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The effect of metronidazole on *Bacteroides fragilis* and *Escherichia coli*

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In partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Science, University of Cape Town.

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CERTIFICATION OF SUPERVISOR

In terms of paragraph 9 of "General regulations for the degree of Ph.D.", University of Cape Town, we, as supervisors of the candidate Gabi U. Dachs, certify that we approve of the incorporation in this thesis of material that has already been published or submitted for publication.

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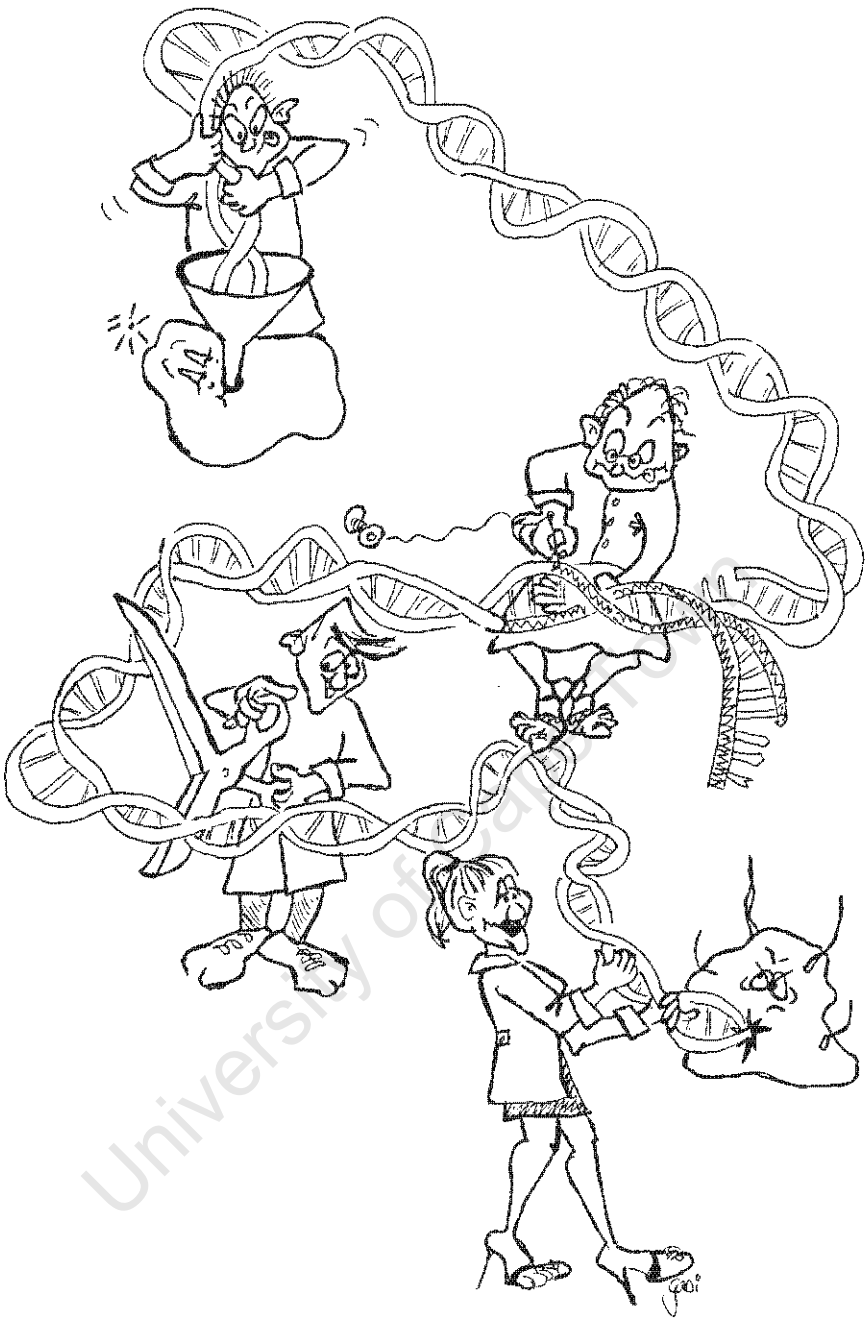

Confidence is the thing you had before you knew better.

Mark Twain

University of California

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Abstract

The antibiotic metronidazole is used extensively in the clinical treatment of anaerobic infections, including those caused by the anaerobic pathogen *Bacteroides fragilis*. Metronidazole is an inert substance that requires reductive activation to become cytotoxic. In its activated form metronidazole induces DNA damage. Relatively little is known about the cytotoxic effects of this drug *in vivo*. The aim of the work reported in this thesis was to analyze the mode of action of metronidazole in living systems. Furthermore, the potential for bacterial cells to develop resistance mechanisms to metronidazole is largely unknown, and therefore the role played by *B. fragilis* genes in influencing the potency of metronidazole was investigated.

The *B. fragilis* Bf2 strain, containing the cryptic plasmid pBFC1, exhibited increased resistance to metronidazole as compared to plasmid-free *Bacteroides* strains. A DNA restriction endonuclease fragment of pBFC1 was cloned on a recombinant plasmid, pMT100, and introduced into the facultative anaerobic bacterium *Escherichia coli* to study the effect of the gene product on the metronidazole susceptibility of the cells.

E. coli provided a convenient *in vivo* system for this investigation, since the organism is a facultative anaerobe, its DNA repair systems are well characterized, and a wide range of *E. coli* repair mutants is available. With the aid of these *E. coli* mutants it was possible to screen a *B. fragilis* genomic library for genes involved in the repair of metronidazole-induced DNA lesions. The mutants also facilitated the *in vivo* study of the nature of metronidazole-induced DNA lesions in *E. coli*, and the investigation of the influence of the cloned *B. fragilis* gene on the repair of these lesions.

The recombinant plasmid, pMT100, conferred both increased resistance and increased sensitivity to metronidazole to different *E. coli* strains. The pMT100 encoded gene product conferred increased resistance to *E. coli* strains with intact RecA and RecBC systems under aerobic and anaerobic conditions. The presence of pMT100 in *E. coli* strains, mutated in either *recA* or *recBC*, decreased the tolerance of the cells to metronidazole under anaerobic conditions. In *E. coli* strains with a functional RecE system, the pMT100 encoded gene product

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conferred increased sensitivity to metronidazole which was independent of the oxygen conditions.

The pMT100 encoded gene product also affected the tolerance of *E. coli* strains to the DNA damaging agent, far UV irradiation. In all *E. coli* strains tested, the presence of the plasmid pMT100 increased the sensitivity of the cells to UV irradiation.

The locus affecting metronidazole resistance, metronidazole sensitivity and UV sensitivity was located on a 1.6 kb DNA fragment which was sequenced. An open reading frame (ORF1) of 195 bp encoded a protein of 64 amino acids with a predicted M_r of 7.3 kDa. In *E. coli*, the gene was expressed from the λ promoter of the vector DNA.

Metronidazole induced DNA strand breaks in *E. coli* cells under aerobic and anaerobic conditions. DNA degradation following metronidazole treatment appeared to be enzyme mediated. The metronidazole-induced DNA strand breaks did not appear to be the only mode of killing, since the extent of DNA degradation did not correlate with the sensitivity of the strains to metronidazole. The presence of pMT100 in *E. coli* strains reduced the amount of DNA degradation induced by metronidazole irrespective of the genotype of the *E. coli* strain or the oxygen conditions.

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Abbreviations

()	designates plasmid-carrier state
A	adenosine
A_{254}	absorbance at 254 nm
aa(s)	amino acid(s)
Ala	alanine (A)
ara	gene encoding arabinose utilization
Arg	arginine (R)
argE	gene encoding arginine synthase
Asn	asparagine (N)
Asp	aspartic acid (D)
ATCC	American Type culture collection
ATP	adenosine 5'-triphosphate
bp(s)	base pair(s)
C	cytidine
Ci	Curie
CsCl	cesium chloride
Cys	cysteine (C)
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetra-acetic acid
EtBr	ethidium bromide
exo	exonuclease
Fd	ferredoxin
G	guanosine
gal	gene encoding galactose utilization
Gln	glutamine (Q)
Glu	glutamic acid (E)
Gly	glycine (G)
gpt	gene encoding guanine phosphotransferase
h	hour(s)
His	histidine (H)
hisG	gene encoding histidine synthase

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hsdS	gene encoding host specificity
hsr	gene encoding host restriction
Ile	isoleucine (I)
ilv	gene encoding isoleucine-valine synthase
kb	kilobase pairs
kDa	kilodalton(s)
kdgK	gene encoding ketodeoxygluconokinase
lac	gene encoding lactose utilization
LB	Luria-Bertani broth
Leu	leucine (L)
leuB	gene encoding leucine synthase
Lys	lysine (K)
Met	methionine (M)
mgl	gene encoding methyl-galactose utilization
min	minute(s)
M_r	relative molecular mass
mRNA	messenger RNA
mtl	gene encoding mannitol utilization
nm	nanometers
ntr	gene encoding nitroreductase
OD_{600}	optical density at 600 nm
ORF	open reading frame
p	plasmid
PAGE	polyacrylamide gel electrophoresis
Phe	phenylalanine (F)
phoA	gene encoding alkaline phosphatase
P_R	rightward promoter (l)
Pro	proline (P)
pro	gene encoding proline synthase
qsr	cryptic lambdoid phage
rac	defective prophage, *recE*
RBS	ribosome binding site
recA	gene encoding RecA
recB	gene encoding RecB
recC	gene encoding RecC
rfbD	gene encoding rhamnose synthetase
RNA	ribonucleic acid

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rpm	revolutions per minute
rpoB	gene encoding RNA polymerase
rpsE	gene encoding ribosomal subunit
rpsL	gene encoding ribosomal subunit, streptomycin^R
s	second(s)
sbcA	suppressor mutation, *recE*$^+$
SDS	sodium dodecyl sulfate
Ser	serine (S)
sp(p)	specie(s)
srl	gene encoding sorbitol utilization
str	streptomycin resistance
supE	suppressor of ochre (UAG) mutations
T	thymidine
TAE	tris-acetate EDTA buffer
thi	gene encoding thiamine synthase
thr	gene encoding threonine synthase
Thr	threonine (T)
Tn	transposon
tonA	gene encoding outer membrane receptor
Tris	Tris(hydroxymethyl)aminomethane
Trp	tryptophan (W)
tsx	gene encoding nucleoside uptake
Tyr	tyrosine (Y)
UV	ultraviolet (light)
uvrA	gene encoding UvrA
uvrB	gene encoding UvrB
uvrC	gene encoding UvrC
v/v	volume/volume
Val	valine (V)
w/v	weight/volume
XGal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
xyl	gene encoding xylose utilization

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Chapter 1
General introduction and literature review

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Chapter 1

General introduction and literature review

The object of the work presented in this thesis was to investigate the mode of action of the antibiotic agent of choice, metronidazole, against the clinically important anaerobic bacterium *Bacteroides fragilis*. The ways in which metronidazole may cause cell death and the role of specific genes of *B. fragilis* in combatting the effect of metronidazole, were analyzed. Since metronidazole attacks the genetic material of the organism, it is necessary to study the DNA repair capacity of the bacterium. The facultative anaerobic bacterium *E. coli* served as a convenient system for this investigation, because its DNA repair systems are well documented. This has facilitated the study of the effect of *B. fragilis* genes on the repair capacity of different *E. coli* strains when they are challenged with metronidazole.

The general introduction will therefore cover three main topics: the physiology and genetics of *B. fragilis* (1.1), the history and mode of action of metronidazole (1.2), and the mechanisms and enzymes of DNA repair (1.3).

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1.1 *Bacteroides fragilis*

1.1.1 Introduction to *Bacteroides*

The existence of organisms to which the presence of oxygen is toxic was first reported by Louis Pasteur. Present day evidence suggests that the first phase of biological evolution occurred in a world that was completely devoid of free oxygen. Oxygen was only produced much later due to the activity of photosynthesizing organisms. Aerobic respiration proved to be exceedingly efficient for the production of energy, but organisms utilizing it had to develop defences against the highly reactive oxygen radicals. Strictly anaerobic bacteria are believed to lack certain components of this defence. Anaerobic microbes comprise a large and diverse group of organisms, that includes both autotrophs and heterotrophs.

Bacteroides strains are obligate anaerobic gram-negative rod-shaped bacteria. Obligate anaerobic bacteria are characterized by their inability to grow in the presence of oxygen, although they may tolerate the exposure to oxygen for a limited period of time. Non-pathogenic strains of *Bacteroides* are common inhabitants of the alimentary tract of warm-blooded animals. *Bacteroides* strains account for about 30% of all fecal isolates and are believed to play an important role in the colonic ecosystem (Salyers, 1984). *B. fragilis* is the most important anaerobic human pathogen, since it accounts for approximately 80% of anaerobic bacteraemias (Gorbach and Barlett, 1974). This opportunistic pathogen can cause a variety of infections, including blood stream infections and abscesses of the brain, lung or abdominal cavity (Salyers, 1984). The treatment of these infections is complicated because *Bacteroides* strains are resistant to a wide range of antibiotics.

B. fragilis is a member of the intestinal tract *Bacteroides* species which are a unique phylogenetic group that diverged from the other eubacteria at an early stage during evolution (Weisburg *et al.*, 1985). The divergence of *Bacteroides* from the eubacterial line may have occurred before the divergence of the purple bacteria, gram positive bacteria and cyanobacteria.

The ATPase encoding gene, which encodes a proton translocating ATP-synthase, of *B. fragilis* has been cloned and sequenced (Amann *et al.*, 1988). The sequencing data was used to support the conclusions on the phylogenetic relationship of *B. fragilis* to other bacteria drawn by Weisburg *et al.* (1985).

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1.1.1.1 *Bacteroides* physiology

The colonic *Bacteroides* strains are characterized by their relative tolerance to oxygen and the ability to ferment a variety of different sugars. *Bacteroides* strains are believed to be responsible for most of the polysaccharide digestion in the human colon (Salyers, 1984).

A levanase encoding gene (*scrL*), encoding an enzyme able to hydrolyze fructose polymers, has been cloned from *B. fragilis* (Scholle *et al.*, 1990). It was shown that ScrL enabled *E. coli*, which does not contain an endogenous sucrase, to utilize sucrose as its sole carbon source. The gene shows sequence homology to a cloned *Bacillus subtilis* levanase (Blatch and Woods, Dept. of Microbiology, University of Cape Town (U.C.T.), personal communication).

The intestinal *Bacteroides* strains are believed to be responsible for the digestion of much of the dietary fiber in the colon, thereby providing substrates for the other colonic bacteria and the host (Salyers, 1984).

The colonic *Bacteroides* cannot utilize peptides or amino acids as the sole carbon source. Proteolytic activity has, however, been detected during stationary phase growth on defined media (Riepe *et al.*, 1980). Proteolytic activity in *Bacteroides* is of interest since it has been implicated in pathogenesis (MacFarlane *et al.*, 1992). Proteolytic enzymes of other bacteria have been shown to be important in disease states, and the *B. fragilis* protease is believed to destroy the human brush border enzymes (Riepe *et al.*, 1980). *B. fragilis* constitutively produces at least three different types of peptidases (Gibson and MacFarlane, 1988). Research on the production of proteases in batch cultures of *B. fragilis* showed that during exponential growth most of the proteolysis was cell associated, whereas during stationary phase, the majority of proteolytic activity was secreted (MacFarlane *et al.*, 1992).

The enzyme neuraminidase is found in many pathogenic bacteria, including *B. fragilis*, and has been implicated in the pathogenicity of these bacteria (Russo *et al.*, 1990). The gene encoding the enzyme in *B. fragilis* was cloned and sequenced. The neuraminidase is a glycohydrolase, which cleaves sialic acid residues found on the surface of eukaryotic cells, resulting in the disruption of host functions.

The glutamine synthetase from *B. fragilis* has been characterized and found to be markedly different from the two known forms of glutamine synthetase found in eukaryotes and in other prokaryotes (Southern *et al.*, 1986, 1987; Hill *et al.*, 1989). These findings support the results of Weisburg *et al.* (1985) which place the *B. fragilis* in a unique phylogenetic group.

The absolute growth requirements of this anaerobe include iron in the form of haemin, vitamin B_{12} and a reducing agent such as cysteine. The growth of *B. fragilis* on haemin containing media is inhibited by ferric and ferrous iron chelators (Rocha *et al.*, 1991).

The *Bacteroides* membrane has an unusual lipid composition. Studies have shown that the *B. fragilis* cell surface is more hydrophobic than that of other

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gram-negative bacteria (Kobayashi *et al.*, 1991). This might explain the relative sensitivity of *B. fragilis* to hydrophobic antimicrobial agents, such as lincomycin and clindamycin.

Even though *B. fragilis* is a potent pathogen, the cell envelope lipopolysaccharide layer is less toxic than that of other enteric bacteria (Salyers, 1984). The capsular material of the cell is therefore thought to contain the virulence factor of *B. fragilis* (Kasper, 1976). The capsular material has been shown to promote the formation of intra-abdominal abscesses even in the absence of viable bacteria (Onderdonk *et al.*, 1977). The capsule composition is complex, due to a variety of sugars and two distinct polysaccharides (Pantosti *et al.*, 1991). The structures of the two polysaccharides were recently elucidated (Baumann *et al.*, 1992).

Oyston and Handley (1991) have suggested that carbohydrate residues on the cell surface, which possibly form part of the capsule, are involved in adhesion to epithelial cells and in haemagglutination.

In many pathogens the production of novel outer membrane proteins (OMP) was induced *in vitro* under iron limiting conditions, but no induction of significant quantities of *B. fragilis* OMP was observed after growth in the mouse peritoneal cavity (Patrick and Lutton, 1990).

The relationship between the colonic bacteria and their host is complex. Using the Ames test it has been shown that mutagens are produced by some species of *Bacteroides* (Van Tassel *et al.*, 1982). The incidence of colonic cancer was found to correspond with the occurrence of secretion of mutagens by *Bacteroides* strains within the population. On the other hand, *Bacteroides* strains produce enzymes that reduce the mutagenicity of several nitro-compounds (Kinouchi and Ohnishe, 1983). It is unclear what effect these activities have on the human host.

1.1.1.2 Environmental stress and DNA repair in *B. fragilis*

The physiological responses of *Bacteroides* to environmental factors have been reviewed by Woods and Jones (1986). These authors pointed out that most experimental work performed in the laboratory is done on exponentially growing cells, whereas the bacteria of the human intestine live under starvation conditions and environmental stress, where little or no growth occurs.

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Studies on *Bacteroides* have shown that they are difficult to mutate by normal means (Droffner and Yamamoto, 1983). Mutagenesis is not a passive process, and requires the intervention of a cellular system that processes damaged DNA in such a way to produce mutations (Walker, 1984). It is interesting to note that in *Salmonella typhimurium* the error-prone repair system, which allows mutation to occur, is not expressed under anaerobic conditions (Droffner and Yamamoto, 1983). This was shown to be due to the lack of expression of certain genes, the products of which are involved in DNA recombination. In *E. coli* nonmutability was achieved by the disruption of either the *recA*, *lexA* or the *umu* genes (Walker, 1984). It is possible that *B. fragilis* lacks components of an analogous mutagenesis system, and it would be interesting to screen *Bacteroides* for these genes.

Oxygen and its radical derivatives are toxic to all living organisms. Bacteria have evolved to become more tolerant (obligate aerobes) or less tolerant (obligate anaerobes) to oxygen. Several strict anaerobes have been shown to become adapted to oxygen exposure after subculturing under laboratory conditions (Willis, 1969), and a strain of *B. fragilis*, Bf-2, was tolerant to oxygen for up to six hours (Jones and Woods, 1981). The enzyme superoxide dismutase (SOD) was proposed to be the key enzyme that distinguished aerobes from anaerobes (McCord *et al.*, 1971), but SOD has since been isolated from several anaerobic bacteria. In *Bacteroides* the SOD content increases dramatically from low levels under anaerobic conditions, to high levels under aerobic conditions (Gregory *et al.*, 1977). The SOD-encoding gene of *Bacteroides gingivalis* has recently been cloned and sequenced (Nakayama, 1990).

Hydrogen peroxide, which is a stable oxygen radical, was found to damage DNA *in vitro*, by liberating all four bases from the sugar-phosphate backbone (Rhaese and Freese, 1968). Exposure of *Bacteroides* to oxygen radicals either blocked (Glass *et al.*, 1979) or decreased (Schumann *et al.*, 1983) macromolecular synthesis and inhibited amino acid uptake (Stevenson, 1979).

Far UV irradiation is an environmental stress factor that causes DNA damage in the form of intra-strand pyrimidine dimers. *B. fragilis* was shown to be more sensitive to far UV irradiation under aerobic conditions than under anaerobic conditions (Jones *et al.*, 1980). Exponentially growing cells were also more sensitive than stationary phase cells. The effect of oxygen was not specific to UV damaged cells, since the same effect was observed in *Bacteroides* treated with

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N-methyl-*N'*-nitrosoguanidine, ethylmethane sulphonate, acriflavin and mitomycin C under aerobic conditions (Slade *et al.*, 1984).

Cells irradiated with far UV usually show a greater survival when they are held in a liquid buffer and are allowed to repair the DNA damage before plating, than those plated directly. This phenomenon is called liquid holding recovery (LHR). In *B. fragilis*, LHR of irradiated cells occurs only under aerobic conditions, whereas anaerobiosis inhibits LHR (Jones and Woods, 1981). It is interesting to note that repair deficient *E. coli recA* mutant strains also exhibit no LHR (Ganesan and Smith, 1968).

Following irradiation, *B. fragilis* cells exhibit rapid and extensive DNA degradation similar to that observed in *E. coli recA* mutants (Schumann *et al.*, 1983). *B. fragilis* DNA synthesis, and to a lesser extent RNA and protein synthesis, is sensitive to UV irradiation. It has therefore been suggested that the inhibition of DNA degradation and DNA synthesis by oxygen and far UV irradiation could be the reason for the increased UV sensitivity of *B. fragilis* under aerobic conditions (Woods and Jones, 1986).

