulated by low levels of UV irradiation used in this experiment to stimulate induction of possible repair proteins, and this could perhaps be taken as a reason for the low levels of protein expression observed in irradiated cells. However, this leaves the lack of adequate protein

banding in non-irradiated cells unexplained. The existence of such protease(s) can also be suggested as the possible explanation for the accumulation of low molecular weight proteins shown by S26 (Fig.4), although this may simply be a facet of the mutation that has been engendered in this mutant.

Very little is at present known about protein induction in Streptomyces. The mycelial nature of the organism has caused its own problems where UV irradiation is concerned. However, since UV is traditionally used in similar studies on other organisms, it was also used as the agent of induction here. A possible alternative to UV, such as mitomycin C, could perhaps be considered. The similarity of action of this antibiotic to UV light has alredy been described in Chapter 2. Other alternatives are agents such as EMS or MMS. Cells could be grown in liquid culture, with low levels of one or the other of these agents added after approximately one day of growth in order to induce protein synthesis. The cells could then be grown further in the presence of low levels of such an agent and used subsequently to test for protein induction. This type of process would certainly be easier to standardise and to control when compared to the perhaps uncertain reproducibility of UV irradiation.

Chapter 6

Comparative studies on the effect of different mutagens on S.cattleya wild type and mutant strains

Summary

Due to their changed natures with respect to the DNA repair systems as compared to the wild type organism, it could be expected that the mutants would show correspondingly different reactions to mutagenesis. This was tested using three different mutagens, namely, ethylmethanesulfonate (EMS), methylmethanesulfonate (MMS) and N-methyl-N'-nitro-N-nitrosoguanidine (NTG). The reactions of the three mutants and the wild type S.cattleya were tested against a range of concentrations of these mutagens. Two specific aspects were concentrated on: the survival rate of the strains with increasing concentrations of the mutagen, and the mutation rate (or percentage of mutants among the survivors). S26 showed a low survival rate with all three mutagens tested. However, in all cases R6 and R12 showed a higher survival rate than the wild type although with MMS the differences are the least marked. NTG had by far the most powerful effect on all the strains, where both survival and mutagenesis rates were concerned. The mutagenesis curves of

R6 after treatment with all three mutagens remained very similar to one another.

6.1 Introduction

Early studies of mutation induction in the sixties were mostly concerned with simple cases of chemical mutagenesis in phages, but recently the emphasis has shifted onto bacteria. Due to the very fact that bacteria possess a complete self contained machinery concerned with self reproduction and maintenance, it was only to be expected that chemical mutagenesis in bacteria was going to be different from any known earlier (Kondo et al., 1970). One of the reasons for this difference is that in the bacterial system, with its higher genome complexity, detection of mutation is not done directly at DNA level but rather through some change in the phenotypic characteristics of surviving individuals.

In their study on chemical mutagenesis, Kondo et al. (1970) used four E.coli strains possessing different DNA repair capacities to compare mutational frequencies of various mutagenic agents. The four strains used were the wild type organism, with normal DNA repair capacity; an Exc^- strain, unable to excise dimers; a Res^- strain, unable to resynthesise the excised portion, and thus defective in the second step of excision as Exc^- is in the first; and a Rec^- strain, defective in recombinational repair. Seven mutagenic

treatments were used in this study, but only the three used in the current study will be commented on here. There is evidence that the molecular environment at the replicating point of the DNA could be responsible for the different mutagenic specificities of chemicals. In previous studies quoted by Kondo et al. (1970) it has been reported that all three kinds of error that occur in E.coli - repair errors, replication errors and recombinational errors - are responsible for the generation of mutations in E.coli. In the study under discussion (Kondo et al., 1970), NTG induced considerable yields of mutations in the Rec$^-$ strain of E.coli. The same strain showed a high frequency of mutation when treated with EMS, but not with MMS. This lack of mutagenesis by MMS does not appear to be due simply to the effect being masked by the high lethality that MMS causes in this strain because the Res$^-$ strain, with a similar sensitivity, does show a high mutation yield after MMS treatment. NTG and EMS were the most powerful mutagens tested as the least mutable strain used in the study (the Rec$^-$ strain) is mutable by these chemicals and the maximum mutant yields are higher for these chemicals than for the others tested. These similarities imply that the mechanisms of action of NTG and EMS could be different in some essential manner from those of other mutagens, although the two themselves may not necessarily be similar to one another. Kondo et al.(1970) classified the mutagenesis patterns they observed with the various mutagens they tested

into mutagenesis types. MMS is a Type II mutagen, and its action involves recombinational error caused by unexcisable damage. EMS is classed as being a Type V mutagen, as it induces mutations due to replication errors. NTG is something of an intermediate, falling under a heading of Type II+V. This mutagen induces mutation via a mixture of replication and recombination errors.

Type II mutagenesis: there is evidence that unexcisable damage is responsible for MMS-induced mutations of thebase-change type. However, mutation does not appear to be the major cause of MMS lethality. Depurination or strand breakage may lead either to cell death or to the premutation single strand gaps, described earlier in Chapter 1. As an alternative, recombination may be directly provoked through action of restriction (or similar) enzymes by base modification such as purine alkylation.

Type V mutagenesis: although MMS and EMS are usually classified together as acting at the level of alkylation of purines in the DNA, they show sufficiently different mutagenic patterns in E.coli to be classed as different types of mutagens. For example, the Rec^- strain of E.coli was almost as highly mutable as the other strains when EMS was used but not when MMS was the mutagen. It is proposed that EMS damages non-DNA material which in turn leads to error-filled progeny DNA, even if the parent DNA is intact.

Possible candidates for such material are membrane factors, DNA polymerases or other relevant enzymes in the replication pathway. Alternatively EMS could produce error promoting damage in DNA which is then passed on to the daughter DNA. Kondo et al. (1970) support the former hypothesis.

Type II and V mutagenesis: NTG is one of the most powerful mutagens known. The pattern of mutagenicity it causes is seen to be similar to that of EMS; however, reduction of mutagenesis in the E.coli Rec^- strain (as compared to the wild type) was much more pronounced with NTG. It is also a more powerful prophage inducer. NTG action is proposed to be a combination of those of EMS and MMS. There is evidence that NTG induces mutations through reaction with the DNA replicating point, but Kondo et al. (1970) believe that NTG affects the molecular environment of the replication fork rather than the DNA itself.

Thus, base change mutation in E.coli may be induced via both recombination and replication errors. The former are assumed to arise from spontaneous or damage-provoked recombination inducers directly (UV, NTG, MMS) or indirectly. The latter is assumed to occur due to damage to DNA the replication machinery after mutagen treatment.

A similar study to that quoted above was performed by Ishii and Kondo (1974) more recently. Many of the conclusions of

this study are no more than confirmations of those of Kondo et al. (1970) in the earlier work, but there are a few novel aspects. It was concluded here that recA-dependent repair of DNA damage or errors in replication enhanced by damage to the replication system or template strands produces deletions as well as base changes. From dose response curves, the mutagenic efficiency of EMS was shown to be higher than that of NTG. MMS was not investigated but presumably falls somewhere lower down on the scale. These authors also addressed themselves to the questions of whether the mutagens studied have specificity for inducing deletions or base changes, and whether the dose response curves obtained reveal the mechanism of action of the mutagens. It was seen that the class of mutagen into which MMS falls preferentially induces deletions, EMS was seen to produce base pair substitutions more often, and NTG, once again, appears to be intermediate. It was suggested that an explanation for the multi-hit curves of mutagenesis and killing by EMS and NTG may be that they act indirectly on cellular machinery responsible for DNA repair; because there may be several such non-DNA targets per cell, indirect chemical mutagenesis can easily display multi-hit response curves. In the above study, the earlier types of mutagenesis proposed by Kondo et al. (1970) are somewhat modified, and EMS and NTG were classified together as mutation inducers that act without the participation of the recA gene, through replication errors enhanced by damage to the DNA repair-

independent replication machinery. NTG was seen as possibly reducing the fidelity of the replicative system. The suggestion that NTG acts indirectly is supported by the fact that it reacts with proteins, and further support for this idea is offered by its postulated action on the molecular environment of the DNA replication rather than on the DNA itself. However, there is also evidence that both NTG and EMS mutagenesis depends, at least partly, on repair-dependent factor(s); this implies that direct damage of DNA may still take place (Ichii and Kondo, 1974). EMS can cause direct mispairing; and the mutagenesis by NTG and EMS may be due to the combined effects on DNA replication of damage sustained by the replication machinery and the template strand.

In Micrococcus radiodurans sensitivity to the mutagenic action of NTG has been reported, but the organism is at the same time resistant to the mutagenic action of many other DNA-damaging agents, including ionizing and UV irradiation (Rebeyrotte, 1983). The absence of an SOS error-prone DNA repair system, which could lead to mutations via misrepair processes, has been invoked to explain this phenomenon. The sensitivity to NTG has been postulated to be due to the lack of an inducible error-free DNA repair process, which in E.coli cells cultivated with a sublethal concentration of an alkylating agent induces a resistance to future exposure to such an agent in such cells, both with respect to lethality

and mutagenic effects it may engender (the adaptive response; discussed more fully in Chapter 1). This type of adaptive system has been shown to be lacking in M.radiodurans by the action that NTG has on the organism, but there is evidence that the organism has a constitutive repair system (Rebeyrotte, 1983). Haemophilus influenzae has also been shown to lack an adaptive repair system (Kimball, 1980; quoted by Rebeyrotte, 1983). An adaptive response to NTG is, however, seen in Bacillus subtilis (Hadden et al., 1983).