The isolation of repair-deficient mutants of *B. fragilis* has enabled Abratt *et al.* (1985) to characterize the error-free repair system of *Bacteroides*. One mutant showed increased sensitivity to mitomycin C, but had the same sensitivity to far UV irradiation as the wild-type, whereas the second mutant showed increased sensitivity to UV and moderate sensitivity to mitomycin C. The constitutive dimer excision systems of the wild-type and the mitomycin C-sensitive strains were shown to function efficiently under both aerobic and anaerobic conditions, whereas the UV-sensitive mutant displayed decreased excision capabilities (Abratt *et al.*, 1986). Under aerobic conditions far UV induced fewer metabolically regulated strand-breaks in the wild-type *B. fragilis* than under anaerobic conditions (Abratt *et al.*, 1990). The mitomycin C-sensitive mutant, and more significantly the UV sensitive-mutant, had reduced levels of UV-induced strand-breakage as compared to the wild-type.

The survival of phages after treatment with DNA-damaging agents (phage reactivation) is studied as an indication of the DNA repair capacity of the host, since the phage does not encode its own repair system. Phage reactivation following UV irradiation in *B. fragilis* has been observed under anaerobic

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conditions and was found to be inhibited by chloramphenicol (Slade *et al.*, 1983a). Phage-reactivation systems were also shown to be induced by aerobic conditions and hydrogen peroxide (Slade *et al.*, 1983b). The peroxide induced phage reactivation system differed from both the UV and oxygen induced phage reactivation systems. The three inducible phage reactivation systems appeared to be error-free repair systems since they did not result in mutagenesis (Woods and Jones, 1986).

Although there were indications that *B. fragilis* lacked a *recA*-controlled error-prone SOS processing system, a *recA*-like gene that complemented the repair functions of a *E. coli recA* mutant strain was cloned from *B. fragilis* by Goodman *et al.* (1987). These authors showed that antiserum prepared against the *E. coli recA* protein cross-reacted with the *Bacteroides recA* gene product. Furthermore, the *recA* gene sequence showed significant homology to the *recA* genes from other bacteria, although no SOS box, characteristic of LexA-controlled promoters, could be identified upstream of the gene (Goodman and Woods, 1990). Bacterial DNA repair systems are reviewed in more detail in section 1.3.

Although no direct evidence for an SOS-like damage processing system could be found, it has been shown that the treatment of *B. fragilis* with DNA damaging agents did result in the induction of a range of proteins of different sizes. The production of two large proteins (M_r of 70 000 and 90 000) was a common feature after treatment with far UV irradiation, oxygen and hydrogen peroxide (Schumann *et al.*, 1984).

The stress on bacteria caused by high temperatures induces the production of a set of proteins, called heat-shock proteins. Other stimuli, including ethanol and anoxia, also induce the heat-shock response, which is therefore believed to be a general cellular adaptation to stress (Daniels *et al.*, 1984). In *B. fragilis*, proteins of a different size to those induced by DNA damaging agents were induced by heat (Schumann *et al.*, 1982; Goodman *et al.*, 1985). Furthermore, elevated temperatures did not induce phage reactivation.

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1.1.2 *Bacteroides* genetics

1.1.2.1 Cryptic plasmids

Small plasmids of between 3-7 kb in size are widespread among the *Bacteroides* and plasmids of up to 150 kb have been detected (Salyers, 1984). Most of the small plasmids could be assigned to one of three homology classes by Southern blot analysis (Callihan *et al.*, 1983). Plasmids could further be separated into subclasses by size and restriction enzyme digestion patterns. Homology class I plasmids were approximately 2.7 kb, class IIa 3.9 kb, class IIb 4.8 kb, class IIc 6-7 kb and class III 5.6 kb in size. The plasmids were proven to be compatible, since a member of each of the three classes could be found in a single isolate.

The small cryptic plasmids are likely to be mobilized by the conjugative *Bacteroides* elements (Shoemaker *et al.*, 1986b) or other mobilizing plasmids, since they are believed to be too small to be self-mobilizing (Salyers *et al.*, 1987).

A small cryptic plasmid, pBFC1, has been detected in *B. fragilis* Bf-2, which is the strain investigated in this thesis. *B. fragilis* Bf-2 could not be cured of pBFC1 by treatment with elevated temperatures, acridine orange or ethidium bromide (Southern, 1986).

Some large cryptic plasmids are also found to be widespread among *Bacteroides* (Mays and Johnson, 1979). A 34.5 kb plasmid from *B. fragilis* showed homology to plasmid DNA from eight other *Bacteroides* strains that contained plasmids ranging in size from 34 to 60 kb. A 35 kb plasmid from *B. thetaiotaomicron* similarly displayed homology to plasmid DNA from six strains of *Bacteroides* that contained plasmids of 27 to 35 kb in size.

Cryptic plasmids have been detected in 50% of clinical *B. fragilis* group isolates (Nagy *et al.*, 1990). No correlation was detected between the presence of the plasmids and resistance to ten antibiotics (penicillin, ampicillin, cefoxitin, mezlocillin, piperacillin, chloramphenicol, clindamycin, erythromycin, tetracycline, metronidazole) and six different heavy metal ions (sodium arsenate, sodium arsenite, cadmium nitrate, lead nitrate, mercuric nitrate, zinc nitrate). The strains could not be cured of the plasmids with the use of ethidium bromide or acridine orange (Nagy *et al.*, 1990).

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1.1.2.2 R plasmids of *Bacteroides*

Antibiotic resistance genes have been identified on some of the larger natural plasmids (R plasmids) of *Bacteroides*, and four plasmids have been studied extensively. Tally *et al.* (1979) showed that the 41 kb plasmid pBF4 (also designated pIP410 (Magot *et al.*, 1981)) from *B. fragilis* conferred resistance to clindamycin, erythromycin and streptogramin when transferred to other *B. fragilis* strains. The 14.6 kb plasmid pBFTM10 from *B. fragilis* was found to be very similar to pCP1, which was isolated from *B. thetaiotaomicron*, and both conferred clindamycin and erythromycin resistance (Tally *et al.*, 1979; Guiney *et al*, 1984b). The 81 kb *Bacteroides ovatus* plasmid, pBI136, conferred clindamycin resistance (Smith and Macrina, 1984).

The plasmids required cell-to-cell contact on a solid surface for transfer, and mating was not detected in liquid medium (Tally *et al.*, 1982, Odelson *et al.*, 1987). Transfer was DNase insensitive, but sensitive to chloroform treatment of the donor growth medium. Mating was not mediated by pili or other surface appendages.

Hybridization studies of the R plasmids showed that there was DNA homology in the clindamycin-erythromycin resistance encoding region, but less homology was detected outside of this region (Shimell *et al.*, 1982).

The plasmid pBF4 moved at high frequency among isogenic strains of *Bacteroides*. The resistance to erythromycin-clindamycin encoded by pBF4 was not expressed in *E. coli* (Guiney *et al.*, 1984b, Shoemaker *et al.*, 1985). By isolating spontaneous deletion mutants of pBF4, that had lost their ability to confer clindamycin resistance, it was possible to map the locus of the clindamycin-erythromycin resistance gene (Welch and Macrina, 1981). The region encoding clindamycin-erythromycin resistance on pBF4 was sequenced and the gene encoding resistance was designated *ermF* (Rasmussen *et al.*, 1986). It encodes a rRNA methylase. The gene is flanked by two copies of the insertion element IS*4352*.

The plasmid pBF4 was shown to contain a DNA restriction endonuclease fragment that conferred non-inducible tetracycline resistance to aerobically grown *E. coli* strains, whereas pBF4-containing *B. fragilis* and anaerobically grown *E. coli* strains were tetracycline sensitive (Guiney *et al.*, 1984a). This cryptic

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determinant is dissimilar to another tetracycline resistance transfer system, Tc^r ERL, located in the host organism.

The aerobic tetracycline resistance on pBF4 has been characterized by Speer and Salyers (1988). It was shown that although the gene was transcribed under anaerobic conditions, the gene product was non-functional. Spent media in which cells containing the gene had been grown anaerobically prior to sterilization, supported the growth of tetracycline sensitive strains under aerobic conditions. This result indicated that the tetracycline resistance gene product detoxified the drug. It was shown, by tracing radiolabeled tetracycline, that the cells were not sequestering the drug (Speer and Salyers, 1989). The resistance was due to the chemical modification of tetracycline, since the endproduct was chemically different to tetracycline. The aerobic tetracycline resistance gene was sequenced and designated *tetX* (Speer *et al.*, 1991). Sequence analysis and experimental data, showing the requirement for NADPH, identified *tetX* as a gene encoding a NADP-requiring oxidoreductase.

The genes on pBF4 for resistance to tetracycline under aerobic conditions and erythromycin-clindamycin resistance are part of the transposon Tn*4351* (Shoemaker *et al.*, 1985). Tn*4351* can transpose from a plasmid to the chromosome of *Bacteroides* where it can generate auxotrophic mutations (Shoemaker *et al.*, 1986a). Tn*4351* can mediate the integration of the broad-host-range-mobilizing plasmid R751 into the chromosome if the plasmid is part of the transposon-containing construct. R751 excises from the chromosome by recombination of the flanking repeat sequences of Tn*4351* to form non-replicating circles which are mobilizable by the conjugative tetracycline resistance element Tc^r ERL (Shoemaker and Salyers, 1987).

Tn*4351* was found to be closely related to Tn*4400*, which was originally contained on pBFTM10 (Smith and Gonda, 1985). Tn*4400* conferred a lower aerobic tetracycline resistance than Tn*4351*, which was due to a lower level of expression of the gene (Speer *et al.*, 1991). In Tn*4351*, a four-base pair difference upstream of the *tetX* gene is part of a strong promoter consensus sequence, which was absent in Tn*4400*.

The drug resistance determinant on pBFTM10, Tn*4400*, transposes in both *E. coli* and *Bacteroides* (Robillard *et al.*, 1985). *E. coli-Bacteroides* shuttle vectors

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constructed from pBFTM10, which lacked *E. coli* mobilization regions, could be mobilized among *E. coli* and *Bacteroides* by IncP plasmids or a conjugal *Bacteroides* tetracycline element (Shoemaker *et al.*, 1986a,b). The transfer frequency was increased when the *Bacteroides* donor was pre-treated with low doses of tetracycline.

Two genes that encoded the transfer functions for the plasmid, *btgA* and *btgB* (*Bacteroides* transfer gene), were identified on pBFTM10 (Hecht *et al.*, 1991). The two genes were also required for mobilization by the broad-host-range-mobilizing plasmid R751 and made pBFTM10 self-transferable.

The plasmids pCP1 and pBFTM10, similar to pBF4, encoded a tetracycline resistance gene that was only expressed under aerobic conditions (Guiney *et al.*, 1984a). Unlike *tetX* the aerobic resistance involves an energy-dependent saturable reduced uptake of tetracycline (Park *et al.*, 1987). The *Bacteroides* resistance determinant was assigned to a new class, TetF, since efflux systems, that had been described before, functioned under both aerobic and anaerobic conditions.

The transposon Tn*4551* on pBI136, which, apart from for the *erm* resistance determinant and the direct repeat sequences, is dissimilar to the transposons reported on pBF4 and pBFTM10, confers resistance to clindamycin and erythromycin (Smith and Spiegel, 1987). Sequence analysis revealed that the transcription regulatory sequence of the resistance gene (*ermFS*) was located within the direct repeat sequences (Smith CJ, 1987). The ErmFS protein differed from the ErmF protein of pBF4 by only one amino acid. DNA regions of high homology among the plasmids pBF4, pBFTM10 and pBI136 are shown in Figure 1.1.

A conjugal plasmid that conferred resistance to chloramphenicol has been described by Martinez-Suarez *et al.* (1985). The 39.5 kb plasmid contains a gene encoding a chloramphenicol acetyltransferase.

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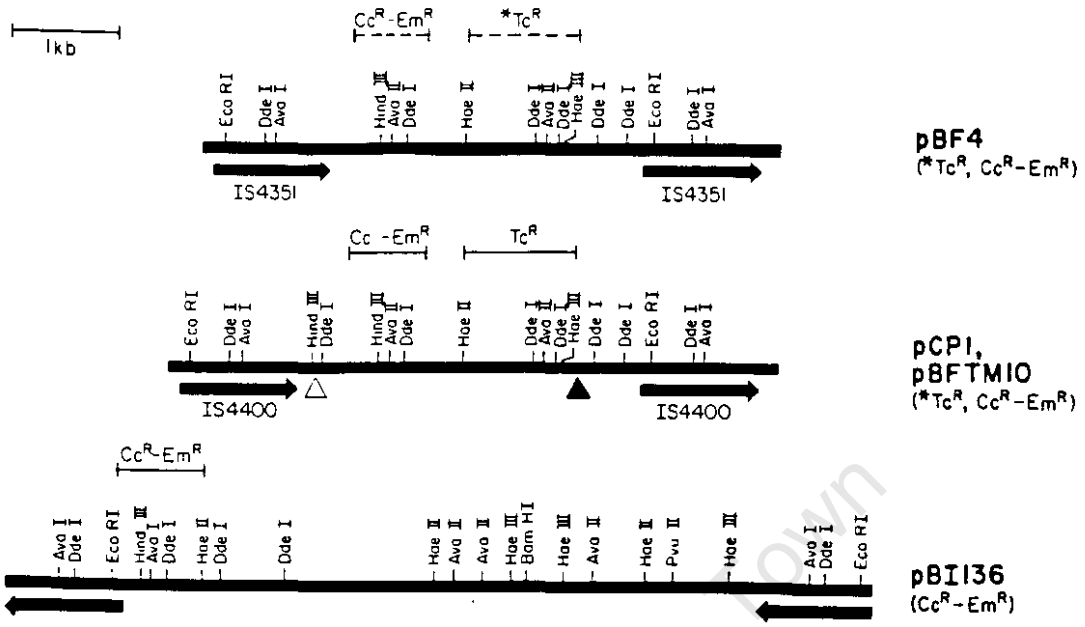

Fig. 1.1. Comparative restriction maps of the clindamycin erythromycin resistance regions of the plasmids pBF4, pBFTM10 (pCP1) and pBI136. The approximate locations of the resistance genes are indicated. The arrows show the location and orientation of the direct repeat sequences. The triangles under the pBFTM10 map designate areas that differ from the corresponding region on pBF4 (Salyers *et al.*, 1987).

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1.1.2.3 Conjugal elements and plasmid independent transfer of resistance

Transfer of antibiotic resistance that was not associated with a plasmid was first described by Macrina *et al.* (1981). Transposable elements are found commonly in *Bacteroides* and are present on the R plasmids, where they contain the erythromycin-clindamycin resistance determinant.

A cryptic conjugal transposon, Tn*4399*, was able to confer transferability in *cis* to a transfer negative pBFTM10 plasmid by inserting in different positions on the plasmid (Hecht and Malamy, 1989). An insertion element, IS*942*, was detected upstream of the metallo-β-lactamase gene from *B. fragilis* (Rasmussen and Kovacs, 1991). The insertion element, IS*4351*, previously exclusively found to be associated with the clindamycin resistance genes, *erm*, was shown to also occur independent of *erm* in *B. fragilis* (Fletcher and Macrina, 1991).

The transfer of clindamycin and erythromycin resistance by pBF4 and pBFTM10 has been shown to sometimes be accompanied by the transfer of tetracycline resistance. The conjugal transfer of tetracycline resistance was not plasmid encoded. The frequency of conjugational transfer of pBFTM10 and the tetracycline resistance element was increased when the donor was exposed to clindamycin or tetracycline before mating (Malamy and Tally, 1981). Several different tetracycline and tetracycline-erythromycin resistance elements that were found to be related, but not identical, have been reported by Shoemaker *et al.* (1989). The conjugal elements are believed to be chromosomal, because plasmid DNA was not found to be consistently associated with tetracycline resistance transfer.

At least two types of conjugative elements exist: those that transfer only tetracycline resistance and for which the transfer frequency is enhanced by pregrowth in tetracycline, and those that transfer both tetracycline and clindamycin-erythromycin resistance and for which the transfer frequency is not affected by pregrowth in tetracycline (Salyers *et al.*, 1987).

The tetracycline resistance elements Tc^r ERL and Tc^r Em^r DOT have been studied in some detail. Besides having the ability to self-transfer among *Bacteroides* strains, Tc^r ERL can recognize a mobilization region on pBFTM10 (Shoemaker *et al.*, 1986b) and is also able to mobilize members of the cryptic plasmids (Valentine *et al.*, 1988). The transfer was shown to be enhanced by pregrowth in a subinhibitory concentration of tetracycline. Low-copy number, plasmid-like DNA forms were detected in strains harboring the Tc^r ERL element (Shoemaker and Salyers, 1988). The production of two distinct forms, NBU1 and NBU2 (nonreplicating *Bacteroides* units), was controlled by the Tc^r ERL element. NBU1 and NBU2 were produced after tetracycline treatment, but not after treatment with UV light, mitomycin C, inactive tetracycline or after thymidine starvation. NBU1 and NBU2 are not part of the conjugal element, but are normally integrated in the chromosome of selected *Bacteroides* species. Conjugal elements other than Tc^r ERL can mediate the production of one of the two forms, termed Plf (production of plasmid-like forms) (Shoemaker and Salyers, 1988). The NBU's were shown to be not only excised by the Tc^r elements but also transferred and integrated into the chromosome of the recipient (Bedzyk *et al.*, 1992).

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In Tc^r Em^r DOT the genes responsible for the Plf activity were localized to a region adjacent to the tetracycline resistance gene within the conjugal element (Stevens *et al.*, 1990). DNA sequence analysis revealed two genes, *rteA* and *rteB* (regulation of tetracycline resistance elements), which are arranged in a single operon that includes the tetracycline resistance gene, *tetQ* (Nikolich *et al.*, 1992, Stevens *et al.*, 1992). It was shown that insertions within the *tetQ* gene both abolished the Plf activity and element self-transfer. This evidence further substantiates the idea that the three genes are part of an operon and that the DNA region is essential for transfer. The sequences of *rteA* and *rteB* exhibit homologies to known genes encoding two-component regulatory systems (Stevens *et al.*, 1992).

By using Southern hybridization in combination with transverse alternating field electrophoresis it was shown that most known Tc^r elements are approximately 70-80 kb in size, except for Tc^r Em^r 12256, which is 150-200 kb in size (Bedzyk *et al.*, 1992). It was further shown that the insertion of the elements within the chromosome was not random, and neither was the integration of NBU's, which was distant from the integration site of the Tc^r element.

Conjugal transfer systems that transfer tetracycline and penicillin resistance were also detected in the oral, black-pigmented *Bacteroides* species (Guiney and Bouic, 1990). A tetracycline resistance element of a rumen bacterium, *Prevotella ruminicola*, was found to be related to that of a colonic *Bacteroides* and was able to transfer to the colonic *Bacteroides*, demonstrating that natural transfer between these two genera is possible (Shoemaker *et al.*, 1992).

1.1.2.4 Conjugation, transformation and transduction

Until recently the only successful method of introducing foreign DNA into *Bacteroides* was by mating and therefore several *E. coli-Bacteroides* shuttle vectors have been constructed (Guiney *et al.*, 1984b; Shoemaker *et al.*, 1985; Smith, 1985; Valentine *et al.*, 1988; Pheulpin *et al.*, 1988). A list of the available shuttle vectors and their origins, adapted from Odelson *et al.* (1987), is shown in Table 1.1.

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Table 1.1. Chimeric *Bacteroides* plasmids.

Plasmid, Size	Phenotype[a]		Replicon		Ref.
	E. coli	*Bact.*	*E. coli*	*Bact.*	
pDP1, 19 kb	Ap^rTc^r	MLS^r	pBR322	pCP1	Guiney *et al.*, 1984b
pE5-2, 17 kb	Su^rTc^r	MLS^r	RSF1010	pB8-51[b]	Shoemaker *et al.*, 1985
pFD176, 7.3 kb	Ap^rβgal	MLS^r	pUC19	pBI143[b]	Smith, 1985
pBI191,[c] 5.3 kb	none	MLS^r	none	pBI143	Smith, 1985
pVAL-1, 11 kb	Ap^rTc^r	MLS^r	pBR328	pB8-51	Valentine *et al.*, 1988
pKBF367-1, 14.7 kb	Ap^rTc^r	MLS^r	pBR322	pBF367	Pheulpin *et al.*, 1988

[a] *Bact.*, *Bacteroides*; Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; MLS^r, clindamycin/erythromycin resistance; Su^r, sulphanilamide resistance; βgal, β-galactosidase activity.

[b] pB8-51 and pBI143 are small cryptic *Bacteroides* plasmids.

[c] pBI191 is a *Bacteroides* cloning vector and does not replicate in *E. coli*.

The vectors described in Table 1.1 are conjugative shuttle vectors with the exception of the two plasmids developed by Smith, which are vectors used for transformation. These shuttle vectors can be mobilized from *E. coli* to *Bacteroides* by the broad-host-range plasmids RK2 (eg. pDP1) or R751 (eg. pE5-2). The frequency of transfer of the vectors was shown to be increased greatly when matings were performed under aerobic conditions (Shoemaker *et al.*, 1986a,b).

A derivative of pVAL-1, in which the *Bacteroides* replicon has been deleted, can be utilized as a suicide vector that delivers the transposon Tn*4351*, containing the *erm* gene and any gene of interest that has been cloned into it, to the *Bacteroides* chromosome (Valentine *et al.*, 1988). Besides transfer from *E. coli* to *B. fragilis*,

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pVAL-1 has also been employed successfully to introduce a xylanase gene from a ruminal into a colonic *Bacteroides* (Whitehead and Hespell, 1990).

Despite the fact that *Bacteroides* shuttle vectors have been developed from large plasmids to smaller ones that are more convenient to use, genetic manipulation of *Bacteroides* can be problematic. One of the major obstacles concerns the expression of cloned genes.

As a result of the early evolutionary divergence from the other eubacteria, *Bacteroides* have evolved a unique genetic mechanism of gene control (Smith *et al.*, 1992). Genes from other gram-negative bacteria are generally not expressed in *B. fragilis* (Guiney *et al.*, 1984a,b; Shoemaker *et al.*, 1985; Feldhaus *et al.*, 1991; Smith *et al.*, 1992; Abratt *et al.*, 1992). This was first noticed during the use of shuttle vectors carrying *E. coli* antibiotic resistance genes which were not expressed in *Bacteroides*.

Gene expression has been investigated in depth with the use of expression vectors and promoter probe vectors (Smith *et al.*, 1992). The expression vectors were constructed so as to incorporate the IS*4351* promoter regions, that were shown to control the expression of the erythromycin resistance gene in pBF4 (Rasmussen *et al.*, 1986). A chloramphenicol acetyltransferase (cat) gene was tested and expression in *Bacteroides* was dependent on the presence of the IS*4351* encoded promoter activity (Smith *et al.*, 1992). The promoter-less cat gene was employed in a promoterprobe vector to test known *E. coli* promoters (Ptet, Ptac, Ptrc, Psyn, P1P2*rrnB*) in *B. fragilis*. No chloramphenicol resistance was detected in *B. fragilis* using any of these promoters.