In a study on Salmonella typhimurium a similar approach of comparing the response of a group of mutated strains of the organism to several mutagens was taken (Shanabruch et al., 1983). MMS was shown to be $recA^+$-dependent, and EMS to be $recA^+$-independent. The mutated strains were all shown to have a high sensitivity to mutagenesis by EMS,MMS,and NTG, but the sensitivity to killing by these agents was not increased. Spontaneous mutation rates were also increased in the mutants. While EMS and MMS showed increased mutagenesis rates merely as increases in the slopes of their graphs, NTG showed a different type of curve with very few mutants induced at low doses and a very sharp increase in mutant accumulation with increasing dosages of the NTG. This resulted in a curve where the other graphs were essentially linear. This was the situation for the wild type; in the mutated strains tested mutability was higher and the dose

response curves were approximately linear in all cases. This raised the possibility that the mutants may be deficient in a saturable, highly accurate and mutagenesis-preventing repair system shown by the wild type. The authors present several models that could explain the effects of the mutagens tested. In the first model, an alkylated base opposite a normal base is recognised and removed; but due to several reasons this is not likely to occur (Shanabruck et al., 1983). In model II, a normal base opposite a lesion is recognised and removed. Again, for various reasons involving the mechanism of recognition of modified bases and the inability to quite explain the mechanism of action of NTG in this context, this model is unlikely. Model III involves recognition and processing of mismatched normal bases that are generated as a consequence of mutagen treatment. This is taken as being the model of choice, as it can account for different types of action exhibited by different mutagens. However, it must be noted that these models are not necessarily mutually exclusive but may be acting in concert, for mutagenesis a process that is extremely complex.

With regard to the eukaryotic situation, two studies similar to to the ones performed above on E.coli and S.typhimurium were done with Saccharomyces cerevisiae (Prakash, 1974; Prakash and Higgins, 1982). Two genes, rad6 and rad9, that confer sensitivity to radiation in yeast were shown to also reduce the number of mutations induced in this organism by

EMS, MMS and NTG. Yeast therefore appears to require a functional repair system for mutation induction by chemical agents (Prakash, 1974), as the repair capacity of organisms determines not only their survival after exposure to DNA damaging agents but also their response to the mutagenic effect of such agents. Mutations induced by the so-called radiomimetic chemical mutagens such as MMS seemed to depend on the same repair system required for radiation induced mutations. However, a functional repair system was required for the induction of mutation by non-radiomimetic chemical mutagens such as EMS and NTG. Mutability in S.cerevisiae strains carrying the mutant genes rad6 or rad9 was greatly reduced when compared to the wild type strain; thus, these genes, not directly comparable to any found in E.coli, are essential for chemically induced mutations in yeast.

Correlations exist between sensitivity of organisms to radiation and sensitivity to certain chemical agents. Such correlations exist in bacteria, including E.coli, B.subtilis and S.typhimurium, as well as in eukaryotic cell cultures. Similar ties, between UV light and 4-nitroquinoline-1-oxide and between X-rays and MMS, have also been shown to occur in yeast. This suggests that the mutation induction by at least the radiomimetic chemical agents should depend partly on the same genes which control UV and X-ray mutagenesis. Prakash (1974) showed that mutations induced by the nonradiomimetic agents such as EMS and NTG also depend on genes involved in

repair. It was concluded that the establishment of induced mutations (including those induced by NTG and EMS) requires the repair functions, but that different mechanisms are involved in the production of spontaneous mutations and induced ones. It looks as though the RAD6 locus in yeast, and probably the RAD9 locus as well, is involved in error prone DNA repair. Whatever the nature of mutants defective in these loci, it is clear that both loci are closely concerned with induced mutations.

In the second study, mutants in the rad52 epistasis group have been studied (Prakash and Higgins, 1982) and the effect of EMS on these mutants was examined. Alkylating agents such as EMS and NTG had, according to evidence in earlier work, been thought to be independent of a functional repair system as such. They are both recA-independent mutagens. These ideas have, however, been based mostly on studies in E.coli; both in this and in the previous study it has been established that just such a functional repair system is essential for EMS mutagenesis in yeast. A large number of genes is seen to affect EMS-induced transitions from GC to AT in stationary phase diploid yeast (Prakash and Higgins, 1982). In addition to the rad6 gene studied earlier, a plethora of other genes that mediate similar effects were uncovered in this study. Many of these mutant genes were also deficient in DNA repair. In rad6-1 mutants, postreplication repair of UV induced damage was defective

and MMS-induced single strand breaks were repaired only to a limited extent while double strand breaks were not repaired. In the rad52 epistasis group, the members are characterised by sensitivity to effects of MMS and ionising radiation (Prakash and Higgins, 1982). The rad6 and rad52 loci belong to only one epistasis group for the repair of EMS induced damage although they belong in different epistasis groups concerned with repair of damage by UV light. Presumably repair of EMS induced damage would take place before DNA synthesis in stationary phase cells, and misrepair here would lead to mutations. Thus mutants defective in repair of EMS induced lesions could also affect mutations induced by EMS.