Bacteroides phages have been isolated, but these have proven to be ineffective for transducing purposes because they are lytic (Booth *et al.*, 1979).

Reports on transformation of *Bacteroides* are rare. A method using calcium shock described by Burt and Woods (1977) was tested by Salyers (1984) and coworkers, but with the use of this protocol pBF4 or pBFTM10 could not be introduced into *Bacteroides*. It has been suggested that the secretion of DNases by *Bacteroides* could explain the difficulty of transformation (Rudek and Haque, 1976). A technique employing polyethylene glycol is limited to the transformation of selected *B. fragilis* strains (Smith, 1985).

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The efficiency of transformation can be reduced by the action of restriction enzymes. *E. coli* DNA has been found to be restricted in *B. fragilis* (Salyers *et al.*, 1987). Conjugation has advantages over transformation with regard to restriction barriers, since the plasmids are introduced into the cell during conjugation in a single stranded form which is not amenable to restriction.

Recently, transformation of *Bacteroides* with a variety of plasmid DNAs was made possible by the use of electroporation techniques (Smith *et al.*, 1990). The frequency of transfer was dependent on the source of the DNA. Plasmid DNA isolated from isogenic strains transformed the cells at a higher frequency than plasmid DNA isolated from other *Bacteroides* species. Plasmid DNA isolated from *E. coli* was inefficient in the transformation of *B. fragilis*. The choice of antibiotic resistance markers has been shown to also influence the recovery of transformants.

1.1.3 *Bacteroides* antibiotic resistance

The pattern of *Bacteroides* antibiotic susceptibility is changing constantly and needs to be monitored on a regular basis. Studies conducted in the early 1960's showed that nearly all *B. fragilis* strains were sensitive to tetracycline. However, since the first report of resistant strains in the mid 1960s, the occurrence of tetracycline resistance has increased, to approximately 60% of all isolates, in the early 1980s (Tally and Malamy, 1982). A summary of recent reports on the susceptibility of *B. fragilis* to selected antibiotics in different countries is shown in Table 1.2.

B. fragilis is frequently isolated from infections where an abscess develops as a host response. The abscess acts as a barrier to antimicrobial action and therefore the effect of subinhibitory concentrations of antibiotics on *B. fragilis* was investigated (Ferreira *et al.*, 1989). The antibiotics (metronidazole, clindamycin, chloramphenicol, penicillin G) tested at one half MIC all considerably reduced cell growth.

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Table 1.2. Antibiotic resistance of the *B. fragilis* group

Origin[a]	Year	Pen.[b] 4µg	Chlr.[b] 16µg	Clind.[b] 4µg	Tet.[b] 8µg	Met.[b] 8µg
UK[c]	1987	25-100	0	<10	40	0
US[d]	1988	nd[h]	0	5	nd[h]	0
California[e]	1991	92-100	0	8-22	nd[h]	0
Korea[f]	1992	94	0	24	85	0
Canada[g]	1992	97	0	9	nd[h]	0

[a] Country or place were investigation was held.

[b] The percentage of isolates resistant to the breakpoint concentration of the antibiotic, which is based on the criteria of the National Committee for clinical Laboratory Standards. (pen, penicillin G; chlr., chloramphenicol; clind., clindamycin; tet., tetracycline; met., metronidazole).

[c] to [g] (Fox and Phillips, 1987; Cuchural *et al.*, 1988; Appleman *et al.*, 1991; Lee *et al.*, 1992; Bourgault *et al.*, 1992; respectively)

[h] not determined

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The effect of the antibiotics at half-MIC on the *B. fragilis* neuraminidase activity, which is considered a virulence factor, was tested. The neuraminidase activity remained unchanged when the cells were treated with metronidazole and clindamycin, was reduced when the cells were treated with chloramphenicol, and was increased when the cells were treated with penicillin G. It is therefore important to achieve MIC levels of an antibiotic at the site of infection when combating *Bacteroides*, otherwise the treatment could increase the virulence of the bacteria.

Anaerobiosis has different effects on different antimicrobial agents. It disrupts the uptake of agents such as the aminoglycosides, the quinolones and novobiocin, it favours agents such as metronidazole and tetracycline, and it has no effect on agents such as penicillin, chloramphenicol, clindamycin or rifampin (Brook, 1991). The mode of resistance to a range of antibiotics by *Bacteroides* is summarized in Table 1.3.

Table 1.3. Mode of antimicrobial resistance in *Bacteroides*

Antibiotic	Mode of resistance	Reference
β-lactam[a]	membrane-bound β-lactamase	Tally & Malamy, 1982
β-lactam[a]	uptake barrier	Olsson *et al.*, 1979
chloramphenicol	nitroreductase	Louie *et al.*, 1977
chloramphenicol	acetyl-transferase	Britz & Wilkinson, 1978
aminoglycoside[b]	absence of transport	Bryan *et al.*, 1979
clind./eryth.[c]	rRNA methylase	Thakker-Varia *et al.*, 1985
tetracycline	ribosomal modification (class Q)	Nikolich *et al.*, 1992
metronidazole	reduced oxidoreductase activity	Ingham *et al.*, 1978

[a] The β-lactams include imipenem, penicillin and cefoxitin.

[b] The aminoglycosides include streptomycin and gentamycin. All *Bacteroides* strains are naturally resistant to the aminoglycosides.

[c] Resistance to clindamycin in *Bacteroides* is usually associated with resistance to erythromycin and streptogramin, which is called the macrolide lincosamide-streptogramin (MLS) resistance.

A class of β-lactamases, class B, requiring a metal cofactor has been reported in a limited number of bacterial species and the gene encoding resistance, *ccrA*, was cloned from *B. fragilis* and sequenced (Rasmussen *et al.*, 1990, 1991, Thompson and Malamy, 1990). The enzyme encoded by *ccrA* is able to inactivate nearly every class of β-lactam antibiotics and is insensitive to β-lactamase blocking agents, such as clavulanic acid. The metallo-β-lactamase, CcrA, was

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characterized by Yang *et al.* (1992) and shown to be inactivated by ion chelators and activated by clavulanic acid.

Transfer of resistance to ampicillin among *B. fragilis* has been shown to be dependent on the presence of the tetracycline or clindamycin transfer elements (Butler *et al.*, 1980).

MLS resistance, encoded by *erm* genes, is due to the methylation of adenine residues in the 23S rRNA, which reduces the affinity between the antibiotic and the 50S ribosomal subunit (Thakker-Varia *et al.*, 1985).

Different modes of resistance to tetracycline in microorganisms have been reported. The tetracycline resistance of classes A-E, F and L is due to the decreased accumulation of tetracycline by the resistant cells, resistance of classes M-O and Q is due to the protection of the ribosome and resistance of class X is due to the detoxification of tetracycline (Speer *et al.*, 1991). Except for class Q resistance, which was detected only in the *Bacteroides* genus, the resistance due to efflux of tetracycline and ribosomal modification was found in bacteria other than *Bacteroides*, (Nikolich *et al.*, 1992). Classes F and X resistance genes originate from *Bacteroides* but only function in aerobically grown bacteria.

A molecular survey has been conducted on the tetracycline and clindamycin resistance of clinical *Bacteroides* isolates (Fletcher and Macrina, 1991). In this study the cloned *ermF* and *tetQ* genes were used as DNA hybridization probes to test the occurrence of these genes in the isolates. The study showed that the *tetQ* gene was common in the tetracycline resistant strains, whereas the occurrence of *ermF* was low. A previously uncharacterized inducible clindamycin resistance was detected in more than half of the clindamycin resistant isolates.

1.1.3.2 Metronidazole resistance

Metronidazole is an antibiotic with selective toxicity on anaerobic organisms. It is an inert substance that needs to be chemically or enzymatically reduced to form the active toxic compound. The mode of action of this drug is discussed in more detail in section 1.2. Since metronidazole was used extensively in the work described in this thesis, the resistance to this drug is discussed in detail below.

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Although metronidazole has been used widely in the treatment of anaerobic infections, very few reports on resistance to this drug exist (see Table 1.2.). Because the identification of anaerobic bacteria is complicated, the sensitivity to a 5 µg metronidazole disc is widely used as a preliminary identification marker (Sprott and Kearns, 1988).

The first confirmed report of a highly resistant (MIC 64 µg/ml) strain of *B. fragilis* was by Ingham *et al* (1978). The mode of resistance in this strain (designated *B. fragilis* TAL1313 by Tally *et al.* (1979) or recently *B. fragilis* NCTC 11295) has been extensively studied. The resistance could not be transferred by filter mating and no extrachromosomal DNA could be identified (Tally *et al.*, 1979; Breuil *et al.*, 1989). The metronidazole resistant strain had a decreased ability to take up the drug as well as a decreased capacity to reduce metronidazole to its active compound (Tally *et al.*, 1979). The resistant strain also grew more slowly than sensitive strains and was killed less rapidly by metronidazole (McLafferty *et al.*, 1982). *B. fragilis* NTCT 11295 displayed the same relationship between the lethal action of metronidazole and the accumulation of its inactive metabolite, acetamide, as sensitive strains. This indicated that the strain had a decreased ability to reduce the drug, but that it was as sensitive to the reduced compound as susceptible strains.

It has been proposed that metronidazole resistance in *B. fragilis* NTCT 11295 was due to reduced pyruvate ferredoxin oxidoreductase activity (Britz and Wilkinson, 1979). The pyruvate ferredoxin oxidoreductase activity of *B. fragilis* NCTC 11295 was shown to be lower when the strain was grown on media containing metronidazole, than when it was grown on metronidazole free media (Inoue *et al.*, 1988). The lactate dehydrogenase enzyme was shown to compensate for the reduced oxidoreductase activity and decreased ATP regeneration system (Narikawa *et al.*, 1991).

Several other cases of metronidazole resistant *Bacteroides* have been reported, but only a few could be verified. Rotimi *et al.* (1979) have isolated a *B. distasonis* with a reported MIC of 64 µg/ml from an untreated patient. Independent studies confirmed this report (Eme *et al.*, 1983). A resistant *B. fragilis* clinical isolate, *B. fragilis* 396-82, was reported by Eme *et al.* (1983) and the findings have been confirmed by Dublanchet *et al.* (1986). Six more strains with reduced sensitivity to nitroimidazoles that showed a stable resistance were identified by Dublanchet.

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Metronidazole resistant *Bacteroides* strains were also reported by MacWalter and Baird (1983), Sprott *et al.* (1983), Lamothe *et al.* (1986), and Sprott and Kearns (1988), but no further studies on the strains have been reported. An article by Applebaum and Chatterton (1978) reported that 21% of *Bacteroides* sp. isolated in the King Edward Hospital in South Africa were resistant to metronidazole, but no further studies on these strains have been reported. Interestingly, the *B. fragilis* Bf-2 strain studied in this thesis was isolated in the King Edward Hospital.

The nature and identification of other reportedly resistant strains is disputed. The metronidazole resistance phenotype of four *Bacteroides* strains isolated by Acar *et al.* (1981) proved to be unstable after subculture (Eme *et al.*, 1983). Tabaqchali *et al.* (1983) argued that a clinical isolate reported by Britz and Wilkinson (1979) is a nutritionally aberrant form of *E. coli*. Other possibilities of error in the isolation of metronidazole resistant strains have been identified as being due to either poor anaerobiosis (Milne *et al.*, 1978) or light inactivation of metronidazole sensitivity discs (Jones and Scott, 1977).

A strain of *Bacteroides vulgatus*, which displayed moderate resistance to nitroimidazoles (metronidazole MIC 2-4 µg/ml), was characterized by Breuil *et al.* (1989). A plasmid, pIP417, of this strain could transfer resistance to a sensitive strain, *B. fragilis* 638, where it increased the metronidazole resistance to 16-32 µg/ml. pIP417 was mobilized to other strains of *B. fragilis* by a co-resident plasmid, pIP418. Another plasmid that confers nitroimidazole resistance has been identified and the resistance determinant was found to be different from that of pIP417 (Reysset *et al.*, 1992). The regions involved in transfer of the plasmids were similar.

The susceptibility of *B. fragilis* and *E. coli* to metronidazole has been used extensively in this thesis both to investigate the cytotoxicity of this antibiotic and to study DNA repair mechanisms. The history and mode of action of metronidazole is reviewed in section 1.2.

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1.2 Metronidazole

1.2.1 History of metronidazole

The discovery of metronidazole and its activity against a wide spectrum of organisms had an enormous impact on the medical community. In this section on the history of metronidazole I will briefly try to summarize the fascinating evolution of this chemical. Information was mainly obtained from 'Flagyl, the story of a pharmaceutical discovery' by James A. McFadzean.

In 1977 Catterall wrote that in the fifties, 'at the majority of clinics there were queues of sad looking unhappy women...with wet and stained underwear and poor morale'. No effective cure could be offered to these patients infected with a parasite, *Trichomonas vaginalis*. The laboratories of Rhone-Poulenc in Paris set out to find a cure and eventually in 1955 the search was fruitful when a crude extract of a streptomyces culture was shown to kill the parasite (Despois *et al.*, 1956). The active ingredient was azomycin. Following this discovery, many related compounds were synthesized, and in 1957 metronidazole was first made (Fig. 1.2.)(McFadzean, 1986). Metronidazole, or Flagyl, displayed the best balance between toxicity to the parasite and safety in animals.

Following many successful animal trials, the most interesting trial of metronidazole was conducted in a female prison in London, where the inmates were as isolated as possible from members of the opposite sex and therefore at a lower risk of reinfection (McFadzean, 1986). The cure rate was 100%. After extensive clinical evaluation orally administered metronidazole was introduced for prescription use in 1960 in France and the UK.

This could well have been the end of a successful, but short, story of metronidazole. However, due to the careful observations of a dentist, David Shinn, other pathways for metronidazole were opened (McFadzean, 1986). Shinn had treated a patient for ulcerative gingivitis using the then common treatment of chromic acid and peroxide. The patient showed a miraculous recovery within four days. Not believing in miracles, Shinn questioned the patient and discovered that she was on treatment for trichomoniasis. He subsequently

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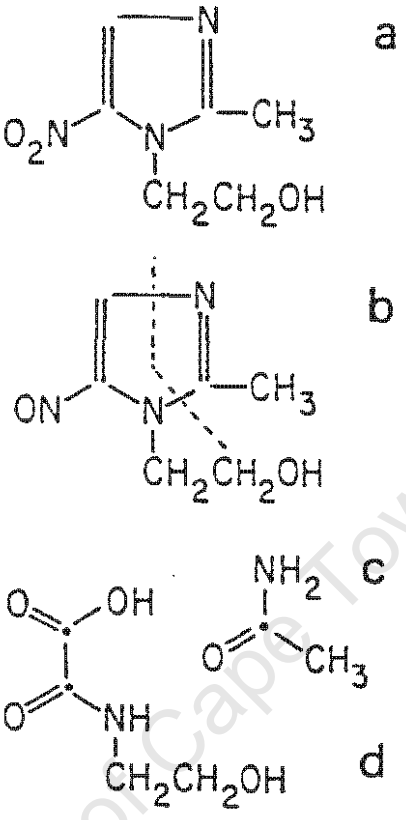

Fig. 1.2. Metronidazole and its derivatives. Chemical formulas of metronidazole (a); its nitroso derivative (b); acetamide (c); and 2-hydroxyethyl oxamic acid (d) (Mueller, 1983).

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treated six more patients suffering from gingivitis with metronidazole and all showed the same instant recovery (McFadzean, 1986).

One of the causative agents of ulcerative gingivitis is an anaerobic bacterium, *Bacteroides fusiformis*. A subsequent study on the effect of metronidazole on anaerobes was met with little interest by microbiologists and clinicians. At that stage anaerobic bacteria were mainly associated with rare diseases, such as the *Clostridium*-caused tetanus and gas gangrene (McFadzean, 1986). Although it was known that 20% of the weight of wet faeces consisted of viable bacteria, it was believed that the majority were *E. coli*. Drasar *et al.* in 1966 first observed that

98% of viable bacteria of the large intestine belonged to the *Bacteroides* group. Still, the medical importance of anaerobic bacteria was not accepted. Anaerobes were not cultured routinely and cultures from wound infections always yielded enough known aerobic pathogens to account for sepsis (McFadzean, 1986).

The May and Baker (distributors of metronidazole in the UK) metronidazole research team seemed to be in a blind alley, summed up by HRH the Duke of Edinburgh who said: 'May and Baker - ah yes, you were the company with a drug looking for a disease' (McFadzean, 1986).

Anaerobic organisms, however, had been identified in a few serious infections and increasing numbers were recognized as the techniques for cultivating anaerobes improved. Treatment with metronidazole proved effective against anaerobic isolates from the faeces of patients that contaminated amputation stumps (Parker, 1969; McFadzean, 1986). More reports that demonstrated the effectiveness of metronidazole in treating post operative sepsis followed (McFadzean, 1986). After clinical trials in 1978 several London hospitals concluded that it was unethical to withhold metronidazole treatment when undertaking hysterectomy operations.

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The most important facts that emerged, were that anaerobes were the only organisms of importance following abdominal surgery, anaerobes were derived from the patients own flora, and metronidazole was active against all these anaerobic organisms (McFadzean, 1986). Metronidazole was proven to be effective in all types of abdominal surgery and also in non-surgically induced anaerobic infections, involving both prokaryote and eukaryote organisms.

In 1972, by which time metronidazole had been administered to millions of patients, a report appeared, challenging the drug's safety (Rustia and Shubik, 1972). Studies with mice had shown that vast quantities of metronidazole could cause tumors. Although further investigations conducted with hamsters and rats did not support these findings, a petition was launched in the USA to remove the indication for trichomoniasis from the label, and let the 'female patient learn to live with her parasite' (McFadzean, 1986). The petition was rejected. In the more than 25 years since the discovery of metronidazole no evidence of the induction of tumors by this drug in the most relevant animal, the human, was found (Beard *et al.*, 1979; Roe, 1983). Uneventful recovery has been reported following suicide

attempts involving the digestion of up to 12 g of metronidazole (Kucers and Bennet, 1987).

Metronidazole and its derivatives have even found a niche in cancer treatment. Since the introduction of radiotherapy, attempts have been made to find a chemical capable of increasing the sensitivity of malignant cells to radiation. Oxygen was found to be effective, but absent in the anoxic parts within the tumor due to insufficient blood supply. Treatment of cancer patients with high doses of metronidazole plus radiation resulted in a delayed relapse of several months as compared to the control patients, which were treated with radiation alone (Urtusan *et al.*, 1977). The Gray Laboratory demonstrated that metronidazole had a chemotherapeutic effect in the absence of radiation (Willson, 1977). Recently, mainly the derivatives of metronidazole are used in cancer treatment (McFadzean, 1986).

May and Baker Ltd. were awarded the Queen's award for Technological Achievement in 1982 following their role in the development and production of metronidazole (McFadzean, 1986).

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1.2.2 Mode of action of metronidazole

Metronidazole itself is devoid of activity. The fact that it is a prodrug, which is inert and is only changed into an active drug at the site of action, was fortuitous and not designed (McFadzean, 1986). Only actively growing and metabolically active cells are susceptible. The action of metronidazole will be discussed in the following section by dividing it into four basic steps: drug entry (1.2.2.1), reductive activation (1.2.2.2), toxic effect (1.2.2.3), and exit of end products (1.2.2.4) (Mueller, 1983).

1.2.2.1 Entry of metronidazole

It has been suggested that metronidazole entry occurs by passive diffusion with no selectivity for anaerobic versus aerobic cells. There is no direct evidence for this theory, and therefore active transport cannot be ruled out (Ings *et al.*, 1974). In anaerobic cells the metabolic modification of the drug would decrease intracellular concentrations of metronidazole and thereby increase the transmembrane concentration gradient (Mueller, 1983).

Significant accumulation of ^{14}C-labeled metronidazole in *Trichomanas* (Ings *et al.*, 1974; Mueller and Lindmark, 1976) and anaerobic bacteria (Tally *et al.*, 1978) has been observed. Non sensitive cells did not exhibit this accumulation, but metronidazole was shown to diffuse into these cells. It was suggested, that since some label left the cell, the measured accumulation of metronidazole was an underestimate (Mueller, 1983).

1.2.2.2 Activation of metronidazole

Metronidazole is specifically toxic to anaerobes and facultative bacteria growing under anaerobic conditions, and not toxic to aerobes, because it interacts with biochemical processes which are not present in aerobic cells (Ingham *et al.*, 1980; Edwards, 1980). It is generally accepted that the activation of metronidazole involves the reduction of the chemical's nitro group (Mueller, 1983). The active compound is highly unstable and has, therefore, not been characterized. The multistep reduction of metronidazole is irreversible (Chen and Blanchard, 1979), except for the first reductive step which is reversible in the presence of oxygen (Mueller, 1983).

The mechanism of reduction has not been elucidated completely, but is believed to occur in single electron transfer steps either via active reduction by cellular enzymes, or via electron transfer from reduced electron carriers (Edwards, 1980; Mueller, 1983). The reducing systems of obligate anaerobes will be discussed first, since these organisms were originally believed to be the only ones susceptible to metronidazole treatment (Edwards, 1980). The facultative bacteria, using *E. coli* as an example, will then be discused in detail, because *E. coli* strains were used to elucidate the action of metronidazole throughout the work described in this thesis.

The role of enzymes in the reduction of metronidazole, collectively called metronidazole reductases or nitroreductases, has been postulated for several years (Tally *et al.*, 1978; Chrystal *et al.*, 1980; McLafferty *et al.*, 1982). The importance of the pyruvate dehydrogenase enzyme in the reduction of metronidazole was demonstrated by the characterization of metronidazole resistant *B. fragilis* and *Clostridium perfringens* mutants (Narikawa and Nakumara, 1987; Sindar *et al.*, 1982). The mutants, produced by chemical mutagenesis, all showed reduced levels of pyruvate dehydrogenase activity.

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The redox potential of metronidazole is similar to that of ferredoxin, and it has been suggested that enzymes capable of transferring electrons to ferredoxin could also transfer electrons to metronidazole (O'Brien and Morris, 1972). In the clostridia, the two enzymes that are important in the electron transfer pathway and that require ferredoxin are pyruvate-ferredoxin-oxidoreductase and hydrogenase. It has been noted that the addition of metronidazole to *T. vaginalis* and *Clostridium acetobutylicum* inhibited the ability of the cells to produce hydrogen gas (Edwards and Mathison, 1970; O'Brien and Morris, 1972). The hydrogenase enzyme of *Clostridium pasteurianum* was subsequently shown to accept metronidazole as a replacement substrate for protons, which results in the production of reduced metronidazole and oxidized ferredoxin (Church *et al.*, 1988, 1990).

The enzyme pyruvate-ferredoxin-oxidoreductase has been shown by Narikawa (1986) to also be involved in the reductive activation of metronidazole. The author tested 41 clinically important species, covering 18 different genera, and found that the distribution of metronidazole susceptibility was the same as that of pyruvate-ferredoxin-oxidoreductase activity.

Non-enzymatic transfer of electrons to metronidazole by electron carriers has been suggested (O'Brien and Morris, 1972). Metronidazole inhibits hydrogen production by the hydrogenase enzyme by serving as an alternative electron acceptor that is preferred to protons (Chen and Blanchard, 1979).

It has been shown *in vitro* that metronidazole can accept electrons from reduced ferredoxin in the absence of any other enzymes (Lindmark and Mueller, 1976). Direct electron transfer from ferredoxin to metronidazole was also implicated by the inhibition by metronidazole of photosynthesis, where ferredoxins play a vital role (Edwards *et al.*, 1974). Interestingly, metronidazole was shown to kill *Rhodopseudomonas acidophila* in the light, but not in the dark (Edwards *et al.*, 1974). This bacterium grows chemo-organotrophically in the dark, and photo-organotrophically in the light.

Conventional testing methods showed that metronidazole had only limited activity against facultative organisms, even at metronidazole concentrations far exceeding those obtainable in serum (Prince *et al.*, 1969; Benazet *et al.*, 1970; Ralph

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and Clarke, 1978; Onderdonk *et al.*, 1979). Prince *et al.* (1969) did report, however, that *E. coli* was slightly more sensitive to metronidazole than other facultative bacteria. Ingham *et al.* (1980) demonstrated a decrease in the number of viable *E. coli* cells following treatment with metronidazole at 10 µg/ml, which is a therapeutically attainable concentration, and a significant decrease following treatment with 100 µg/ml metronidazole under strict anaerobic conditions.

Onderdonk *et al.* (1979) have conducted a series of *in vivo* and *in vitro* studies on the effect of metronidazole on mixed infections of *B. fragilis* and *E. coli*, and concluded that the anaerobe produced an active metronidazole derivative which diffused to the facultative organism, killing it. Investigations by Soriano *et al.* (1982) supported these findings. Conflicting reports by Chrystal *et al.* (1980) indicated that the presence of *E. coli* enhanced the bactericidal effect of metronidazole against *B. fragilis*. They concluded that *E. coli* was producing an active compound to which *B. fragilis* was exceptionally sensitive. Further work by Ingham *et al.* (1980, 1981) led to an explanation for the discrepancies. They showed that the presence of *B. fragilis* decreases the oxidation reduction potential of the growth medium, which in turn favoured the activation of metronidazole. It was concluded that *E. coli* could reduce metronidazole in an environment with a sufficiently low redox potential. On account of the influence of oxygen, Ingham and Sisson (1988) disputed reports by Moran *et al.* (1987), who proposed enhanced killing of *E. coli* due to an active compound produced from metronidazole by *B. fragilis*. It was suggested that the toxic intermediate would be too unstable to diffuse to other cells and cause cell death.

The enzyme of *E. coli* believed to be responsible for the reduction of metronidazole is involved in the reduction of nitrates and chlorates (Yeung *et al.*, 1984). Mutant *E. coli* strains impaired in their DNA repair capacity (see section 1.2.2.3) have been shown to be more sensitive to metronidazole than the wild type strains, except when the repair mutants contained another mutation, that of the nitroreductase. In this case the mutants reverted to the susceptibility of the wild type. Sensitivity to metronidazole, therefore, involves both the ability to reduce metronidazole, and the susceptibility of the target molecule, DNA, to the reduced intermediate.

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1.2.2.3 Metronidazole cytotoxicity and DNA damage

In spite of metronidazole's healthy safety record (see history of metronidazole), it was shown by the Ames test to be mutagenic (Rosenkranz and Speck, 1977; Demeo *et al.*, 1992). This is an indication that metronidazole can affect the genetic material of the cell.

Since the biologically active reduction products of metronidazole have not been isolated, the mechanisms involved in cytotoxicity are not completely understood. It is widely accepted that DNA is the main target of metronidazole, but an increasing number of reports suggest that other targets within the cell are similarly important.

Two initial *in vivo* studies identified DNA as the main target for activated metronidazole. Metronidazole was shown to inhibit the uptake of labeled thymidine in *T. vaginalis* and *Clostridium* strains (Ings *et al.*, 1974), and was shown to inhibit DNA synthesis in *Clostridium* strains (Plant and Edwards, 1976). It was suggested that the decrease in DNA synthesis was due to degradation of the DNA, which was subsequently demonstrated *in vitro* by Edwards (1977). Reduced metronidazole was added to isolated DNA under anaerobic conditions and the effect of the drug on the nucleic acid was studied. Reduced metronidazole decreased the melting temperature of DNA and changed the melting profile, but not the cooling profile. This indicated that metronidazole was neither an intercalator, since these tend to increase the melting temperature, nor was it a cross-linking agent, because it did not alter the cooling profile. Alkaline and neutral density gradient centrifugation indicated the appearance of single and double strand DNA breaks following metronidazole treatment.

This study, however, has been criticized since metronidazole had been reduced by sodium dithionite, which itself causes strand breaks. The *in vitro* investigations were repeated using electrochemical reduction techniques (Edwards *et al.*, 1978; Knight *et al.*, 1978; Rowley *et al.*, 1979). The results supported the findings of Edwards (1977). Metronidazole decreased the viscosity of DNA, decreased the thermal renaturation and melting temperature of DNA, increased the relative amount of single stranded DNA as shown by hypoxiapatite chromatography, decreased the molecular weight of DNA as shown on agarose gels, and decreased the amount of intact helix in DNA as demonstrated by the binding of an intercalator. The damaged DNA sedimented

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to a less dense region in sucrose sedimentation gradients. These findings supported the theory that metronidazole caused strand breakage as a principal mechanism of toxicity.

Other *in vitro* studies demonstrated that only reduced metronidazole reacted with DNA and that reduced metronidazole binds to nucleic acids (LaRusso *et al.*, 1977). A number of *in vitro* studies confirmed the binding of radioactivity from radiolabeled metronidazole to DNA when it was reduced in the presence of DNA (LaRusso *et al.*, 1978; Kedderis *et al.*, 1989). However, only minute amounts of the original amount of radioactivity were retrieved.

Ludlum *et al.* (1988) have described the dithionite-dependent formation of an unstable adduct between a nitroimidazole and guanosine. Recently a nitro radical anion derivative from metronidazole, which has been proposed as the reductive intermediate responsible for cytotoxic action, was synthesized and found to interact with DNA (Tocher and Edwards, 1992). The half life of the anion was reduced by the addition of nucleic acid. The derivative was able to discriminate and was shown to prefer AT rich DNA.

An *in vivo* investigation by Malliaros and Goldman (1991) failed to reveal binding of metronidazole to DNA. Metronidazole labeled in two positions, in order to detect binding of any portion of the metronidazole molecule, was employed to study the interaction between metronidazole and *E. coli* DNA. Only small amounts of radioactivity were associated with the extracted DNA. The authors attributed the binding to radiolabeled impurities, since the addition of unlabeled metronidazole did not affect the amount of radioactivity bound to DNA. Other possible explanations are that the extraction procedure dislodged bound metronidazole, or that metronidazole interferes with DNA without binding to it.

The first DNA repair enzyme implicated in the effect of metronidazole was a deoxyribonuclease. The action of DNase-1 was inhibited *in vitro* when the DNA substrate was treated with metronidazole, and the size of oligonucleotides produced from DNA by DNase-1 was increased by 50% (Knight *et al.*, 1978).

The most convincing argument for metronidazole activity on DNA came from *in vivo* studies conducted by Yeung *et al.* (1984) and Jackson *et al.* (1984). The

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authors showed that *E. coli* strains with impaired DNA repair systems were more sensitive to metronidazole than DNA repair proficient strains. UV sensitive *E. coli* mutants were highly susceptible to metronidazole, indicating that metronidazole caused lesions that were similar to those caused by UV light. An *E. coli* mutant defective in 3-methyl-adenine-DNA glycosylase did not exhibit increased sensitivity (Yeung *et al.*, 1984). This indicates that the mismatch repair system of *E. coli* was not involved in the repair of metronidazole induced lesions.

A further analysis of the report by Jackson *et al.* (1984) indicated that the effect of multiple DNA repair mutations on the survival of *E. coli* strains after metronidazole treatment was multiplicative. Comparisons of the MIC to metronidazole of the wild type strains and each of their DNA repair mutant derivatives showed the following. Relative to the parental strain, the effect of the loss of a functional *uvrA* gene resulted in a decrease in MIC to 1/4, the loss of the *lexA* gene resulted in a decrease to 1/16, and the loss of the *recA* gene resulted in a decrease to 1/60 of the MIC of the mutant strain as compared to the repair wild type strain. It could therefore be predicted that the loss of both the *uvrA* and the *recA* genes would result in a decrease in MIC to 1/240 (1/4 times 1/60) of that of the wild type MIC, which was proven to be the case. Similarly, the loss of the *uvrA* and *lexA* genes resulted in a decrease in metronidazole MIC to 1/64 (1/4 times 1/16) of the wild type MIC.

Using radiolabeled metronidazole it has been shown that metronidazole interacted not only with DNA, but with a range of macromolecules of the cell. Thirty percent of the labeled drug was bound to intracellular components, the distribution of which paralleled that of protein (Mueller and Lindmark, 1976). In *Clostridium* strains, metronidazole was not shown to bind to RNA or effect RNA synthesis (Plant and Edwards, 1976).

The physiological effect of high concentrations (>100 times MIC) of metronidazole on *C. pasteurianum* has been investigated by Church *et al.* (1991). At the concentration used (10 μg/ml), 99.9% of the cells were killed within 5 min of the addition of the drug. Cell death was accompanied by the cessation of the production of fermentation end products and a significant increase in the protein content of the supernatant. The rapid killing could not be explained by the relatively slow cytotoxic effect on DNA. The authors therefore proposed that cell lysis is another mode of killing by metronidazole (Church *et al.*, 1991).

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The immediate effect of metronidazole on the cells had not been described before, although killing within a few hours had been reported. Investigations on anaerobic pathogenic bacteria showed a rapid killing within 2-4 h by metronidazole at 10 µg/ml (Tally *et al.*, 1978). Metronidazole caused a sharp growth end point in *B. fragilis* and *E. coli* cultures accompanied by cell elongation (Skarin and Mardh, 1981; Jackson *et al.*, 1984). Levett (1991) reported rapid (within 4-8 h) bactericidal effects on vegetative *Clostridium difficile* by a range of metronidazole concentrations greater than the MIC.

Morphological evidence of damage to various cell components has been detected in *T. vaginalis* exposed to metronidazole, with a disintegration of the cells at high drug concentrations (Buchner and Edwards, 1975). Recent microscopic studies on the ultrastructure of several periodontopathic bacteria, including *Bacteroides*, showed that subinhibitory concentrations of metronidazole cause morphological changes and cell lysis accompanied by cell elongation (Novaes *et al.*, 1991). The most noticeable impact of metronidazole was the significant reduction in the number of ribosomes in the cytoplasm.

Similar investigations were performed on *B. fragilis* (Cavalcanti *et al.*, 1991). Subinhibitory concentrations of metronidazole induced cell filamentation and loss of cytoplasm, and enhanced the cell's surface anionogenicity and hydrophobicity. These alterations to the cell surface are likely to favour the interaction between the pathogen and phagocytes.

It is therefore clear that although DNA remains the favoured target of metronidazole, other significant targets in the cell are affected, and this must be taken into account in the study of metronidazole.

1.2.2.4 Inactive end products

The end products of the reduction of metronidazole consist of fragments of the molecule which lack cytotoxic activity (Mueller, 1983). The main products that have been detected are 2-hydroxyethyl oxamic acid and acetamide (Fig. 1.2.) (Koch *et al.*, 1979; Koch and Goldman, 1979).

Since the cytotoxic intermediate of metronidazole could not be studied, Chrystal *et al.* (1980) have investigated the accumulation of acetamide and used this as an

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indication of the amount of the toxic compound produced. The relationship between bacterial survival and the formation of acetamide in *B. fragilis* and *E. coli* in the presence of metronidazole was linear at low metronidazole concentrations. Differences between anaerobic and facultative bacteria indicated that the antimicrobial action of metronidazole depended on both the capacity of the microorganism to produce the intermediate and their specific susceptibility to the toxic intermediate (Chrystal *et al.*, 1980; McLafferty *et al.*, 1982).

In order to understand the mode of action of metronidazole at the molecular level, it is essential to study the ways in which bacterial cells can deal with DNA damage. Although the response of *B. fragilis* to metronidazole is the aim of this research, it is convenient to use the bacterium *E. coli* as a tool in the investigation of metronidazole, since, unlike *B. fragilis*, its repair systems are well studied and vital mutant strains are available. DNA repair systems of the bacterium *E. coli* are therefore reviewed in section 1.3.

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1.3 DNA repair

The DNA repair system of a cell has to deal with a wide variety of insults to the genetic material, even in the absence of specific DNA damaging agents. In a typical mammalian cell, for example, many tens of thousands of spontaneous lesions occur daily, each of which would be lethal in the absence of DNA repair systems (Friedberg, 1985). However, in a typical *E. coli* cell only one in 10^{10} bases is mutated per generation (Drake, 1969). This accuracy is achieved both by a very reliable replication of the genome as well as the surveillance of the genome by a host of DNA repair enzymes. Interestingly, spontaneous mutations have been shown to occur primarily in the region of protein-DNA recognition sequences (Halliday and Glickman, 1991).

The molecular basis of the DNA repair mechanisms of the obligate anaerobic bacterium *B. fragilis* is not completely understood. The bacterium contains a RecA-like protein and appears to contain an excision repair system (Goodman *et al.*, 1987; Abratt *et al.*, 1990). In the work described in this thesis, *B. fragilis* genes involved in DNA repair were cloned both to investigate the molecular basis of metronidazole damage and to extend the studies on the DNA repair mechanisms of this pathogenic bacterium. In order to understand bacterial DNA repair, the *E. coli* DNA repair systems are reviewed below.

Because the literature in the field of DNA repair is vast, to say the least, this review will concentrate on the repair mechanisms and enzymes that could possibly be involved in the repair of DNA lesions caused by metronidazole. Metronidazole is believed to primarily cause single and double strand DNA breaks (Edwards *et al.*, 1978; Knight *et al.*, 1978; Rowley *et al.*, 1979; Edwards, 1986). In the next sections the following topics will be covered: the SOS response of the cell (1.3.1), the excision repair system (1.3.2), the recombinational repair system and the enzymes involved (1.3.3), and mutagenic repair (1.3.4). Due to the availability of many excellent and extensive reviews (Walker, 1984, 1985, 1987; Friedberg, 1985; Cox and Lehman, 1987; Smith, 1988; Weinstock, 1987; Clark, 1991) only the most recent discoveries will be discussed in detail.

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1.3.1 The SOS response

Treatment of *E. coli* and *B. fragilis* cells with metronidazole has been shown to cause cell elongation (Skarin and Mardh, 1981; Jackson *et al.*, 1984). This is a stress response and an indication that the SOS response of the cells has been induced.

The *E. coli* SOS response is the induction of a complex set of cellular responses following the exposure of the bacterial cells to agents or conditions which cause DNA damage or disrupt DNA synthesis. These include: increased DNA repair capacity, enhanced mutagenesis, filamentation of cells, cessation of respiration and DNA degradation (Kushner, 1987). The SOS system is an elaborate network of at least 20 different genes, which represents approximately 0.5% of all *E. coli* genes, under the control of the RecA and LexA proteins (Walker, 1987; Ennis *et al.*, 1989). The SOS system includes the genes encoding the excision repair system, *uvrA,B,C,D,* and the genes encoding the recombination repair system, *recB,C,D,E,F.* The genes of the bacterial DNA repair system, their gene products and the main functions of the enzymes are listed in Table 1.4 for reference purposes.

The complex SOS response of *E. coli* can be summarized as follows (Walker, 1987). In an uninduced cell the LexA protein binds to the operator/promotor sequence of a range of unlinked DNA repair genes, including those of the *lexA* and *recA* genes, and represses their expression. This control is finely tuned, since the repression is not 100% efficient and varies considerably from one gene to the next. The degree of repression depends on the strength of binding, the number of binding sites, and the strength and the number of the promoter sites. For example, the LexA protein binds tightly to the *recA* operator, and weakly to the *uvrA* and *lexA* operator sites (Walker, 1984). The *uvrB* gene, on the other hand, has two promoters, only one of which is LexA-controlled, which results in a high basal level of UvrB present in uninduced cells (Sancar *et al.*, 1982). This complex arrangement ensures that the system is not activated by very small amounts of inducing signal. Relatively weak signals would therefore fully induce the excision repair system, but would not result in the accumulation of large amounts of RecA protein.

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Table 1.4 The genes and gene products of the DNA repair system (Walker, 1984; Weinstock, 1987; Luisi-DeLuca *et al.*, 1988; Smith, 1988; Van Houten, 1990).

Locus	Gene product	Function	LexA control
lexA	LexA	repressor of repair gene expression	yes
uvrABC	UvrABC	excision repair, recognition and incision	yes
uvrD	helicase II	excision repair, removal of DNA oligomer with lesion	
recA	RecA	recombination, strand exchange regulation of LexA, coprotease activation of UmuD, coprotease controls exo V activity	yes
recBCD	Exo V	repair of double strand breaks recombination, creates substrate for RecA	yes?
recE	Exo VIII	repair of double strand breaks recombination, creates substrate for RecA	no
recF	RecF	recombination, daughter strand gap repair promotes RecA activation	no
umuDC	UmuDC	mutation repair, inhibits proofreading of polymerase	yes

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An inducing signal, which is thought to be comprised of RecA binding to a single stranded region of DNA and to a nucleotide triphosphate, activates RecA in a reversible fashion (Walker, 1987). The single stranded region occurs in gaps caused by the replication fork encountering a lesion, which is believed to activate RecA (Roberts and Devoret, 1983). Replication of the damaged template enhances, but is not required for the generation of the signal (D'Ari and Husiman, 1982).

Activated RecA then mediates the cleavage of the LexA repressor, which leads to the derepression of the DNA repair genes under the control of LexA. For the RecA mediated cleavage of LexA to occur, the RecA protein must be activated; overproduction of RecA is not sufficient to cause the induction of the SOS response (Clark, 1982). As the amount of intact LexA proteins decreases, various SOS genes, including *recA*, are expressed at increased levels.

One of the physiological responses to stress is the induction of filamentous morphology of the bacteria. The expression of *sulA* (also termed *sfiA*) is under the LexA/RecA control (Cole, 1983). The *sulA* gene product appears to function as an inducible inhibitor of septation. The *lon* gene product affects the rate of degradation of the *sulA* encoded protein (Mizusawa and Gottesman, 1983).

After the damaged stretch of DNA has been repaired, which eliminates the inducing signal, the RecA protein returns to its inactive state. The continued synthesis of the LexA repressor leads to the repression of the SOS genes, thereby restoring the cell to the uninduced state.

A set of about 14 proteins, that are not under the control of the LexA repressor, but the production of which is induced by the exposure to DNA damaging agents has been identified (Lesca *et al.*, 1991).

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1.3.2 Excision repair

DNA damage can generally be repaired efficiently by using the complementary strand as a template. During excision repair, an oligonucleotide containing the DNA damage is excised from one strand and replaced by copying the correct data from the undamaged second strand. Excision repair occurs in the non-replicating regions of the chromosome, where the enzymes encounter double stranded DNA. In the regions of the chromosome where single stranded regions of DNA occur, recombinational repair is required (see section 1.3.3).

E. coli uvrA mutant strains have been shown to be more sensitive to metronidazole than the *uvr*$^+$ strains (Yeung *et al.*, 1984; Jackson *et al.*, 1984). It is therefore apparent that the excision repair system is involved in the repair of metronidazole induced DNA lesions.

Excision repair by the UvrABC complex has been extensively studied and is possibly the best understood DNA repair system to date (see Van Houten, 1990; Lin and Sancar, 1992; for recent reviews). The *uvrA*, *uvrB*, *uvrC* and *uvrD* genes encode the subunits of the endonuclease responsible for the excision of UV induced pyrimidine dimers and other DNA lesions (Walker, 1987). The expression of the *uvrA* and *uvrB* genes is controlled by the SOS signal. Conflicting results concerning the control of the expression of the *uvrC* gene have been presented, for, although the upstream region contains several putative LexA binding regions, none bound LexA *in vitro* (Van Houten, 1990).

The UvrA protein contains a zinc finger DNA binding motif and is the damage recognition subunit of the UvrABC enzyme (Van Houten, 1990). Purified UvrA protein was shown to bind to UV irradiated DNA more efficiently than to non-damaged DNA (Seeberg and Steinum, 1982). The UvrA protein contains two damage recognition domains, one of which also stabilizes the protein-DNA complex at the site of damage (Claassen and Grossman, 1991). UvrA contains two functional ATP-binding sites (Thiagalingam and Grossman, 1991). ATP hydrolysis was shown to be required for the UvrA function which discriminates between damaged and undamaged DNA. The UvrA proteins exist as dimers in solution, and the ratio of monomers to dimers is affected by ATP (Van Houten, 1990). Interestingly, the LexA binding site of the *uvrA* gene is believed to also regulate the divergently transcribed *ssb* gene (Brandsma *et al.*, 1983).

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The UvrA and UvrB subunits interact in solution to form a $UvrA_2UvrB$ complex (Orren and Sancar, 1989). The ATP dependent activity of the UvrAB complex on double stranded DNA generates positive and negative supercoils (Koo *et al.*, 1991). This limited helicase activity of UvrAB is possibly involved in the detection of damaged sites in the DNA, or in the prepriming of the damaged site for UvrC incision.