A feature of EMS mutagenesis, shown both in yeast and in E.coli, is its specificity in the preferential induction of GC to AT transitions. To account for this specificity in yeast, as well as for the effect of the various rad mutations on EMS mutagenesis, it has been suggested that EMS mutagenesis in this organism arises during repair of apurinic sites on a DNA strand when the opposite strand has an unexcised O^6-alkylguanine and overlaps the gap formed during excision of the apurinic site. During the repair process, it is possible that thymine could be inserted opposite the alkylated base, thus rendering that base mutagenic when replicated by the repair complex (Prakash and Higgins, 1982). The rad50 and rad51 mutants in S.cerevisiae

do not appear to be very sensitive to lethal effects of EMS. It is possible that the products of these genes only function during repair of potentially mutagenic lesions, which, by occurring at comparatively low frequencies, have a correspondingly small effect on viability.

A study of mutagenic repair in Streptomyces has been performed, and has been used as the basis of the work presented here. Streptomyces fradiae was used as the test strain by Stonesifer and Baltz (1985). These workers used a S.fradiae strain defective in repair of lethal damage induced by MMS and NTG, but showing nearly normal sensitivity to EMS-induced lethality. The strain shows substantially decreased mutability by all three mutagens. These multiple traits appear to have been caused by a single mutation. The mutated gene was designated as mcr-6 and its product appears to control an error prone (mutagenic) DNA repair system. Mediation of EMS mutagenesis by an error prone DNA repair pathway in this organism, as compared to the direct mispairing seen in E.coli, implies that the more complex Streptomycetes have evolved more efficient error avoidance mechanisms than those shown to exist in lower forms of bacteria. These mechanisms appear to be more related to those observed in S.cerevisiae.

In some respects, pleiotropic effects of this mutation in S.fradiae resemble those of E.coli recA mutants. However,

significant differences exist, too (Stonesifer and Baltz, 1985). EMS mutagenesis in the Streptomyces mutant was only about 1% of that shown by the wild type. The authors believe that O^6-ethylguanine residues in S.fradiae may induce mutations by an error-prone repair pathway; in this way the mcr-6 mutation in this organism is similar to the rad6 mutation in S.cerevisiae. It would be interesting to determine if the similarities extend further than this, as the rad6 mutants in yeast are distinguished from the recA mutants of E.coli by being able to undergo recombination. The question whether the S.fradiae mcr-6 mutant is able to do this is as yet still open.

It is logical to assume that, due to their very large genome sizes, the Streptomycetes have evolved more sophisticated policing mechanisms with regard to the state of their DNA and, consequently, more sophisticated methods of rectifying damage. EMS, for example, may induce lesions blocking normal replication that have to be repaired before replication resumes; at low levels of EMS, a constitutive error free repair mechanism may be all that is required to do so, with, subsequently, low levels of mutagenesis and instability because the integrity of the genome is not disrupted. A second system, error prone and similar to that found in E.coli, could be induced when the level of EMS increases and causes too much damage for the primary system to adequately repair. Alternatively, EMS-induced lesions may not block

replication but may, instead, be misread more frequently at higher doses of EMS after the induction of a response that renders the replication machinery more error prone (Stonesifer and Baltz, 1985).

The idea that mutagenicity by direct base pairing decreases as genome complexity increases and that the increase in genome size engenders more stringent error surveillance during normal DNA replication is supported by the observation that in Streptomyces EMS mutagenesis is seen to occur mostly via an error prone DNA repair pathway. It is also seen from the accumulated data that, frequently, dominant and relatively error free repair pathways in bacteria are supplemented by error prone mutational repair pathways in times of environmental stress (Stonesifer and Baltz, 1985). It is just such a situation that has been uncovered in S.cattleya in this study.

6.2 Methods

6.2.1 EMS mutagenesis protocol

This protocol is based on the method reported by Stonesifer and Baltz (1985).

Liquid cultures of the four strains studied were grown in Malt 3 Broth as described previously (Section 5.2.1). The cultures were allowed to reach early stationary phase. The cells were harvested by centrifugation at 19000rpm for 10 minutes in an SS34 rotor and resuspended thoroughly in 13ml 0.2M potassium phosphate buffer (pH 7.0). Each cell suspension was divided into four 3ml aliquots, the remaining 1ml of suspension being kept as a control. To the four aliquots, EMS was added to 0.5%, 1.0%, 2.0% and 3.0% final concentrations, respectively. The samples were incubated at 37°C for 10 minutes, after which the reactions were terminated by diluting them 1/10 into 0.16M sodium thiosulphate. A series of dilutions of the resulting samples were plated on Malt 3 Agar plates to check survival rates of treated cells. To test the mutagenesis rate, undiluted cell suspensions from each concentration of EMS tested in every strain were plated onto Malt 3 Agar containing 10ug/ml rifampicin, and mutagenesis was scored by calculating the percentage of resistant mutants obtained from the number of

survivors observed. (The MIC of rifampicin was determined in a preliminary experiment, and found to be between 6-7ug/ml for the wild type, 4ug/ml for R6, 2-3ug/ml for R12 and 0.8-1.0ug/ml for S26.)