The UvrB subunit contains a cryptic nucleotide binding site and shares homology with UvrC (Van Houten, 1990). In solution the UvrB protein was shown to exhibit no ATPase activity, and only the formation of the UvrAB complex induced increased ATPase activity. Site directed mutagenesis of the cryptic ATPase encoding site on UvrB eliminated the incision activity of the UvrABC complex (Seeley and Grossman, 1989). UvrB does not bind to DNA in

solution except in the presence of UvrA (Kacinsky and Rupp, 1981). Studies by Lin and Sancar (1992) have shown that the UvrB subunit is responsible for the incision 3' of the DNA lesion.

UvrC displays DNA binding activities and shows a preference for single stranded DNA (Sancar *et al.*, 1981). It shows no affinity for UvrB in solution and no selectivity for damaged DNA, but binds specifically to the UvrB-damaged DNA complex (Lin and Sancar, 1992). The UvrC subunit contains the active site required for the incision 5' of the damaged site. The carboxyterminal part of the protein was shown to be sufficient to interact with the UvrB-DNA complex and induce the incision (Lin and Sancar, 1991).

The *uvrD* gene encodes the helicase II which is responsible for the removal of the excised oligonucleotide (Van Houten, 1990). Plasmid pUC8 containing approximately one nick per molecule can be completely unwound by helicase II (Runyon and Lohman, 1989). Mutants that either lack the UvrD protein or that contain a truncated protein, which retains the ATP binding site of the protein, were sensitive to DNA damaging agents, and exhibited elevated levels of spontaneous mutation accompanied by increased recombination (Washburn and Kushner, 1991).

A model for the mechanism of excision repair has been proposed (Lin and Sancar, 1992) (Fig. 1.3) as follows. (1) Prior to damage recognition, the ATP-mediated UvrA dimers bind to UvrB. (2) The complex binds nonspecifically and transiently to undamaged DNA, causing localized unwinding. The UvrA dimer acts to target the UvrB subunit to the site of damage. (3) The binding of the UvrAB complex to the damaged site causes the DNA to kink. (4) The UvrA dimer is released as the UvrB subunit forms a stable complex with the partially unwound damaged DNA. The released UvrA dimer can then direct another UvrB molecule to another DNA lesion. (5) The UvrB-DNA complex creates a suitable substrate for the UvrC subunit. (6,7) The presence of UvrC causes the ATP-dependent cleavage of the phosphodiester bonds of the DNA backbone at points 3' and 5' to the lesion. (8) The damage-containing oligonucleotide and UvrC are released from the DNA by the action of UvrD (helicase II). (9) DNA polymerase I releases UvrB from the gapped DNA and fills the gap. (10) DNA ligase joins the newly synthesized oligonucleotide to the DNA backbone.

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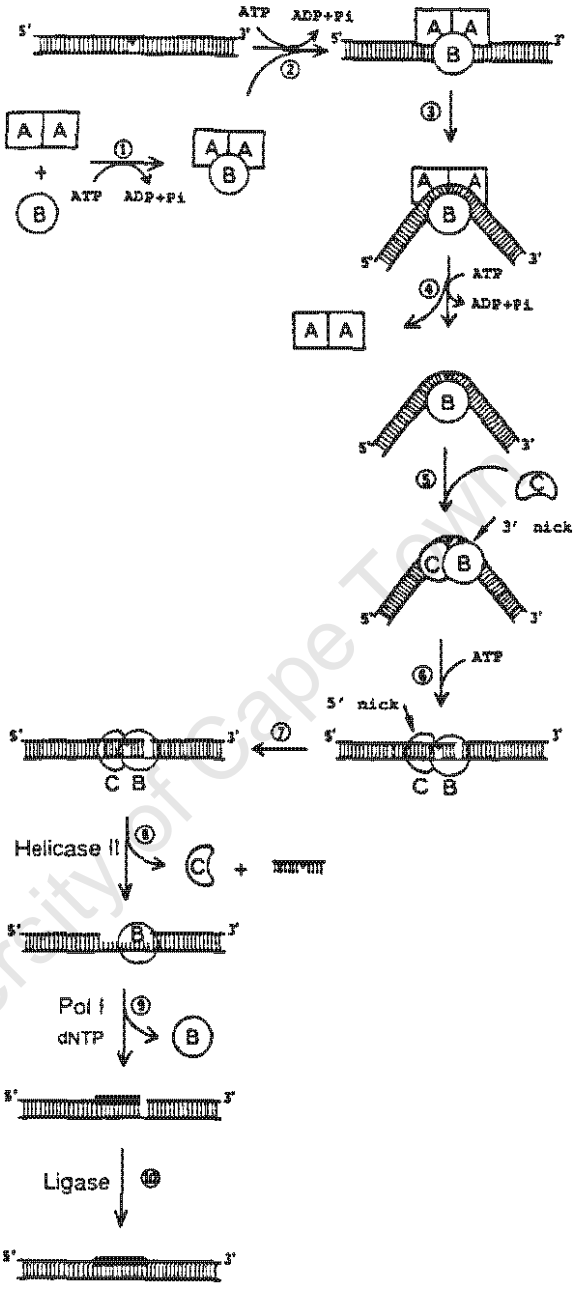

Fig. 1.3. Model for the reaction mechanism of the (A)BC excinuclease. The subunits of the excinuclease are indicated, UvrA (A), UvrB (B), UvrC (C) and UvrD (helicase II) (Lin and Sancar, 1992).

The UvrABC enzyme recognizes and repairs a wide variety of DNA lesions. It has been suggested that the enzyme complex functions as a molecular caliper

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which measures the dimensions of the DNA helix and subsequently recognizes damage-induced distortions of the DNA (Van Houten, 1990). The UvrABC enzyme can identify DNA containing covalent modifications, bulky substitutions, localized unwinding, DNA bends or kinks, altered charge distributions around the bases, and changes in the structural dynamics of the DNA helix. Lesions which are not substrates for the enzyme include base mismatches, extrahelical bases and sequence specific bends (Van Houten, 1990).

The UvrABC complex has been shown to repair some of the hydrogen peroxide induced DNA lesions (Hagensee and Moses, 1986; Goerlich *et al.*, 1989). Peroxide releases the four bases from the DNA backbone, causes modification of the bases, cleaves the DNA to produce strand breaks, inhibits DNA replication and stimulates repair synthesis. Although most of the damage consists of modified bases, and is therefore a suitable substrate for excision, it is possible that the UvrABC excision repair system may recognize and repair strand breaks.

Following the treatment with DNA damaging agents two types of repair patches have been observed. The majority were short (13 to 25 nucleotides) and are believed to have been generated by the excision repair complex (Van Houten, 1990). The few long repair patches (1600 to 2000 nucleotides) have been shown to be generated by recombinational repair.

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1.3.3 Recombinational repair

If a noncoding DNA lesion occurs in a single stranded gap, the information required for its repair must come from a separate, homologous DNA molecule. The homologous DNA strand is produced during replication and is used in recombinational repair. For this reason recombinational repair is also termed post-replication repair. It has been argued that DNA repair is the most important function of homologous recombination (Cox, 1991).

Some DNA repair genes have been extensively studied in the context of their role in recombination, rather than their role in repair. Recombination is initiated by single or double stranded discontinuities in the DNA, which can either be a result of DNA damage or can be created by the action of the RecBCD enzyme

(Walker, 1987). The models proposed to explain recombinational repair are discussed in section 1.3.3.1.

Recombinational repair utilizes the function of more than 12 recombination proteins, which can be grouped into three pathways, namely RecBCD, RecE and RecF, all of which are RecA dependent. The function of the main recombination enzymes in short term survival, that is in DNA repair, will be discussed in sections 1.3.3.2 - 1.3.3.5. For a review on the other (recG, recJ, recN, recO, recR, recQ) recombination enzymes see Clark (1991).

1.3.3.1 Models of recombinational repair

Recombinational repair is believed to be involved in the increased tolerance of the cell to DNA damage, which includes single stranded gaps, and single and double stranded DNA breaks. These lesions are either induced directly by the DNA damaging agent, or are the result of the replication fork encountering a noncoding lesion, which results in a gap in the opposite strand. Studies have recently shown that similar sized gaps are produced in the leading and lagging daughter strands of UV irradiated DNA when the lesions are not removed by excision repair (Wang and Chen, 1992). Three models of recombinational repair are discussed in detail below. In the models, the RecA function of promoting strand exchange is an absolute requirement.

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DNA synthesis is discontinuous and proceeds by the formation of Okazaki fragments. During replication this can result in single stranded regions of DNA opposite unrepaired noncoding DNA lesions. These gaps can be filled by genetic recombination using the intact new homologous strand (sister strand), which is termed gap filling repair (West *et al.*, 1981) (Fig. 1.4.). The repair of daughter strand gaps is believed to require the RecF, but not the RecBC protein (Smith and Sharma, 1987). This process of recombinational repair dilutes out the DNA lesions through successive rounds of replication, rather than repairs them. This tolerance to DNA damage results in increased survival of the cell and is therefore loosely termed 'repair' (Walker, 1987).

Sargentini and Smith (1992) proposed a model for RecBCD dependent repair of double stranded breaks. The model depicts the unwinding of the DNA by the RecBCD enzyme at a double stranded break, which results in single stranded regions. These single stranded loops allow the RecA directed invasion into

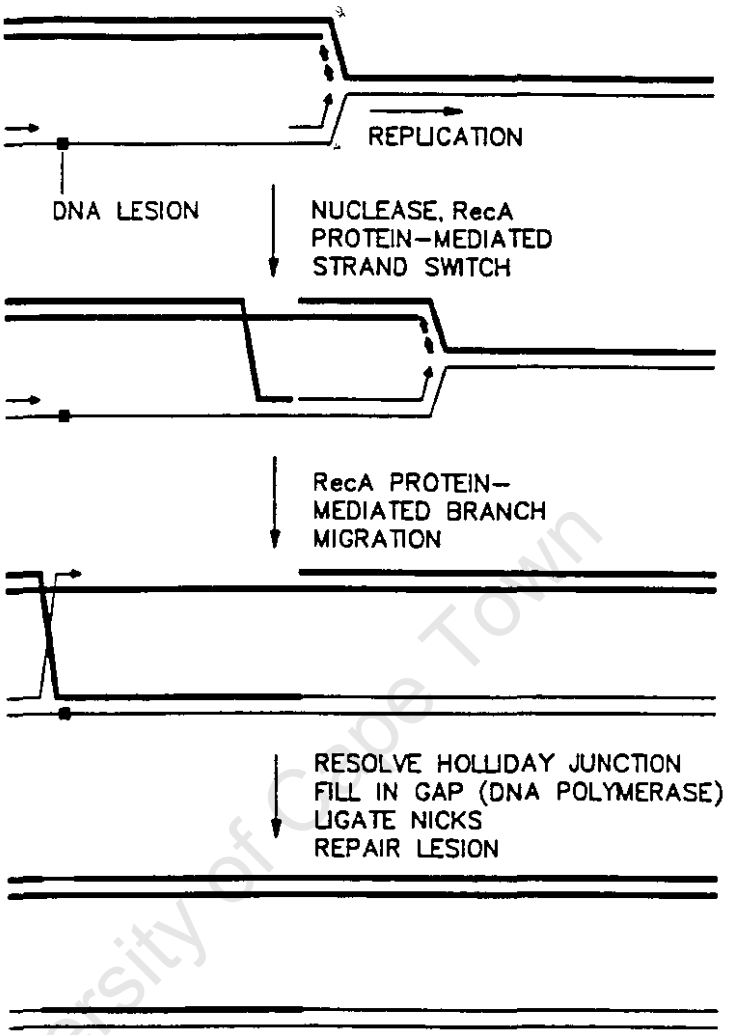

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Fig. 1.4. Model of sister strand gap repair mediated by RecA (West *et al.*, 1981; Cox, 1991).

homologous sister-DNA sequences, which leads to the accurate repair of double stranded breaks.

Nussbaum and coworkers (1992) proposed a similar model for RecE dependent, double stranded break-induced recombination repair (Fig. 1.5.). The exonuclease activity of Exo VIII enlarges the double stranded break (A) to produce a gap with a single stranded DNA overhang (B). This single stranded region participates in a homologous recombination reaction with the undamaged sister strand and primes repair synthesis (C,D). The migration of the crossover junction extends

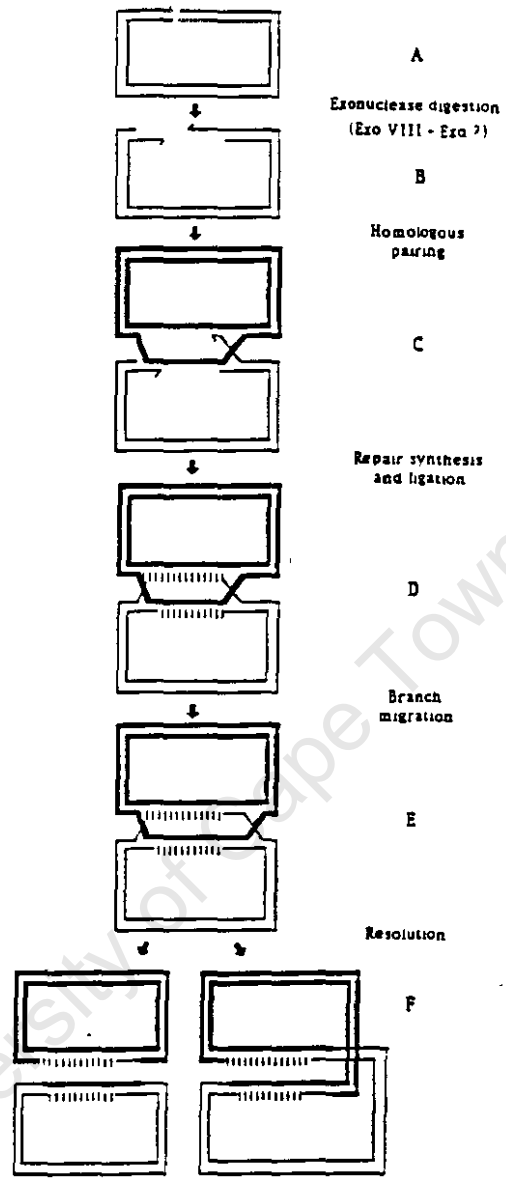

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Fig. 1.5. Model of RecE-dependent recombinational repair of double stranded DNA breaks. Damaged substrates are shown as thin lines, intact substrates in heavy lines and newly synthesized DNA as discontinuous lines (Nussbaum *et al.*, 1992).

the heteroduplex region (E). The resolution of the junction is endonuclease mediated to yield crossover and non-crossover products (F).

Wang and Smith (1983) proposed a model, termed sister duplex recombination, to explain the repair of double stranded breaks associated with UV induced lesions (Fig. 1.6.). The authors propose that the RecF pathway functions in gap filling repair, whereas the RecBCD pathway is responsible for sister duplex recombination. In an excision repair deficient mutant, the sister duplex recombination pathway is believed to be functional when daughter strand gaps, mediated by UV irradiation followed by replication, are converted at random to double stranded breaks. The sister duplex recombination mechanism then leads to the segregation of DNA lesions onto one strand. This can generate one intact lesion free strand of DNA per replication cycle.

1.3.3.2 *recA*

The *recA* gene is highly conserved among many evolutionarily divergent bacteria. This is remarkable, since the molecular system that has the recA protein as its central enzyme first evolved over 1.5 billion years ago (Roca and Cox, 1990).

E. coli strains lacking a functional RecA protein have been shown to be more sensitive to metronidazole than DNA repair wild type strains (Yeung *et al.*, 1984; Jackson *et al.*, 1984). The RecA system therefore appears to be required for the repair of metronidazole induced DNA damage and will be discussed in some detail.

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The RecA system plays an important role in the survival of the cell. Although *recA* deletion mutants exist, 50% of the cells in an *E. coli recA* mutant cell culture are non-viable with another 10% of cells containing no DNA (Capaldo *et al.*, 1974). *RecA* mutants also exhibit spontaneous DNA degradation (Clark, 1973).

The RecA protein is very versatile. It promotes DNA strand exchange and, when activated, mediates the autodigestion of several repressors, such as LexA and the λ repressor, and other proteins, such as UmuD (Walker, 1984; Burckhardt *et al.*, 1988). Both the strand exchange function and the coprotease activity require ATP and are active when the RecA polymer adopts a filamentous form (McEntee, 1992). The active site for the RecA coprotease activity is believed to be the contact region between two adjacent RecA molecules within the RecA filament (Story *et al.*, 1992). RecA also inhibits the degradation of DNA by the *recBCD* encoded exonuclease V (Pollard and Randall, 1973; Brcic-Kostic *et al.*, 1991). Under

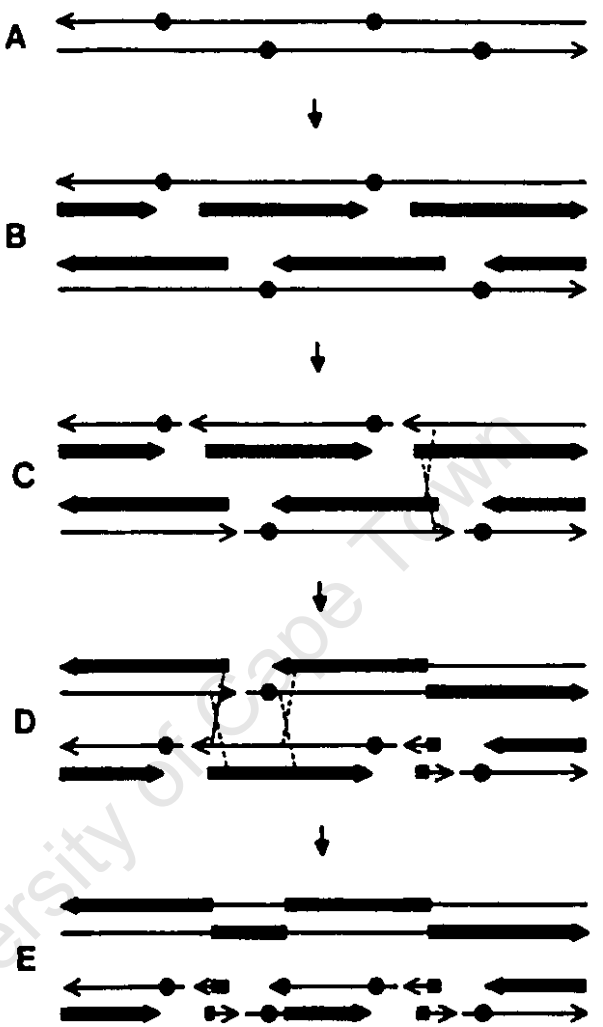

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Fig. 1.6. Model for the RecB-dependent repair of double strand breaks. Following treatment with DNA damaging agents (A), DNA replication in an excision repair mutant is discontinuous opposite lesions (B). These daughter strand gaps can be converted at random to double strand breaks. These breaks can initiate reciprocal recombination events at regions of homology between the two duplex DNA molecules (C and D). The continuation of this type of recombination can generate one intact, lesion-free molecule of DNA per deviding cell by segregating out DNA lesions (E) (Wang and Smith, 1983).

specific pH conditions *in vitro*, RecA causes the unwinding of double stranded DNA (Wu *et al.*, 1983).

The RecA mediated strand exchange consists of the following three phases: (1) RecA binds cooperatively to a gapped duplex DNA leaving the bases free to react with a second molecule of DNA, (2) the RecA/DNA complex binds to a duplex DNA molecule, and (3) the RecA protein promotes unidirectional branch migration (Cox and Lehman, 1981). *In vitro* experiments have shown that a complex consisting of single stranded DNA and single stranded binding (SSB) protein is a substrate for RecA-promoted strand exchange (Weinstock, 1987). The nucleation of RecA proteins on the DNA molecule and the formation of joint molecules, which is the prerequisite to strand exchange, is enhanced by the removal of secondary structures from the gapped DNA (Chow *et al.*, 1992). The presence of the SSB protein removes hairpin formations from single stranded DNA, and thereby stimulates DNA strand exchange.

Recent *in vitro* studies have shown that the combination of purified RecA, DNA and the newly discovered RuvC protein are sufficient to produce recombinant DNA molecules (Dunderdale *et al.*, 1991). Cell-free extracts produced from *ruvC* mutant strains were shown to lack the resolution activity that is associated with wild type strains. The RuvC protein acts as a resolvase by specific endonucleolytic cleavage of the RecA mediated strand exchange intermediate. *Ruv* mutants are sensitive to DNA damaging agents, but show only a slight decrease in recombination efficiency (Benson *et al.*, 1991). In addition to *ruvC*, two more genes, *ruvA* and *ruvB*, are part of the LexA controlled *ruv* locus (Walker, 1984). The RuvA protein binds preferentially to single stranded DNA (Shiba *et al.*, 1991). It has been proposed that the RuvAB complex reduces secondary structure in the DNA and promotes branch migration during recombination.

The RecA protein binds to a variety of DNA forms, including linear, circular and gapped DNA molecules, with a preference for single stranded DNA molecules (Radding, 1982). The RecA filament has the capacity to bind three single DNA strands in the filament groove (Mueller *et al.*, 1990). Single stranded gaps opposite a DNA lesion following DNA replication are therefore the ideal substrate for RecA mediated strand repair.

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RecA molecules have been shown *in vitro* to form extended filaments containing hundreds of RecA monomers along the DNA molecule (Dombroski *et al.*, 1983; Leahy and Radding, 1986; DiCapua and Mueller, 1987). This filament is believed both to facilitate strand exchange, which is its vital role in postreplication repair, and to exclude other DNA binding proteins. The nucleation of RecA molecules on the DNA extends and unwinds the DNA helix. The DNA in RecA/DNA filaments is extended 150% relative to the B-form DNA (Stasiak and Di Capua, 1982).

The filament formation of the RecA protein is energetically expensive (Cox, 1991). The number of RecA molecules, which is approximately 1000 molecules per cell, is increased by 20-fold after UV induced DNA damage (Weinstock, 1987), and approximately 100 molecules of ATP are hydrolyzed for every base pair of heteroduplex DNA formed in a RecA mediated strand exchange reaction (Brenner *et al.*, 1987). This would seem wasteful given that in homologous recombination, the substrates and products are equivalent in terms of base pairs and the reaction is isoenergetic (Cox, 1991). Furthermore, eukaryotic proteins that promote homologous DNA strand exchange without filament formation or ATP hydrolysis have been described (Craig, 1988; Moore and Fishel, 1990). The principal function of RecA, therefore, must be to promote strand exchange past DNA lesions that would cause the blockage of DNA synthesis and cell death if they were not repaired (Cox, 1991). ATP hydrolysis by the RecA protein has been shown to be required for driving strand exchange through regions of heterology (Roselli and Stasiak, 1991).

Another function of the RecA is believed to be required for the replication of the DNA past DNA lesions, since the polymerases (I and III) of the cell have very efficient proofreading abilities (Kushner, 1987). It has been shown that, in SOS induced cells, a different form of polymerase I, with a lower fidelity, exists (Lackey *et al.*, 1982). It has been proposed that the binding of RecA relaxes the fidelity of the DNA polymerase. RecA was also shown to inhibit the 3' to 5' exonuclease activity of polymerase III, which is the proofreading mode of the polymerase (Lu *et al.*, 1986).

RecA is actively involved in mutagenesis both via the LexA/RecA control of the *umuC,D* genes, and due to the activation of UmuD (Walker, 1984; Shinagawa *et al.*, 1988; Woodgate and Sedgwick, 1992)(Section 1.3.4). It has also been suggested

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that the UmuDC proteins are targeted to the DNA lesion by RecA (Bailone *et al.*, 1991).

The *recA* gene has been examined in great detail in order to elucidate the function of RecA at the molecular level. Ennis *et al.* (1989) have analyzed different *recA* mutations and reported that the RecA promoted functions for SOS mutagenesis and those for repressor cleavage are genetically distinct. Wang and Tessman (1986), however, suggested that the domains for coprotease and recombinase activities overlap around residue 204. The *in vitro* and *in vivo* analysis of *recA* mutants produced by site directed mutagenesis at residue 204 has demonstrated that this region is involved in both functions (Cazaux and Defais, 1992). Because of this it was suggested that the site is involved in the unwinding of double stranded DNA and could allosterically direct conformational changes in the protein.

The RecA protein has been crystalized (Story *et al.*, 1992). The structure consists of a major domain, which binds the DNA, and the smaller amino- and carboxyterminal domains, which stabilize the polymer. From the crystallization data it was proposed that the protein may change its configuration upon ATP binding (Story and Steitz, 1992).

Truncated RecA proteins have also been studied. A mutant *recA* gene that encoded a carboxyterminal truncated protein showed increased affinity for single and double stranded DNA and conferred repair efficiency and recombinational capability to *recA* mutant strains (Tateishi *et al.*, 1992). It has therefore been suggested that the carboxyterminus of the protein regulates the binding of RecA to DNA.

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1.3.3.3 *recBCD*

The RecBCD enzyme is important in the maintenance of cell viability during normal growth (Capaldo-Kimball and Barbour, 1971). The presence of a functional RecBCD system is an absolute requirement for the repair of DNA double strand breaks (Sargentini and Smith, 1986). Since metronidazole is believed to induce single and double stranded DNA breaks (Knight *et al.*, 1978), the enzyme responsible for the repair of double stranded breaks is examined in more detail.

Although it appears that the RecBCD pathway is part of the SOS response, conflicting reports exist as to whether the *recBCD* genes are controlled by LexA (Weinstock, 1987; Smith, 1988). Using a *lexA*(Ind-) mutant, which produces a LexA repressor which cannot be cleaved, recombination mediated by the RecBCD pathway is significantly reduced (Capaldo *et al.*, 1974). Smith (1988), however, reports that the *recBCD* genes are not under LexA control.

The *recB*, *recC* and *recD* genes encode the subunits of exonuclease V (Weinstock, 1987). As few as ten copies of the RecBCD enzyme exist in an uninduced cell. The RecBCD enzyme is a multifunctional enzyme with an ATP-dependent exonuclease and a helicase activity on double stranded DNA, and an exonuclease and ATP independent endonuclease activity on single stranded DNA (Telender-Muskavitz and Linn, 1982). In the presence of the SSB protein, duplex DNA molecules with unwound single stranded regions are formed *in vitro* by the RecBCD enzyme (Taylor and Smith, 1980).

Duplex DNA is cleaved by the RecBCD enzyme *in vitro* only near specific recombination sites, termed Chi sites (Ponticelli *et al.*, 1985). Homologous recombination dependent on the RecBCD enzyme is stimulated near Chi sites. *In vivo*, Chi site activation of the RecBCD enzyme is inhibited following the treatment with DNA damaging agents (Rinken and Wackernagel, 1992). The induction of the SOS response appears to be responsible for the inhibition, which does not result in an overall decrease in recombination. The inhibition could be overcome by the overproduction of the RecD subunit, but not by the overproduction of the other two subunits of RecBCD. It has been proposed that in SOS induced cells the RecBCD enzyme is converted into a Chi-independent recombination enzyme.

RecBCD is the major enzyme of the recombination system. The RecBCD enzyme acts early in recombination, probably to provide a single stranded substrate for RecA, and also late in recombination, possibly to resolve recombinants (Weinstock, 1987). *In vitro*, the RecBCD enzyme attacks the ends of double stranded linear DNA molecules and the helicase activity generates asymmetric single stranded tails, which can act as substrates for RecA mediated strand transfer (Cox and Lehman, 1987; Braedt and Smith, 1989). The single stranded tails can be hydrolyzed to oligonucleotides by the RecBCD enzyme. The hydrolysis is inhibited by the presence of the SSB protein. Under *in vitro*

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conditions, joint molecules could be formed from homologous duplex DNA molecules in the presence of only purified RecA, RecBCD and SSB proteins (Roman *et al.*, 1991).

The RecBCD enzyme is believed to be the major nuclease involved in the degradation of foreign, linear DNA. It is also involved in radiation-induced DNA degradation, which will be discussed in more detail below (Youngs and Bernstein, 1973). Attack on circular duplexes was shown to occur only if they contained single stranded gaps of at least five nucleotides in length (Muskavitch and Linn, 1982). Mutants that lacked exonuclease activity have been isolated, and their characterization indicated that this activity was not required for recombination (Chaudbury and Smith, 1984). These mutants were found to lack the RecD subunit, which indicated that the RecD subunit contains the exonuclease activity of the enzyme.

Mutants lacking the *recD* gene have been shown to exhibit plasmid instability (Biek and Cohen, 1986). A functional RecA protein was required for the expression of the phenotype. The defect in plasmid maintenance in the mutant strains was shown to be due to increased concatemerization of the plasmids and not due to decreased resolution.

Mutants lacking either the *recB* or *recC* genes have indistinguishable phenotypes (Smith, 1988). The purified RecB subunit has been shown to retain DNA-dependent ATPase activity (Hickson *et al.*, 1985). The RecB subunit of the enzyme functions as the helicase in the recombination activities (Boehmer and Emmerson, 1992).

The repair of double stranded breaks in the DNA induced by gamma radiation or chemicals, such as bleomycin, requires a functional *recB* gene (Wang and Smith, 1983, 1984; Sargentini and Smith, 1992). The RecB-mediated repair of gamma irradiation have been shown to result in the production of long deletions (700-1000 bp) in *E. coli*. These spontaneous deletions were absent in *recB* mutant strains, but were increased in a *recF* mutant strain.

RecA directly controls the exonuclease activity of the RecBCD complex. A recent, elegant study has shown that the degradation of DNA containing gamma radiation-induced lesions by the RecBCD enzyme is controlled by the

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RecA-dependent removal of the RecBCD enzyme from the damaged chromosome (Brcic-Kostic *et al.*, 1991). In spite of the degradative activity of RecBCD, its controlled action has been shown to be essential for the repair of double stranded breaks. *RecA* and *lexA* mutant strains exhibited extensive DNA degradation following radiation. It was concluded that the removal of the RecBCD enzyme from the damaged chromosome is a consequence of the induction of the SOS response.

In order to function correctly, the RecBCD pathway is dependent on the following proteins: the SSB protein, the DNA gyrase, the DNA polymerase I, and the DNA ligase (Smith GR, 1987). The SSB protein promotes recombination by stabilizing single stranded DNA regions during presynapsis and postsynapsis (Lavery and Kowalczykowski, 1992). The gyrase introduces negative supercoils into closed circular DNA molecules. The DNA polymerase I fills gapped DNA and the DNA ligase seals the gaps remaining after strand exchange.

In *E. coli recB* and/or *recC* mutant strains a range of suppressor mutations that restored the resistance of the cell to DNA damaging agents and restored its recombination capacity have been identified. These mutations are termed *sbc* (suppression of *recBC* mutation). Repair efficient *E. coli recBCD sbcA* mutants were shown to produce a new exonuclease, exonuclease VIII (exo VIII), and *recBCD sbcB* mutants lacked an exonuclease, exonuclease 1 (exo I) (Weinstock, 1987). The two pathways governing DNA repair and recombination in the absence of a functional RecBCD enzyme are termed RecE and RecF. These repair pathways will be discussed in the following sections.

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1.3.3.4 *recE*

The *E. coli* AB1157 cell line, which has been used extensively to analyze DNA repair mechanisms, carries a deletion of the *recE* gene (Bachman, 1972). This cell line was found to be more susceptible to metronidazole than other *E. coli* wild type strains (Jackson *et al.*, 1984), indicating a possible involvement of RecE in the repair of metronidazole induced lesions.

Exo VIII is encoded by the *recE* gene, which is part of a prophage, the *rac* locus, on the *E. coli* chromosome (Willis *et al.*, 1983, 1985; Luisi-DeLuca *et al.*, 1988). The RecE pathway is functional in *recBCD* mutants that contain the suppressor mutation *sbcA*. The RecE pathway depends on both a functional RecA and RecF

pathway (Gillen *et al.*, 1981). In a *recB recC sbcA* mutant background, mutations in the *recE* gene reduce the restored levels of recombination, and UV and mitomycin C resistance (Cohen and Laban, 1983; Fouts *et al.*, 1983). The *recJ* and *recO* genes are required in addition to the *recF* gene for efficient recombination in *recBCD sbcA* mutants (Lovett and Clark, 1984; Lloyd *et al.*, 1987). The *recJ* gene encodes an exonuclease with a 5'-3' activity on single stranded DNA (Lovett and Kolodner, 1989).

Most *E. coli* strains commonly used in the laboratory contain a point mutation, *recE939*, that causes a frameshift which eliminates the production of exo VIII (Chu *et al.*, 1989). A number of cloned *sbcA* mutations and deletion mutations were analyzed and found to map to the region of this point mutation. The mutations differed, and either restored the open reading frame of the *recE* gene, fused a functional promoter region to the structural gene, or deleted the region containing *recE939*. A shortened exo VIII encoded by one of the plasmids carrying a deletion mutation, pRAC-3, was a functional exonuclease in spite of the fact that 70% of the N-terminal end of exo VIII was deleted (Willis *et al.*, 1985; Luisi-DeLuca *et al.*, 1988; Chu *et al.*, 1989).

Exo VIII is an ATP independent DNase which *in vitro* has a high 5' to 3' processivity on double stranded linear DNA, but which has no activity on single stranded DNA or circular duplex DNA in either a supercoiled, nicked or gapped configuration (Joseph and Kolodner, 1983a,b).

The effect of double stranded DNA breaks on recombination via the RecE pathway has been investigated (Nussbaum *et al.*, 1992). This work suggested that the exo VIII enzyme participates in recombination by a repair mechanism for double stranded DNA breaks that involves digestion of the DNA from the lesion in a 5' to 3' direction. This would yield 3' single stranded DNA tails which may function as substrates for RecA mediated strand exchange.

Plasmid instability, similar to that observed in *recD* mutants, has been reported in *E. coli recBC sbcA* mutant strains (Biek and Cohen, 1986).

1.3.3.5 *recF*

The RecF pathway is primarily utilized when the main recombination RecBCD pathway has been mutated. It is possible that the RecF pathway would be

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involved in the repair of metronidazole induced lesions, although this has not been established yet.

In a RecBCD mutant, the RecF pathway is believed to function inefficiently, probably because a DNA intermediate of this pathway is sensitive to exo I (Clark, 1973, 1980; Clark *et al.*, 1984). The *sbcB* gene is the structural gene for exo I, which digests single stranded DNA from the 3' end and thereby destroys the substrate for RecA mediated strand transfer. *SbcB* mutations alone were shown to be insufficient in restoring repair proficiency to *recBC* mutants, and are usually accompanied by spontaneous mutations in another locus, *sbcC* (Lloyd and Buckman, 1985). An open reading frame upstream of the *sbcC* gene has been analyzed and was shown to encode a protein involved in DNA repair (Gibson *et al.*, 1992). Mutants of this gene, *sbcD*, improve growth of *recBC sbcB* strains.

The *recF* gene has been sequenced and the gene product purified (Blanar *et al.*, 1984). The only activity of RecF known to date is its single strand binding activity, which has been shown to be cooperative (Griffin and Kolodner, 1990; Madiraju and Clark, 1991). It has been hypothesized that the RecF protein reduces the secondary structure of the DNA to promote RecA activity (Madiraju *et al.*, 1988).

Daughter strand gap repair is dependent on *recF* (Smith and Meun, 1970). It has been suggested that RecF is involved in RecA-dependent repressor cleavage and DNA strand exchange (Clark, 1980). RecA activation following UV irradiation was delayed by mutations of the *recF* gene (Sassanfar and Roberts, 1991). RecF was shown to be specifically involved in an SOS inducing pathway that requires replication. It has been suggested that RecF enhances the activation of RecA into a form which promotes LexA cleavage.

Although *in vivo* studies showed that RecF stimulated RecA activities, *in vitro* investigations using high concentrations of purified RecF, demonstrated an interference of RecA-promoted joint molecule formation by RecF (Madiraju and Clark, 1991). At physiological levels of RecF concentration no interference was detected.

Mutations that eliminate the restored DNA repair capacity of *recBCD sbcB* mutants include *recF*, *recJ*, *recN*, *recO*, *recQ*, *uvrD* and *ruv* (Kolodner *et al.*, 1985;

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Clark, 1991). The *recN*, *recQ* and *ruv* genes of the RecF pathway are regulated by the SOS response.

RecF mutants are selectively sensitive to certain DNA damaging agents, such as far UV irradiation, but are no more sensitive to DNA strand breaking agents than the repair wild type strains (Horii and Clark, 1973; Armegod and Blanco, 1978).

The RecF pathway, which is dependent on RecA, is required for the majority of chromosome-to-plasmid recombination events induced by UV irradiation (Mudgett *et al.*, 1991).

1.3.4 Mutagenic DNA repair

Metronidazole was shown by the Ames test to be mutagenic (Rosenkranz and Speck, 1977; Demeo *et al.*, 1992). Relatively few agents have been shown to directly change the DNA base coding, whereas most mutagens require the involvement of the cellular repair process to induce stable mutations (Walker, 1984; Woodgate and Sedgwick, 1992). The mutagenic or error-prone repair of *E. coli* will be reviewed below, because it is likely to be involved in the repair of metronidazole induced lesions and the manifestation of metronidazole induced mutagenesis.

Mutagenic repair is encoded by the *E. coli umuDC* operon and in addition requires the following proteins: RecA and LexA, DNA polymerase III, and possibly GroEL and GroES (Wood and Sedgwick, 1986). The GroEL and GroES proteins act as chaperones and may assist in the proper folding of the UmuDC enzymes. Polymerase III has been shown to preferentially insert adenine during synthesis past DNA lesions resulting in mutagenesis.

Homologues of the *umuDC* genes are carried on several large plasmids (Walker, 1984; Woodgate and Sedgwick, 1992). These include the *mucAB*, *impCAB* and *samAB* genes from the plasmids pKM101, TP110 and LT2, respectively. The *mucAB* genes have been cloned into the *Salmonella typhimurium* ('Ames') strains and used to enhance the sensitivity of the Ames test for environmental mutagens (Perry *et al.*, 1985).

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The *umuDC* genes, as well as the plasmid borne genes, are under RecA/LexA control (Bagg *et al.*, 1981). The overproduction of the UmuDC proteins was shown to be insufficient to promote mutagenesis. UmuD requires posttranslational modification to be active (Burckhardt *et al.*, 1988). The MucA protein is similarly cleaved to its mutagenically active form (Woodgate and Sedgwick, 1992). The modification consists of the autocatalytic selfcleavage promoted by the activated RecA protein (Shinagawa *et al.*, 1988). The carboxyterminal of the UmuD enzyme, termed UmuD', is the active form which is required for efficient mutagenesis (Nohmi *et al.*, 1988). The carboxyterminal of UmuD shares homology with the equivalent region on LexA.

UmuD' was shown to bind to an activated-RecA-affinity column only in the presence of UmuC (Freitag and McEntee, 1989). The cleavage of UmuD to its active form was, however, not dependent on the presence of UmuC (Bates *et al.*, 1991). It was suggested that UmuC guides UmuD' to the activated RecA protein bound to a DNA lesion, or alternatively, that the Umu proteins only act on DNA coated with RecA protein (Woodgate and Sedgwick, 1992).

E. coli strains defective in *umuDC* exhibit a lack of induced mutability and an increased sensitivity to DNA damaging agents (Walker, 1984). Sensitization to far UV irradiation was shown to be increased in excision repair defective or *recA* strains. In these cells the sensitization is due to the requirement for UmuDC activity for the resumption of DNA synthesis beyond a DNA lesion (Witkin *et al.*, 1987). This activity, however, does not seem to be required in wild type cells (Woodgate and Sedgwick, 1992).

The current model on the function of mutagenic repair is as follows. During replication, the DNA polymerase idles at a non-coding lesion and continually inserts bases which are subsequently removed by the proofreading ability of the enzyme (Fersht *et al.*, 1982). The UmuDC proteins are believed to inhibit the proofreading ability of the polymerase, which results in the insertion of potentially mutagenic bases opposite the noncoding lesion (Bridges *et al.*, 1987).

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1.4 Aims and overview of this thesis

The anaerobic bacterium *B. fragilis* is a medically important human pathogen that accounts for most anaerobic infections. The drug of choice in treating these infections is metronidazole, due to a very low incidence of bacterial resistance and a high success rate. Only limited information is available about the molecular mode of action of metronidazole on bacteria *in vivo* or about the potential for organisms to develop resistance to metronidazole.

The aim of the study reported in this thesis was to investigate the way in which metronidazole induces cell death at the molecular level, and to study the cellular defences against metronidazole induced DNA damage. *B. fragilis* genes affecting the metronidazole susceptibility of the facultative anaerobic bacterium *E. coli* were cloned to study the role played by *B. fragilis* genes in governing the potency of metronidazole treatment (Chapter 2). The molecular analysis of the gene cloned from *B. fragilis* is described in Chapter 3.

E. coli provides an ideal living system for the analysis of the effect of metronidazole. *E. coli* can be grown under the same oxygen conditions as *B. fragilis*, and well characterized DNA repair deficient mutants are available to study the nature and repair of metronidazole induced DNA damage. The use of the *E. coli* DNA repair mutants facilitated the study of the influence of the *B. fragilis* genes on the repair of metronidazole induced DNA damage. The effect of the cloned *B. fragilis* gene on the metronidazole susceptibility of *E. coli* repair proficient and deficient strains was therefore studied (Chapter 4), and the function of the gene product was analyzed (Chapter 5).

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Chapter 2
Cloning of *B. fragilis* genes affecting metronidazole susceptibility in *E. coli*

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Chapter 2

Cloning of *B. fragilis* genes affecting metronidazole susceptibility in *E. coli*

2.0 Abstract

Reduced metronidazole causes DNA damage, the extent of which is determined partly by the repair proficiency of the cell. Resistance to metronidazole was used as a selection method for the cloning of *B. fragilis* genes affecting DNA repair mechanisms in *E. coli*. Genes from *B. fragilis* Bf-2, cloned on a recombinant plasmid pMT100, rendered *E. coli* AB1157 and its *uvrA* derivative more resistant to metronidazole and more sensitive to far ultraviolet irradiation under aerobic conditions. The locus affecting metronidazole resistance and UV sensitivity was located on a 1.6-kb DNA fragment which originated from the small cryptic plasmid pBFC1 present in *B. fragilis* Bf-2 cells.

Metronidazole is inert until it undergoes a reductive activation. A selection system using an *E. coli* strain, F19, with a reduced ability to activate metronidazole was used to select for *B. fragilis* metronidazole activating genes. Seven *E. coli* F19 clones containing different recombinant plasmids were isolated. The plasmids containing *B. fragilis* insert DNA were shown to be dissimilar at the DNA level to a group of 26 plasmids containing *C. acetobutylicum* insert DNA isolated by the same technique. The clone most sensitive to metronidazole under anaerobic conditions was shown to contain a recombinant plasmid identical to pMT100. The locus on pMT100 that conferred metronidazole sensitivity to *E. coli* F19 was located within the same 1.6 kb DNA region that conferred metronidazole resistance and UV sensitivity to *E. coli* AB1157.

The *B. fragilis* Bf-2 (pBFC1) minimal inhibitory concentration for metronidazole was determined and shown to be more than three times greater than that of the plasmid-free strains *B. fragilis* 638 and *B. thetaiotaomicron*.

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2.1 Introduction

The response of the medically important anaerobic bacterium *B. fragilis* to DNA damaging agents has been studied in some detail. *B. fragilis* was shown to be more sensitive to far ultraviolet irradiation under aerobic conditions than under anaerobic conditions (Jones *et al.*, 1980; Jones and Woods, 1981; Slade *et al.*, 1981). Abratt *et al.* (1985, 1986) have isolated and studied DNA repair deficient mutants of *B. fragilis* Bf-2 to investigate the DNA repair mechanisms at the molecular level. *B. fragilis* was shown to have an efficient repair system which removed UV adducts under both aerobic and anaerobic conditions (Abratt *et al.*, 1985, 1986, 1990). These molecular studies were extended by screening a *B. fragilis* gene library in an attempt to isolate *B. fragilis* DNA repair genes.

Metronidazole is reduced under anaerobic or microaerophilic conditions to a cytotoxic derivative that causes DNA damage (Knight *et al.*, 1978; Ings, 1974; Edwards, 1980; McLafferty *et al.*, 1982; Mueller, 1983; Yeung *et al.*, 1984). Cell death occurs when the rate of DNA damage is greater than the rate of the host cell DNA repair. DNA repair deficient mutants of *E. coli* (*uvrA,B,C* and *recA*) were shown to be more sensitive to metronidazole than DNA repair wild type strains (Yeung *et al.*, 1984; Jackson *et al.*, 1984) . The *B. fragilis* gene library was therefore screened in an *E. coli uvrA* mutant for increased metronidazole resistance in an attempt to isolate *B. fragilis* Bf-2 DNA repair genes. The cloning of *B. fragilis* genes affecting metronidazole resistance in *E. coli* described here has been reported (Wehnert *et al.*, 1990).