6.2.2 MMS mutagenesis protocol

The protocol used was almost identical as that for EMS mutagenesis, with a few minor changes. The percentage concentrations of MMS used were 0.05%, 0.1%, 0.25% and 0.4% (Stonesifer and Baltz, 1985). The samples were incubated at 37^{o}C for 30 minutes. The methods of stopping the reaction and of plating the resultant cells, as well as the final analysis of results, were carried out in a manner identical to that described in Section 6.2.1.

6.2.3 NTG mutagenesis protocol

Cells obtained from liquid culture were used for this mutagenesis study instead of spores as used in the previous instance of this type of mutagenesis (Section 2.2.6) in order to obtain a better correlation of results with the other two mutagens tested. The resuspension buffer after the centrifugation step in this case was Tris-malate (pH 9.0). 1mg/ml, 2mg/ml and 3mg/ml concentrations of NTG were used

for the tests. The samples were allowed to shake gently at room temperature for one hour for the reaction to proceed. They were then spun down in corex tubes in a benchtop centrifuge and the pellets resuspended in 20% glycerol. The plating methods and the analysis of results were identical to those described in Section 6.2.1.

6.3 Results

The results of these experiments are shown in Fig. 7.

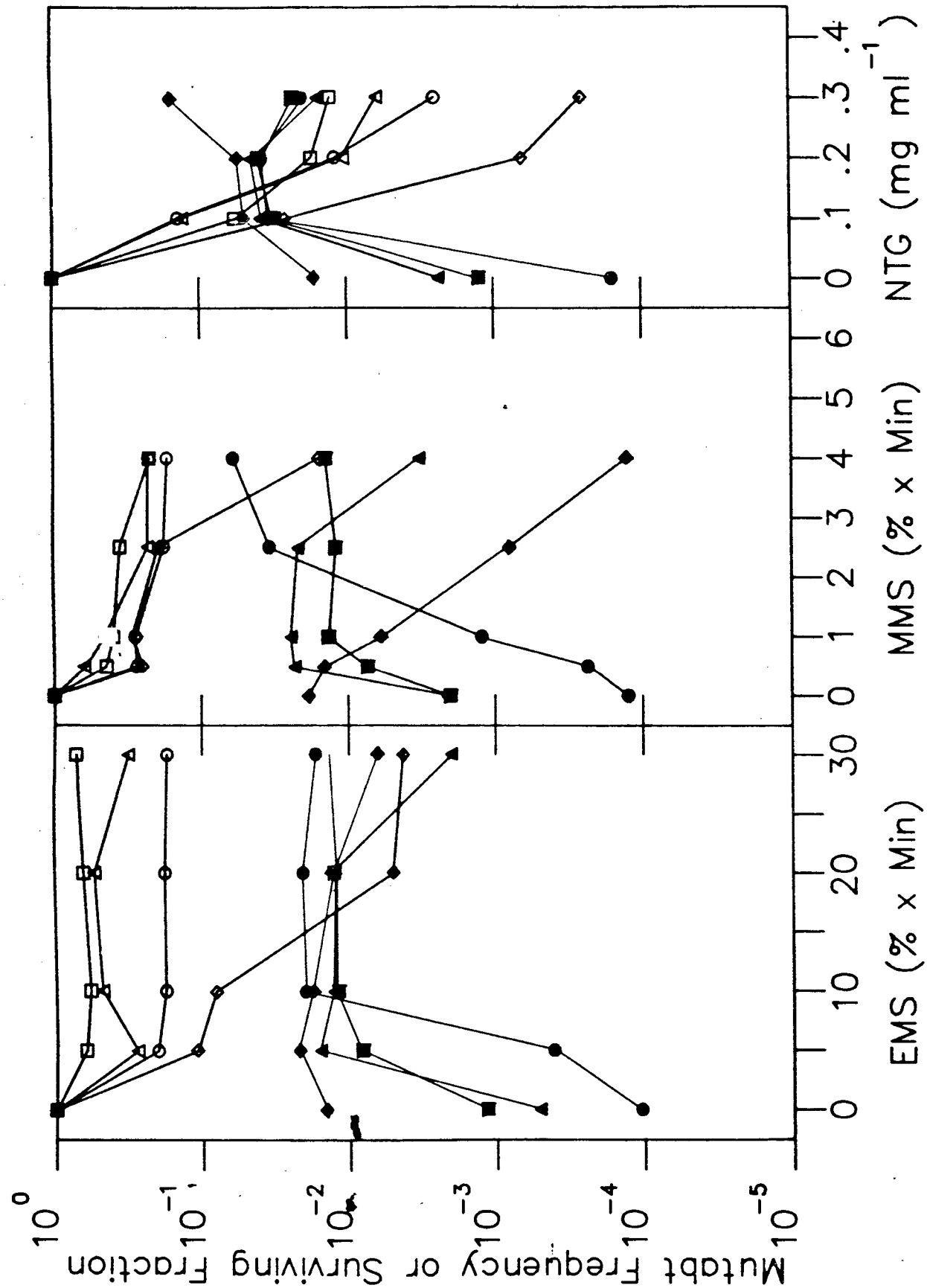
In all cases, S26 showed the most marked drop in survival, although in the case of MMS this only becomes obvious at high dosages (Fig. 7b). With the same mutagen, R6 and R12 show survival rates that do not differ much from that of the wild type, where R12 is observed to have a sharper initial decline but the effect appears to decrease at higher dosages and R6 shows a curve with a slightly steeper overall slope implying increased lethality. Both R6 and R12, however, show significantly increased survival compared to the wild type over all the concentrations of EMS tested, while S26 is very much more susceptible to EMS killing than any of the other strains (Fig. 7a). All strains show a rapid decrease in cell numbers after treatment with NTG, a reaction markedly different from that produced by either of the other two mutagens. An increased survival rate of R6 and R12 compared to the wild type is evident only at higher dosages of NTG (Fig.7c).