It has been shown by Yeung *et al.* (1984) that repair deficient, metronidazole sensitive *E. coli* strains were rendered more resistant to metronidazole when they also lacked the ability to reduce nitrate and chlorate. An *E. coli ntr* mutant has been developed by Santangelo *et al.* (1991) to serve in a selection system to clone metronidazole activating genes. An *E. coli recA* mutant, which was highly sensitive to the reduced toxic intermediate of metronidazole due to its deficient DNA repair system, was mutated using transposon mutagenesis to reduced nitroreductase activity. This mutant had an increased resistance to metronidazole because of a reduced ability to form the toxic compound (Santangelo *et al.*, 1991). This selection system was used in an attempt to isolate metronidazole activating genes from *B. fragilis* Bf-2.

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2.2 Materials and methods

Commonly used materials and methods are described in the Appendix.

2.2.1 Bacterial strains

The *B. fragilis* wild type strain (Bf-2) was described by Mossie *et al.*(1979). It contains the cryptic plasmid pBFC1 of approximately 6kb (J. A. Southern, Ph.D. thesis, University of Cape Town, Cape Town, 1986). *B. fragilis* 638 and *B. thetaiotaomicron* 5482 are plasmid-free strains which have been described previously (Privitera *et al.*, 1979 and ATCC 29148, respectively). The *E. coli* strains used in the cloning and testing of genes that affect metronidazole susceptibility are listed in Table 2.1. The full genotype of the *E. coli* strains is listed in the Appendix.

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Table 2.1. *E. coli* strains

E. coli strain	Relevant genotype	Reference/Origin
DK1		ATCC 35691
AB1157	*uvr*$^+$	ATCC 29055, DeWitt and Adelberg (1962)
AB1886	*uvrA*6 (AB1157 derivative)	Howard-Flanders *et al.* (1966)
C600	*uvr*$^+$	Appleyard (1954)
CC118	*recA*1	Manoil and Beckwith (1985)
F19	*recA*1 *ntr* (CC118 derivative)	Santangelo *et al.* (1991)
F19*	*ntr* (*recA*$^+$ revertant)	Santangelo *et al.* (1991)

2.2.2 Plasmids

The positive selection vector, pEcoR251, is derived from the pCL plasmids described by Zabeau and Stanley (1982). It contains the *E. coli EcoR*1 gene under the control of the λ rightward promoter, the ampicillin resistance gene and the

pBR322 origin of replication. The *EcoR*1 gene product, expressed at high levels by the λ promoter of pEcoR251, is lethal unless insertionally inactivated or regulated by plasmid pcI857 which contains the temperature sensitive λ repressor gene (Remaut *et al.*, 1983).

pBFC1 is a cryptic plasmid isolated from the wild type *B. fragilis* Bf-2 strain. The recombinant plasmid pMT100 and its deletion derivatives contained *B. fragilis* insert DNA cloned into pEcoR251.

The DNA of the 26 *C. acetobutylicum* metronidazole activating clones used in the DNA hybridization experiments was a gift from J. Santangelo (Dept. of Microbiology, U.C.T.).

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2.2.3 Media and growth conditions

Bacteroides strains were grown anaerobically at 37°C using Difco brain heart infusion (BHI) broth or agar, supplemented with haemin, menadione and cysteine (Holdeman and Moore, 1972). Anaerobic experiments were carried out under stringent anaerobic conditions in an anaerobic chamber (Forma Scientific Inc., Marietta, Ohio) containing an atmosphere of oxygen-free N_2, CO_2 and H_2 (85:10:5 by volume). *E. coli* strains were grown aerobically on Luria-Bertani (LB) or yeast tryptone (YT) media, and anaerobically on prereduced YT media, containing 0.5 % glucose and 0.2 % sodium nitrate (YT+g+n) at 37°C. Agar was added to the media at a concentration of 1.5 % (w/v) when necessary. The media were supplemented with ampicillin (100 µg/ml) and metronidazole (0-1000 µg/ml) as described in the text. A solution of sterile NaCl (0.03 M) was used as a diluent.

2.2.4 Construction of the *B. fragilis* genebank and screening for DNA repair genes and metronidazole activating genes

(The genebank was constructed and screened for DNA repair genes by V.R. Abratt, Dept. of Microbiology, U.C.T.). *B. fragilis* Bf-2 DNA was partially digested with *Sau*3A restriction endonuclease, and DNA fragments of 3-10 kb were ligated with pEcoR251 which was digested to completion with *Bgl*II restriction endonuclease. Ligated DNA was used to transform competent *E. coli* DK1 cells. Transformants containing recombinant pEcoR251 plasmids were selected on LB agar containing ampicillin (100 µg/ml).

Plasmid DNA prepared from pools of clones (about 10 000) containing *B. fragilis* DNA was used to transform the *E. coli* AB1886 *uvrA* mutant. Metronidazole resistant transformants were selected on LB agar containing 500 µg/ml of metronidazole under aerobic conditions.

The plasmid DNA prepared from pools of clones containing *B. fragilis* insert DNA was also used to transform the *E. coli* F19 *recA ntr* mutant strain. *E. coli* F19 showed an increased resistance to metronidazole (30 µg/ml) under anaerobic conditions due to the *ntr* mutation as compared to its parental *E. coli* CC118 *recA ntr*$^+$ strain (20 µg/ml). *E. coli* F19 transformants were selected on YT agar supplemented with ampicillin (100 µg/ml). Approximately 2400 colonies were individually transferred onto YT+g+n supplemented with ampicillin (100 µg/ml) or ampicillin plus metronidazole (25 µg/ml) and incubated anaerobically for at least 24 h. Ampicillin resistant colonies that showed increased sensitivity to metronidazole were selected for further study.

2.2.5 Metronidazole assays

Stationary phase *E. coli* cells, diluted to 10^3 -10^4 cells/ml, were plated onto LB or YT agar plates (aerobic) or prereduced YT+g+n agar plates (anaerobic), containing ampicillin (100 µg/ml) and metronidazole (0-1000 µg/ml), and incubated at 37°C. The MIC was determined after 24h for aerobic conditions, and after 48h for anaerobic conditions. *Bacteroides* cells were treated in the same manner as the anaerobic *E. coli* cultures except that they were plated onto prereduced BHI plates containing only metronidazole (0-5 µg/ml).

2.2.6 Ultraviolet (UV) irradiation

Irradiation of *E. coli* cells with far UV (254 nm) under aerobic conditions was carried out as described by Jones and Woods (1981). The irradiated *E. coli* cells were plated onto LB or YT agar supplemented with ampicillin (100 µg/ml).

2.2.7 Preparation of DNA

E. coli plasmid DNA was prepared by the alkali-hydrolysis method of Ish-Horowicz and Burke (1981). *B. fragilis* total DNA was prepared using the high-salt buffer total DNA extraction method of Campbell and Yasbin (1984) (Appendix). pBFC1 was isolated by separating total *B. fragilis* DNA on a CsCl density gradient into chromosomal and plasmid fractions. *E. coli* and

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C. acetobutylicum chromosomal DNA used for the DNA hybridization were gifts from H. Zappe (Dept. of Microbiology, U.C.T.).

2.2.8 DNA hybridization

Total DNA from *B. fragilis* Bf-2, *E. coli* and *C. acetobutylicum* and plasmid DNA was digested to completion with the appropriate restriction endonucleases. The resulting DNA fragments were fractionated by electrophoresis on 0.8 % agarose gels in TRIS-acetate buffer and transferred to a Hybond N^+ nylon membrane (Amersham International) according to the manufacturer's instructions.

Radiolabelled plasmid DNA probes were prepared by nick translation with α-^{32}P dCTP (Rigby *et al.*, 1977) using the nick translation kit of Amersham International. DNA hybridization was performed according to the methods of Southern (1975) and Smith and Summers (1980).

Non-radioactive plasmid DNA probes were labeled by random primed incorporation of digoxigenin-dUTP. DNA labeling and hybridization was performed using the non-radioactive DNA labeling and detection kit (Boehringer Mannheim) according to the manufacturer's instructions.

2.2.9 SDS polyacrylamide gel electrophoresis (PAGE)

SDS PAGE was carried out following the methods of Laemmli (%1970). Crude cell extracts were prepared by pelleting the cells from stationary phase cultures, resuspending the cell pellet in 2x SDS PAGE treatment buffer and boiling the sample for 5 min prior to loading. Proteins from crude cell extracts were electrophoresed at 200 volts constant voltage in a vertical SDS polyacrylamide gel (10%). Gels were stained with Coomassie blue R-250 following electrophoresis to visualize the proteins.

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2.3 Results

2.3.1 Isolation of *B. fragilis* genes involved in DNA repair

2.3.1.1 Cloning of gene(s) from *B. fragilis* that encode increased metronidazole resistance in *E. coli*

DNA repair deficient mutants of *E. coli* (*uvrA,B,C* and *recA*) have previously been shown to be more sensitive to metronidazole than the wild-type strain (Yeung *et al.*, 1984; Jackson *et al.*, 1984). A *B. fragilis* gene library was therefore screened by V. R. Abratt for increased metronidazole resistance in an *E. coli uvrA* mutant, in an attempt to isolate *B. fragilis* DNA repair genes, which could complement the *E. coli* repair deficiency. Recombinant pEcoR251 plasmid DNA containing *B. fragilis* Bf-2 DNA was used to transform the *E. coli* AB1886 *uvrA* mutant, which was unable to grow on LB agar containing 500 µg/ml of metronidazole. An *E. coli* AB1886 *uvrA* transformant was isolated on LB agar containing 500 µg/ml of metronidazole. Plasmid isolation and restriction mapping (see section 2.3.1.4) showed that the transformant contained a recombinant pEcoR251 plasmid. On retransformation the plasmid conferred ampicillin resistance and increased resistance to metronidazole. The recombinant plasmid was designated pMT100 (Fig. 2.1.).

2.3.1.2 Metronidazole resistance assays

The ability of pMT100 to confer metronidazole resistance to *E. coli* AB1886 *uvrA* was determined under aerobic conditions. The resistance to metronidazole of the *E. coli* AB1886 (*uvrA*) strain was increased after transformation with pMT100, whereas transformation with a control recombinant pEcoR251 plasmid (pMT104) (Fig. 2.1.) did not affect the resistance to metronidazole (Table 2.2.). To determine whether the pMT100 insert DNA was encoding a gene(s) that was complementing the *uvrA* mutation of *E. coli* AB1886, pMT100 was transformed into *E. coli* AB1157, the *uvr*$^+$ parental strain of the *uvrA* strain. Transformation with pMT100 increased the resistance of *E. coli* AB1157 to metronidazole (Table 2.2.). *E. coli* C600, another *uvr*$^+$ strain tested, was already very resistant to metronidazole (>1000 µg/ml) and could not be screened for increased metronidazole resistance.

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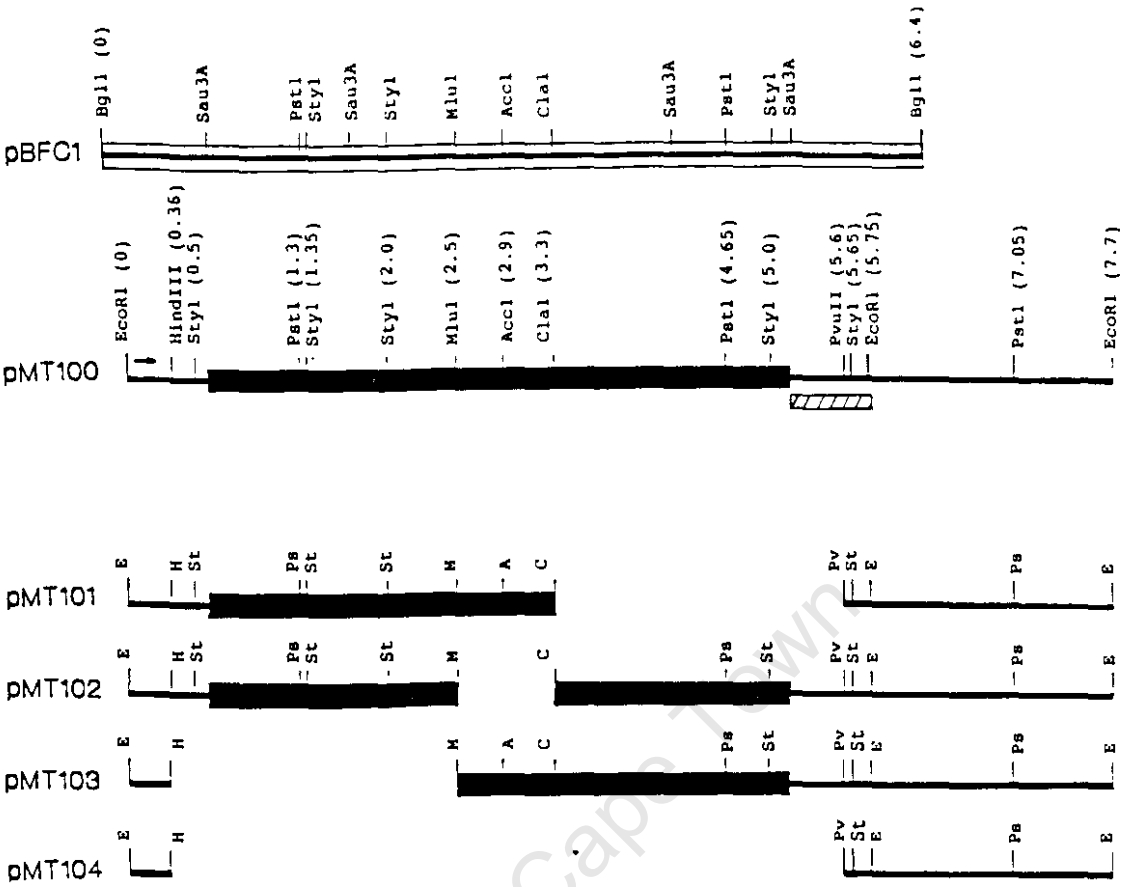

Fig. 2.1. Restriction maps of pMT100, pBFC1 (the *B. fragilis* Bf2 plasmid from which pMT100 was derived), and the pMT100 deletion plasmids pMT101, pMT102, pMT103 and pMT104. The bold bar in pMT100 represents the insert from pBFC1 and the thin bar represents the cloning vector pEcoR251. The hatched area represents an area of spontaneous rearrangements which occurred during cloning.

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Table 2.2. Susceptibility to metronidazole of *E. coli* strains transformed with pMT100.

E. coli strain	Relevant genotype	MIC (μg/ml)[a]
AB1886 (pMT104)[b]	*uvrA*	300
AB1886 (pMT100)	*uvrA*	500
AB1157 (pMT104)[b]	*uvr*$^+$	500
AB1157 (pMT100)	*uvr*$^+$	800

[a] The minimal inhibitory concentration (MIC) to metronidazole was determined on LB agar under aerobic conditions.

[b] *E. coli* strains transformed with the control deletion plasmid pMT104, which showed metronidazole susceptibility similar to those of the plasmid free *E. coli* strains, were used as controls.

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2.3.1.3 Ultraviolet irradiation survival assays

The ability of pMT100 to confer resistance to another DNA damaging agent, far UV irradiation, was investigated. The DNA repair deficient mutant, *E. coli* AB1886, was, however, more sensitive to far UV irradiation after transformation with pMT100 (Fig. 2.2.). The survival of the UV repair proficient *E. coli* AB1157 and *E. coli* C600 strains after UV irradiation was also decreased following transformation with pMT100 (Fig. 2.2.).

2.3.1.4 Restriction endonuclease mapping and deletion studies of pMT100

The restriction map of pMT100 (Fig. 2.1.), which was obtained by complete single or double digestions with restriction endonucleases, indicated that an approximately 5.0 kb DNA fragment was inserted into pEcoR251. The DNA regions controlling the increased metronidazole resistance and UV sensitivity phenotypes were determined in *E. coli* AB1157 and *E. coli* C600 under aerobic conditions by the isolation of pMT100 deletion plasmids pMT101, pMT102 and pMT103 (Fig. 2.1.). Plasmid pMT101 conferred both metronidazole resistance and UV sensitivity in *E. coli* AB1157. The UV sensitivity and metronidazole resistance

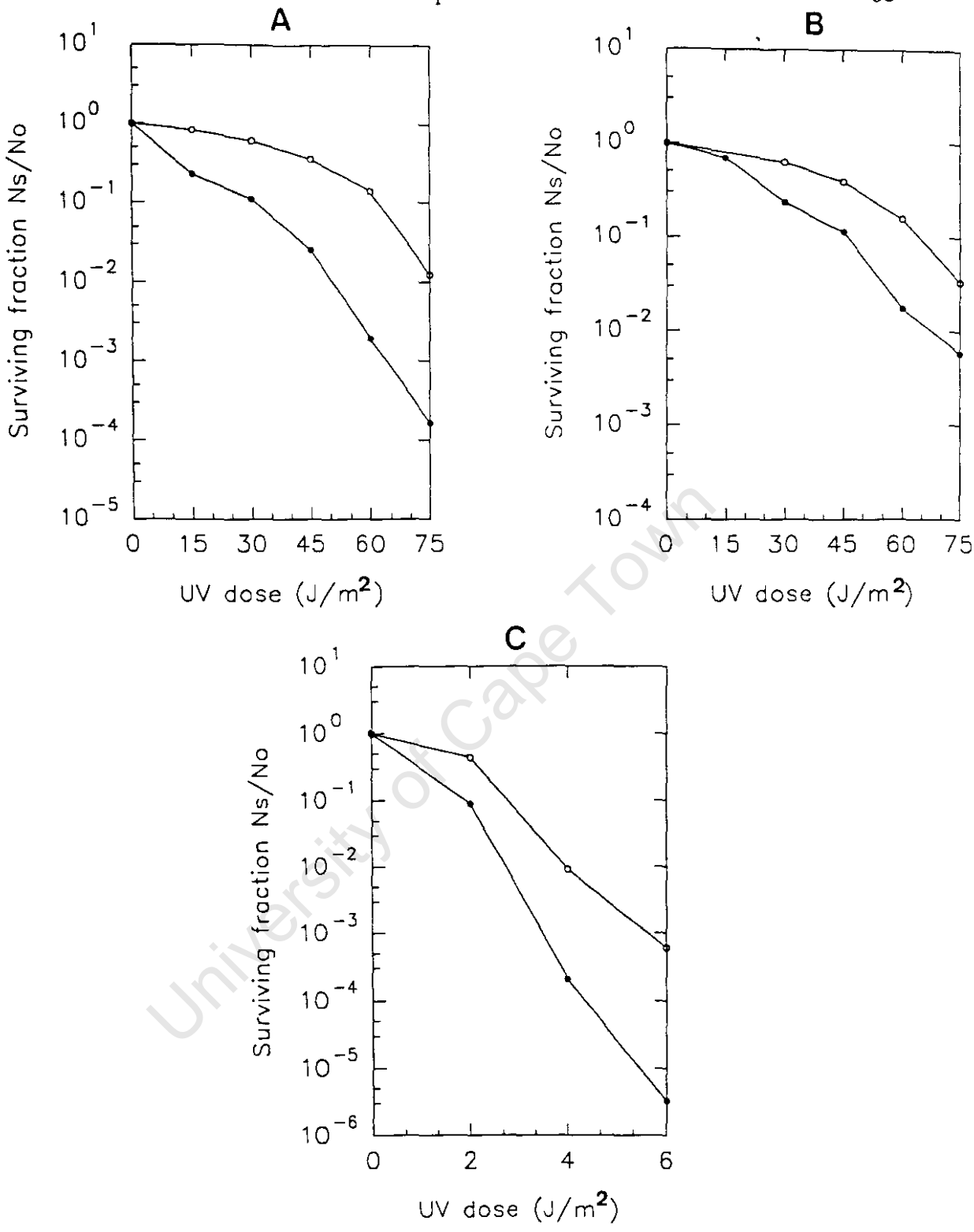

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Fig. 2.2. Aerobic UV survival curves of *E. coli* strains transformed with pMT100 (●) or the control plasmid pMT104 (○). **(A)** *E. coli* AB1157, **(B)** *E. coli* C600, and (C) *E. coli* AB1886 *uvrA*.

assays were also performed with *E. coli* C600 (pMT101) and in this strain pMT101 did not confer UV sensitivity (Wehnert *et al*, 1990). The deletion of the *Cla*1/*Mlu*1 restriction endonuclease fragment in pMT102 resulted in metronidazole resistance and partial UV sensitivity in *E. coli* AB1157. The deletion of a *Mlu*1/*Hind*III restriction endonuclease fragment in pMT103 eliminated both the UV sensitive and the metronidazole resistance phenotypes in *E. coli* AB1157. It was concluded that the loci conferring metronidazole resistance and UV sensitivity were located on the *Mlu*1/*Hind*III restriction endonuclease fragment, which contained 1.6 kb of *B. fragilis* insert DNA.

The pMT100 plasmid was prone to rearrangement on storage in *E. coli* cells. These rearrangements could be detected by *Pst*1 restriction endonuclease digestion, since the rearranged form of pMT100 had usually lost one of the two *Pst*1 restriction endonuclease sites and did not confer the pMT100 encoded phenotype. Plasmid DNA was therefore extracted from pMT100 containing cells and digested with *Pst*1 restriction endonuclease before carrying out metronidazole assays or UV survival assays.