As far as the mutagenesis is concerned, an interesting characteristic of the mutagenesis curves of R6 is that these remain approximately the same after treatment with all

three mutagens. S26 is interesting in that it reacted differently to each mutagen - the mutation rate decreases with increasing dosage in the case of MMS (Fig.7b), increases markedly with increasing dosage of NTG (Fig.7c), and remains relatively unaffected with only a minor decrease in in the mutagenesis rate when treated with EMS. R12 mutagenesis curves mimic, to some extent, the plateaux shown by R6 in all three cases, but in every case the rate of mutagenesis in R12 outstrips the one shown by R6 at high dosages of the mutagen. This effect is most marked in cells treated with MMS (Fig.7b).

Figure 7

Inactivation (open symbols) and mutagenesis to Rif^r (closed symbols) of Streptomyces cattleya wild type (○ ●), MMC^S mutant S26 (◇ ◆), MMC^r mutant R6 (△ ▲) and MMC^r mutant R12 (□ ■) with EMS, MMS and NTG.



6.4 Discussion

In earlier studies, including the one used as the basis for the work performed here (Stonesifer and Baltz, 1985) the reaction of organisms to various chemical mutagens often provided useful information about the state of DNA repair and the mechanisms of the repair of DNA damage in the cell. This has also proved to be the case here.

S26 exhibited a 100-fold increase in the spontaneous rate of mutation towards rif^r phenotype (Fig 7). This could be explained by the presence in this mutant of an active error prone repair system. The three mutagens tested induce progressively less bulky lesions in the DNA (EMS>MMS>NTG). The lack of mutation induction by EMS in S26 could imply that the detection and repair of lesions of the type induced by EMS is performed by the uvrl repair system, which this mutant lacks. MMS induced lesions are probably also acted on by this system - a reduction in the incidence of mutagenesis is seen at higher doses of MMS. The absence of repair in this case is probably the reason for the increasing number of lethal hits observed. NTG, which has a different type of action, causes a different type of lesion; the sharp increase in the mutagenesis rate here would appear to imply that repair of NTG damage may be undertaken by the error

prone uvr2 repair pathway in S.cattleya which is derepressed in this mutant.

The R6 mutagenesis curves remain remarkably similar and almost unaltered through treatments with all three mutagens. R12 mimics these curves to some extent. The lack of any overall change in mutability in these two mutants may indicate that at low dosages the effects of these mutagens on the expression of the error prone repair pathway uvr2 is not significant. At higher mutagen dosages induction does occur, and in this case some incidence of decreased mutability is observed. R12, with its loss of uvr1, is consistently more mutable than is R6, which is to be expected. The increased survival rate of R12 at higher dosages of mutagen is also to be expected, as the dosage increases probably cause induction of the repair system to occur, and while this has the effect of increasing mutagenesis, it also decreases the incidence of fully lethal hits in this strain.

It can thus be seen that the phenotypes assigned to the three mutants in an earlier chapter with respect to their DNA repair capabilities and the DNA repair mutations they possessed hold true, as the results obtained from the mutagenesis studies can be explained in the light of these. The existence of the two DNA repair systems in S.cattleya is indirectly confirmed by this evidence, and tentative roles

in DNA repair within the organism can be assigned to them. The uvrl system, according to the available data, acts on bulky lesions on the DNA while the uvr2 system seems to repair less bulky lesions. The uvrl system could thus be taken as an "error free"-like repair system which recognises and repairs large distortions of the DNA; uvr2, acting as an "error prone"-like repair system, takes care of DNA damage remaining after uvrl has repaired all it can, and appears to be induced in the cell by high levels of DNA damage.

Chapter 7

Conclusion

This study was approached with a view of detecting a possible relationship between genetic instability in S.cattleya and DNA repair systems in that organism, and investigating such a relationship should it be shown to exist. The results of this work were seen as having a direct implication on possible control of genetic instability in this organism by manipulating the DNA repair system(s) it may possess.

Two repair systems have been identified in S.cattleya by the use of three mutants in DNA repair pathways which were generated from this organism, with a possibility that a third system may be present as well. The three mutants, one of which was MMC^S (S26) and the other two MMC^r (R6, R12), were shown to have genetic instability profiles that differed from the wild type S.cattleya and from one another. They were also affected in different ways in their DNA repair capabilities, thus implying that a relationship between the two phenomena does exist. The three mutant strains, as well as the wild type organism, were exposed to

mutagenesis by three different chemical agents, and were shown to exhibit differing reactions to such mutagenesis in ways consistent with their altered genotypes. Further information gained from these experiments served to confirm the existence of the two separate DNA repair systems established in S.cattleya and was helpful in assigning tentative mechanisms of action to these systems. Instability shown by the different strains could be further assessed by their mutability with the chemical agents used. The genetic instability was also examined via the response of the wild type and mutant strains to being grown on media containing divalent metal cations Mn^{2+} and Co^{2+}. R12 showed a large reduction in instability and a simultaneous drop in mutability with increasing dosages of Mn^{2+}. This was one of the tenets on which the presence of a third repair system in S.cattleya is postulated. Of the two systems that have been characterised, one, uvr1, is a caffeine inhibitable, probably error free repair system which does not appear to be implicated to any great extent in the instability phenomenon. The other, uvr2, is seen as playing a major role in instability; it is arsenite inhibitable, and thought to be error prone. R12 has lost the uvr1 repair system by means of its mutation and the instability-inducing uvr2 repair system cannot explain the stabilisation of this mutant in the presence of manganese. Both R6 and R12 also show DNA repair which is actually induced by arsenite over wild type levels. This, together with the data obtained for R12

behaviour in the presence of Mn^{2+}, leads to a tentative suggestion that a third system (uvr3), which is arsenite inducible and largely error free, may also be operating in S.cattleya and may be involved in the instability phenomenon as well, in ways not yet elucidated.

Induction of putative repair proteins was attempted by exposing S.cattleya wild type and mutant strains to low doses of UV light prior to prolonged growth and testing for the presence of such proteins by means of SDS polyacrylamide protein gel electrophoresis. Although some interesting conclusions may be drawn from the profiles obtained for the wild type strain, the experiment was largely unsuccessful because of the very low levels of protein observed with the mutant strains.

At the level reached by this study, further characterisation of the repair systems uncovered in S.cattleya is impossible without pinpointing and cloning the genes responsible for them. It was planned to prepare a genomic library of S.cattleya, and possibly use this to clone into a species of Streptomyces with an established genetic system, which is lacking in in S.cattleya, for further genetic analysis. However, this was precluded by a lack of time. Cloning of the relevant genes is the next step in this on-going study, and will no doubt provide more in-depth information about the link between instability and DNA repair established in

this study.

It has been shown in this work that UV repair deficient mutants exist which can dramatically affect genetic instability in S.cattleya. It is therefore possible to manipulate such instability, via these or other mutants still to be isolated. This has important implications for the stabilization of industrially important antibiotic-producing Streptomyces species, although the actual biochemical pathways of such stabilisation remain to be fully elucidated.

APPENDIX A

ABBREVIATIONS

MMC	:	Mitomycin C
MIC	:	Minimum Inhibitory Concentration
UV	:	Ultraviolet (light)
SDS	:	Sodium Dodecyl Sulphate
PAGE	:	Polyacrylamide Gel Electrophoresis
TEMED	:	N.N,N',N',-tetramethylenediamine
NTG	:	N-methyl-N'-nitro-N-nitrosoguanidine
EMS	:	Ethylmethanesulfonate
MMS	:	Methylmethanesulfonate
w/v	:	weight by volume ratio
v/v	:	volume by volume ratio
TCA	:	Trichloroacetic acid

APPENDIX B

MATERIALS

1. Media

Malt 3 Agar and Broth

Agar		Broth
24g	malt extract	24g
5g	yeast extract	5g
15g	agar (Oxoid no.1)	-
In 1l distilled H_2O Autoclave		In 1l distilled H_2O Autoclave in 100ml aliquots

Nutrient Broth and Nutrient Broth Agar (with additives)

NBA		NB
	Difco Nutrient Broth:	
3.0g	Beef extract	3.0g
5.0g	Peptone	5.0g
15.0g	Agar (Oxoid no.1)	-
5.0g	Glucose (0.5% w/v)	5.0g
1.313g	8mM $Ca(NO_3)_2$	1.313g

In 1l of distilled H_2O
Autoclave

Soft Nutrient Broth Agar (with no additives)

Difco Nutrient Broth:	
Beef extract	3.0g
Peptone	5.0g
Agar (Oxoid no.1)	7.5g

In 1l distiled H_2O
Autoclave

Streptomyces Minimal Medium (Hopwood, 1967)

Agar	10g (if wish solid medium)
L-asparagine	0.5g
K_2HPO_4	0.5g
$MgSO_4.7H_2O$	0.2g
$FeSO_4.7H_2O$	0.01g

In 1l distilled H_2O

Separately: 50%(w/v) glucose

To make: dissolve ingredients (except glucose) in distilled water, adjust to pH 7.0 - 7.2 with NaOH. Pour into flasks in 100ml aliquots; cover with foil and autoclave. Immediately before use, add 2ml of sterile 50% (w/v) glucose solution to amount in flask. Do not leave standing with glucose already added; prone to contamination.

2. SDS-PAGE

Running Buffer (Solution Q)

	10x solution
Tris (0.025M)	60.57g
Glycine (0.192M)	288.26g
SDS (0.1%)	20.00g

In 2l distiled H_2O

Lower Gel Buffer (pH 8.8)

	1l	500ml
Tris (1.5M)	181.17g	90.86g
SDS (0.4%)	4.00g	2.00g

in distilled H_2O

Upper Gel Buffer (pH 6.8)

Tris (0.5M)	30.29g
SDS (0.4%)	2.00g

in 500ml distilled H_2O

Acrylamide Stock

	500 ml	1l
Acrylamide (29.2%, w/v)	146.00g	292.00g
Bisacrylamide (0.8%, w/v)	4.00g	8.00g

SDS Sample Buffer

Glycerol (10% v/v)	10 ml	25 ml
Mercaptoethanol (5% v/v)	5 ml	12.5 ml
SDS (2.3% w/v)	2.3g	5.75g
Tris (0.0625M) pH 6.8	0.3g	0.75g
Distilled H_2O	100 ml	250 ml

Disruption Buffer

0.1% Bromophenol Blue indicator in Sample Buffer (above).

Bromophenol Blue	0.01g
Sample Buffer	10.00ml

Ammonium persulphate

10% (w/v) solution, made fresh every week.

Ammonium persulphate	0.1g
Dist. H_2O	1.0ml

Running Gel Mixture and Stacking Gel Mixture

Running gel mixture		Stacking gel mixture
12.0ml	Acrylamide stock	2.0ml
13.65ml	Lower gel buffer	-
-	Upper gel buffer	3.0ml
8.2ml	Distilled H_2O	7.0ml
160 ul	Ammonium persulphate	64 ul
18 ul	TEMED	13 ul

(for one gel)

Destain

25% (v/v) Methanol
10% (v/v) acetic acid

in 1l distilled H_2O

Coomassie Blue Stain

0.1% (w/v) Coomassie blue or PAGE blue stain in 2l destain

Allow to stir overnight with a magnetic stirrer bar.
Before use, filter with Whatman's No.1 filter paper.

3. Silver Staining Procedure: Chemicals and Solutions

Fixer

Must be made fresh on every occasion

100ml per gel

Methanol (50% v/v)	50ml
TCA (12% w/v)	12g
$CuCl_2$ (2% w/v)	2g

make up to 100ml with distilled H_2O

Solution A

STOCK: 200ml Ethanol
100ml Acetic acid

make up to 2l with distilled H_2O

0.1% $KMnO_4$

Must be made fresh on every occasion

0.015g in 150ml distilled water (for one gel)

10% Ethanol

STOCK: 100ml 99% ethanol in 1l distilled H_2O

0.1% $AgNo_3$

Must be made fresh on each occasion, and kept in the dark or in a foil-wrapped bottle until ready for use.

0.1g in 100ml distilled H_2O (for one gel)

10% K_2CO_3

STOCK: 100g in 1l of distilled H_2O

Fixer

Must be made fresh on every occasion, and kept in the dark until ready to use.

Formaldehyde (0.01% v/v)	10ul
10% K_2CO_3 (stock solution)	20ml

in 100 ml distilled H_2O

4. General

Mitomycin C

Stock solution: 2mg/ml in distilled H_2O
Kept in the dark or in foil wrapped bottle

Rifampicin

Stock solution: 10mg/ml in 50% distiled H_2O
50% ethanol
Filter through 0.22u Millipore filter
Keep at -20°C

Caffeine

Stock solution: 0.05g/ml

Sometimes crystallises out; heat to redissolve crystals before using.

Sodium arsenite

Stock solution: 75mg/ml

Poisonous, therefore assumed sterile. No need to autoclave.

20% (v/v) Glycerol

200ml in 1l distilled H_2O

Autoclave.

0.016M Sodium Thiosulphate

39.71g sodium thiosulphate in 1l distilled H_2O

Potassium phosphate buffer, pH 7.0 (0.2M)
(Williams and Chase, 1968)

Solution A: 0.05M KH_2PO_4
Solution B: 0.05M K_2HPO_4

For 0.2M buffer at pH 7.0:

Solution A: 156ml
Solution B: 244ml

in 1l distilled H_2O

Autoclave each solution separately, and also the final buffer.

0.05M Tris-malate buffer (pH 9.0)

This buffer, used in NTG mutagenesis, was a gift from Dr. R. Kirby (Delic et al., 1970)

5. Materials: Sources

Malt extract	: Merck
Yeast extract	: Biolab
Bacteriological agar no.1	: Oxoid
Mitomycin C	: MPS Laboratories
Rifampicin	: Sigma
Nutrient Broth	: Difco
Caffeine	: Sigma
SDS	: Sigma
EMS	: Sigma
MMS	: Sigma
NTG	: Sigma

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