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2.3.1.5 Origin of the insert DNA fragment in pMT100

The origin of the 5-kb DNA insert in pMT100 was investigated by DNA hybridization between *B. fragilis* total DNA and ^{32}P-labeled pMT100, and between pMT100 and ^{32}P-labeled pBFC1 (Fig. 2.3.). Plasmid pMT100 hybridized to itself and to the *B. fragilis* total DNA. Digestion of pMT100 with *Pst*1 endonuclease resulted in an internal insert DNA fragment of 3.3 kb and as expected this internal fragment hybridized with the equivalent DNA fragment when the *B. fragilis* DNA was digested with the *Pst*1 endonuclease (Fig. 2.3.). Plasmid pMT100 hybridized strongly to a discrete band of uncut *B. fragilis* DNA lying below the bulk chromosomal DNA (Fig. 2.3.). It was suggested that this band was the DNA of the cryptic plasmid pBFC1 present in the *B. fragilis* Bf-2 strain. These hybridization results indicated that the insert DNA on pMT100 hybridized with pBFC1 and not with the *B. fragilis* chromosomal DNA. To determine whether the cloned fragment originated from pBFC1, the plasmid from *B. fragilis* was isolated, labeled with ^{32}P, and hybridized against pMT100 digested with *Pst*1 and *Sty*1 endonucleases (Fig. 2.3.). Labeled pBFC1 hybridized to the 3.3 kb *Pst*1 and to the 3.0 and 0.65 kb *Sty*1 internal restriction endonuclease fragments of pMT100. As expected the restriction maps of the DNA insert of

pMT100 and pBFC1 (J. A. Southern, Ph.D. thesis, University of Cape Town, Cape Town, 1986) were similar (Fig. 2.1.).

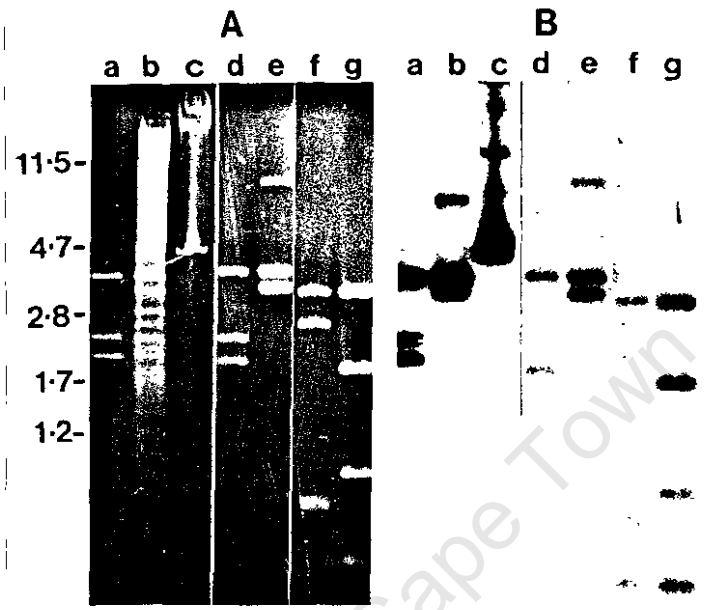

Fig. 2.3. Southern blot analysis of labeled pMT100 to *B. fragilis* total DNA and pMT100 (lanes a-c), and of labeled pBFC1 to pMT100 and pBFC1 (lanes d-g). The agarose gel is shown on the left (**A**) and the corresponding radiograph on the right (**B**). Molecular mass markers are indicated on the left. Digestion of pMT100 with the restriction endonuclease *Pst*1 resulted in an internal insert DNA fragment of 3.3 kb (lane a). Labeled pMT100 hybridized strongly to a 3.3 kb DNA fragment of the *B. fragilis* total DNA digested with *Pst*1 (lane b). pMT100 hybridized to a discrete band of uncut *B. fragilis* DNA below the bulk chromosomal DNA (lane c). Digestion of pMT100 and pBFC1 with *Pst*1 or *Sty*1 resulted in internal insert DNA fragments of 3.3 kb (*Pst*1) (lanes d and e), and 3.0 and 0.65 kb (*Sty*1) (lanes f and g). (**A**) Agarose gel. Lanes (a) pMT100 *Pst*1; (b) *B. fragilis* total DNA *Pst*1; (c) *B. fragilis* total DNA uncut; (d) pMT100 *Pst*1; (e) pBFC1 *Pst*1; (f) pMT100 *Sty*1; (g) pBFC1 *Sty*1. (**B**) Autoradiograph of **A**.

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In control experiments pMT100 was hybridized against *E. coli* chromosomal DNA, and pBFC1 was hybridized against pEcoR251, *E. coli* chromosomal DNA and *C. acetobutylicum* DNA. Only a faint background was detected where labeled

pMT100 plasmid DNA hybridized to DNA of the *E. coli* chromosomal digest. The pBFC1 plasmid did not hybridize to any of the above (results not shown).

The pBFC1 plasmid of *B. fragilis* Bf-2 could be isolated as two distinct conformations when prepared from stationary phase cultures grown from different single colonies; of four DNA preparations performed, two of each conformation were obtained. The one conformation (pBFC1) contained two *Pst*1 restriction endonuclease sites and the other (pBFC1') only one *Pst*1 site. The two plasmids were identical in size and had single sites for the restriction endonucleases *Sty*1, *Mlu*1 and *Bgl*1. pBFC1' was not analysed further and it was not determined which *Pst*1 restriction endonuclease site was lost. The stock culture of *B. fragilis* Bf-2 was not prepared from a single colony and therefore both forms of pBFC1 were maintained.

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2.3.2 Isolation of *B. fragilis* genes involved in activation of metronidazole

2.3.2.1 Cloning of genes from *B. fragilis* that confer increased metronidazole sensitivity to *E. coli*

DNA repair deficient strains of *E. coli* have previously been shown to regain resistance to metronidazole due to a mutation of the nitroreductase gene (Yeung *et al.*, 1984). The *B. fragilis* Bf-2 gene bank was therefore screened in *E. coli* F19 (Santangelo *et al.*, 1991), a *recA ntr* mutant strain, under anaerobic conditions for increased sensitivity to metronidazole in an attempt to isolate metronidazole activating genes. From more than 2400 *E. coli* F19 transformants, ten colonies that showed an increased sensitivity to metronidazole were isolated.

2.3.2.2 Analysis of the metronidazole activating clones

The plasmid DNA of the ten clones that conferred increased metronidazole sensitivity to *E. coli* F19 was extracted and after retransformation the clones retained their metronidazole sensitive phenotypes. Preliminary restriction endonuclease mapping indicated that seven of the ten clones were different and originated from separate cloning events. The seven clones were divided into two classes based on their sensitivity to metronidazole (Table 2.3.). Clones in class I

were very sensitive and did not grow on 5 µg/ml metronidazole, and clones in class II did not grow on 20 µg/ml metronidazole.

Table 2.3. Metronidazole sensitivity of seven *E. coli* F19 clones containing DNA inserts from a *B. fragilis* Bf-2 gene library.

E. coli isolate[a]	MIC (µg/ml)	Sensitivity class[b]
F19 (pMT104)[c]	30	-
CC118 (pMT104)[c]	20	-
F19 (pMAQ21)	<5	I
F19 (pMAI45)	15	II
F19 (pMAW32)	15	II
F19 (pMDA12)	20	II
F19 (pMLA9)	20	II
F19 (pMMA40)	15	II
F19 (pMXF42)	20	II

[a] The *E. coli* F19 isolates contained recombinant plasmids consisting of *B. fragilis* DNA cloned into the vector pEcoR251.

[b] The *E. coli* clones were devided into two sensitivity classes depending on their metronidazole MIC. The MIC's were determined under anaerobic conditions on YT+g+n.

[c] *E. coli* F19 and *E. coli* CC118 transformed with the control plasmid pMT104, which showed metronidazole susceptibility similar to those of the plasmid free *E. coli* strains, were included as controls.

Crude cell extracts of the seven clones were subjected to electrophoresis on SDS-PAGE gels to determine whether there were any extra protein bands. No obvious extra bands could be detected (results not shown).

Southern blot analysis was carried out to determine whether any of the seven plasmids containing *B. fragilis* insert DNA could hybridize to any of the 26 plasmids containing *C. acetobutylicum* insert DNA encoding metronidazole activating genes which had previously been isolated by the same screening technique by Santangelo *et al.* (1991).

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In a preliminary Southern blot analysis, a mixture of the plasmid DNA of the the 26 plasmids containing *C. acetobutylicum* insert DNA was labeled (non radioactive) and used to probe the DNA of the seven plasmids containing *B. fragilis* insert DNA, which had been digested with *Hind*III and *Pst*1 restriction endonucleases. The *Hind*III/*Pst*1 digest was chosen from the initial restriction endonuclease maps of these plasmids since DNA fragments that consisted of only *B. fragilis* DNA were produced. No cross hybridization between the plasmids could be detected (results not shown).

In a more detailed Southern blot analysis the 26 plasmids containing *C. acetobutylicum* insert DNA were grouped in pairs, the DNA labeled to produce 13 probes which were hybridized to 13 nylon filters each containing DNA of the seven plasmids containing *B. fragilis* insert DNA digested with *HinF*1. Restriction endonuclease *HinF*1 was chosen because it is a frequent (four base pair) cutter and was therefore assumed to produce at least one DNA fragment that did not contain vector DNA for each plasmid. As a control pEcoR251 was hybridized to the seven plasmids to show all DNA fragments containing vector DNA. Again, no cross hybridization between the plasmids containing *Clostridium* and the *Bacteroides* insert DNA could be detected (results not shown).

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2.3.2.3 Restriction endonuclease mapping and deletion studies of pMAQ21

Plasmid pMAQ21, which conferred the greatest sensitivity to metronidazole to *E. coli* F19, was chosen for further study. A detailed restriction map, which was constructed using single and double restriction endonuclease digests, indicated that pMAQ21 was identical to pMT100. Transformation of *E. coli* F19 with pMT100 and pMT101 (Fig. 2.1.) conferred increased metronidazole sensitivity (MIC <5 μg/ml) to *E. coli* F19 under anaerobic conditions, whereas transformation with pMT103 and pMT104 did not. Therefore, the locus that conferred metronidazole resistance and UV sensitivity to *E. coli* AB1157 and its *uvrA* derivative under aerobic conditions was the same locus that conferred increased sensitivity to metronidazole to *E. coli* F19 under anaerobic conditions.

2.3.2.4 Metronidazole sensitivity assays

E. coli F19 (pMT100) had an anaerobic metronidazole MIC of <5 μg/ml. To determine whether the pMT100 insert DNA was encoding a gene(s) that was

complementing the *ntr* mutation of *E. coli* F19, pMT100 was transformed into *E. coli* CC118, the *recA ntr*+ parent of *E. coli* F19. The transformants were tested under anaerobic conditions for increased metronidazole sensitivity. Transformation of *E. coli* CC118 with pMT100 increased its sensitivity to metronidazole from 20 µg/ml to less than 5 µg/ml. Transformation of an *E. coli* F19 *recA*+ revertant (*E. coli* F19*) with pMT100 had little effect on the metronidazole sensitivity of the already highly resistant strain (>800 µg/ml) under anaerobic conditions.

2.3.3 Metronidazole susceptibility studies of *Bacteroides* strains

It has not been possible to cure *B. fragilis* Bf-2 of pBFC1 (J. A. Southern, Ph.D. thesis, University of Cape Town, Cape Town, 1986). In a preliminary study the susceptibility to metronidazole of this strain was compared to two plasmid-free *Bacteroides* strains in an attempt to determine a possible role for pBFC1 in metronidazole resistance of *B. fragilis* Bf-2. The MIC for metronidazole in *B. fragilis* Bf-2 was determined and compared to those of *B. fragilis* 638, a strain reported as metronidazole sensitive (Breuil *et al.*, 1989), and *B. thetaiotaomicron* 5482 (Table 2.4.).

Table 2.4. Susceptibility of *Bacteroides* strains to metronidazole

Bacteroides strain	Relevant genotype	MIC (µg/ml)[a]
B. fragilis 638	plasmid free	0.3
B. fragilis Bf-2	pBFC1	1.0
B. thetaiotaomicron	plasmid free	0.3

[a] The MIC of metronidazole was determined on BHI agar under anaerobic conditions.

2.4 Discussion

While plasmid pBFC1 appeared to be cryptic in *B. fragilis* Bf-2 cells, a recombinant plasmid containing a 5.0 kb fragment from pBFC1 carried a locus

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which conferred increased metronidazole resistance and increased sensitivity to ultraviolet irradiation to *E. coli uvrA* and *uvr*+ strains under aerobic conditions. The recombinant plasmid also conferred increased sensitivity to metronidazole to two related *E. coli recA* mutant strains under anaerobic conditions. The locus on pMT100 that conferred metronidazole resistance and UV sensitivity in one case, was the same locus that conferred metronidazole sensitivity in the second case. This was determined by the isolation of pMT100 deletion plasmids.

Resistance to metronidazole could be associated with increased DNA repair capacity, if the cells survive the DNA damage caused by metronidazole due to a competent DNA repair system (Yeung *et al.*, 1984; Jackson *et al.*, 1984). However, the locus on pMT100 also conferred increased sensitivity to another DNA damaging agent, ultraviolet irradiation, in the same *E. coli* strains. Furthermore, the same locus caused decreased survival of two *E. coli recA* mutants after metronidazole treatment under anaerobic conditions. Therefore it was concluded that the locus on pMT100 did not increase the general capacity of DNA repair in the *E. coli* strains.

It was noted that the *E. coli* AB1157 strain had a lower resistance to metronidazole than another DNA repair proficient strain, *E. coli* C600. Jackson *et al.* (1984) similarly reported *E. coli* AB1157 and its derivatives to be more sensitive to metronidazole than another *E. coli* strain, WP2, and its derivatives. A close examination of the genotype of *E. coli* AB1157 and its *uvrA* derivative revealed a possible repair deficiency (deletion of *recE*). *E. coli* C600 was not deleted for *recE*. These findings, together with the observation that pMT100 conferred increased sensitivity to metronidazole to two *recA* mutants, indicated that further analysis of the effect of pMT100 on *E. coli* strains with different DNA repair capacities was required (Chapter 4).

Another mutation in the *E. coli* AB1157 genome, *tsx*, could possibly account for the increased sensitivity to metronidazole as compared to *E. coli* C600 *tsx*+. The *tsx* gene encodes a substrate-specific channel-forming outer membrane protein involved in nucleoside uptake (Bremer *et al.*, 1990). The reduced ability to take up nucleosides could lead to a deficiency of available nucleosides inside the cell, which in turn could retard the DNA repair system and lead to metronidazole sensitivity. It was unclear, however, why these two strains showed the same tolerance to far UV irradiation. It could possibly be accounted for by the

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difference in the DNA repair of metronidazole-induced damage and excision repair of UV induced thymidine dimers.

Other possible causes for increased resistance to metronidazole are a decreased uptake of the drug or a decreased ability of the cells to reduce the drug to its active compound. A metronidazole resistant *B. fragilis* strain was isolated by Ingham *et al.* (1978). This isolate was studied in some detail (Ingham *et al.*, 1978; Tally *et al.*, 1979; McLafferty *et al.*, 1982) and the decreased susceptibility to metronidazole was shown to be due to both the decreased uptake of metronidazole and a decreased conversion of metronidazole to the active compound. Uptake of metronidazole in *E. coli* AB1157 (pMT100) cells therefore required further analysis (Chapter 5).

The nitrate reductase system is believed to be responsible for reducing metronidazole to its active cytotoxic compound in *E. coli* (Yeung *et al.*, 1984). The plasmid pMT100 conferred increased sensitivity to metronidazole to an *E. coli* *ntr*$^-$ mutant and its *ntr*$^+$ parental *E. coli* strain under anaerobic conditions. The increase in sensitivity to metronidazole in the *ntr* proficient strain could be due to an efficient reductase encoded by pMT100, which exceeded the activity of the resident nitroreductase. Metronidazole can be reduced either directly or indirectly by a number of different enzymes, such as a hydrogenase (Church *et al.*, 1990), a pyruvate-Fd-oxidoreductase (Narikawa, 1986), a flavodoxin (Santangelo *et al.*, 1991) and a glutamate synthase (H. Stutz, Dept. of Microbiology, U.C.T., personal communication). However, the plasmid pMT100 also encoded resistance to metronidazole in *E. coli* *uvr*$^-$ and *uvr*$^+$ strains. If pMT100 encoded a metronidazole reductase, it would have been expected to increase the sensitivity of these *E. coli* strains to metronidazole. Therefore, the pMT100 insert DNA did not appear to encode a gene(s) that was activating metronidazole.

The main effect of far UV irradiation on living cells is the induction of DNA lesions (Friedberg, 1985). A recent paper, however, reported that the early effects of UV irradiation included the direct damage of the plasma membrane (Mody *et al.*, 1991). This possibility needs to be taken into account when using UV irradiation as a tool to study DNA repair mechanisms, since not only the DNA is damaged.

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The plasmid pMT100 conferred increased sensitivity to UV to *E. coli* AB1157, its *uvrA* derivative *E. coli* AB1886 and the *uvr*+ *E. coli* C600 strain. Transformation of *E. coli* AB1157 and *E. coli* AB1886 with the deletion plasmid pMT101, a pMT100 derivative that included the *Mlu*1/*Hind*III restriction endonuclease fragment containing the locus conferring UV sensitivity, conferred UV sensitivity to these strains. Transformation of *E. coli* C600 with pMT101, however, did not confer increased sensitivity to UV to the strain. It is possible that in the construction of pMT101 a distant regulatory region that was needed in *E. coli* C600 may have been deleted.

A possible explanation for the increased UV sensitivity of the *E. coli* wild type and *uvr* mutants after transformation with pMT100 could be negative complementation. Negative complementation has been reported by Prudhomme *et al.* (1991). The *hex* genes of *Streptococcus pneumonia*, encoding the mismatch repair system, were introduced into mismatch repair-deficient and wild type *E. coli* strains. The expression of the *hex* genes in the *E. coli* mismatch repair mutant strain did not result in a decrease in mutation rate, and in the *E. coli* wild type strain the resident mismatch repair system was inhibited by the *S. pneumonia* system. The authors suggested that the foreign enzymes bound to the mismatches and thereby protected the lesions from repair by the resident repair system. It is possible that the *B. fragilis* gene on pMT100 encoded a product which interfered with the *E. coli* DNA repair system. This possibility was investigated (Chapter 4).

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Several examples of plasmid genes affecting the UV susceptibility of bacterial strains have been reported:

1. Plasmids isolated from *Bacillus thuringiensis* were found to increase the sensitivity to UV irradiation of vegetative cells and spores of *B. thuringiensis* and *B. cereus* (Benoit *et al.*, 1990). A cloned protoxin gene, which, when introduced on a high-copy-number shuttle vector into a plasmid-cured *B. thuringiensis* strain, increased the UV sensitivity of the spores. The authors, however, concluded that the sensitivity to UV was not due to a specific *B. thuringiensis* plasmid gene or gene product, because the removal of the protoxin containing spore coat did not increase the spores resistance to UV, and the DNA fragment containing the protoxin encoding gene was considered too small to contain another gene.

2. The bacterial SOS system consists of certain DNA repair genes and is inducible by DNA damage. A gene (*psiB*) encoding a SOS inhibitor has been reported to occur on different conjugative plasmids (Golub *et al.*, 1988; Bagdasarian *et al.*, 1986). The presence of the *psiB* gene on the plasmid R100.1, however, did not increase the sensitivity of *E. coli* cells to UV irradiation (Bagdasarian *et al.*, 1980). In the case of the F sex factor the SOS inhibition was only observed when the *psiB* gene was expressed on a high copy number plasmid (Golub *et al.*, 1988).

3. The *muc* region on naturally occurring plasmids was shown to increase the susceptibility of *E. coli* cells to mutagenesis (Mortelmans and Stocker, 1976; Walker, 1984). Analogues to the *muc* genes, the *umuC* and *umuD* genes present on the *E. coli* chromosome, are required for the mutagenic process induced by UV (Walker, 1984). It has been suggested that the *muc* and *umuC/D* proteins enhanced the cell's ability to carry out SOS processing (Walker, 1984) and were required for the resumption of DNA synthesis beyond a photoproduct in the DNA (Bridges and Woodgate, 1985). Mutants in the *umu* genes were not mutatable by most mutagens, including far UV irradiation (Walker, 1984). The introduction of the plasmid pKM101 containing the *muc* gene into an *E. coli umu* mutant increased the strains susceptibility to mutagenesis and increased the resistance to UV irradiation.

No system that displays effects similar to those of pMT100 on the *E. coli* strains studied in this work had been reported prior to the termination of this investigation. More recently, however, a plasmid derivative of pKM101 was reported to sensitize some *E. coli* strains to far UV irradiation (Little *et al.*, 1991). These findings are discussed in Chapter 6.

Sequence analysis of the region of *B. fragilis* DNA on pMT100 was undertaken in an attempt to elucidate the mechanisms controlling the observed phenotypes. The results of the molecular analysis of pMT100 are reported in Chapter 3.

The plasmid pMT100 and the *B. fragilis* plasmid it originated from, pBFC1, were detected in two different conformations. Both the rearranged form of pMT100, and the second conformation of pBFC1, pBFC1', had lost one of the *Pst*1 restriction endonuclease sites. A similar phenomenon in the well-studied R plasmid, pBF4, has been described (Matthews *et al.*, 1991). The plasmid was found to exist in two non-equimolar conformations, which differ by an inversion

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of a large fragment of DNA resulting in a new restriction endonuclease digestion pattern. The relative amounts of the two conformations were influenced by the induction with tetracycline of a conjugal chromosomal tetracycline resistance element (Matthews *et al.*, 1991). The different conformations of pMT100 and pBFC1 were not investigated further and therefore their significance is unknown.

The DNA hybridization studies performed to determine whether any of the seven plasmids containing *B. fragilis* insert DNA, which encoded metronidazole sensitivity, were similar to any of the 26 plasmids containing *C. acetobutylicum* insert DNA isolated by the same technique revealed no similarity at the DNA level. The G+C content of *Clostridium* DNA is approximately 30% as compared to almost 50% in *Bacteroides* DNA. It was perhaps not surprising to demonstrate no cross-hybridization between these two groups of clones. These hybridization results did not exclude the possibility that functionally similar gene products were selected from *Clostridium* and *Bacteroides*.

The MIC for metronidazole in *B. fragilis* Bf-2 was more than three times that of two plasmid-free *Bacteroides* strains. This result indicated the possible involvement of pBFC1 in metronidazole susceptibility of the strain. Breuil *et al.* (1989) published a report on a small plasmid from *B. vulgatus*, a strain moderately resistant to metronidazole (2-4 µg/ml). The plasmid pIP417, when introduced into *B. fragilis* 638, increased the resistance to metronidazole from 0.5-1 µg/ml to 16-32 µg/ml. The cloned *B. fragilis* gene from pBFC1 was therefore introduced on a shuttle vector into the two plasmid-free *Bacteroides* strains to illucidate the role of pBFC1 in *Bacteroides* (Chapter 4).

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Chapter 3
Molecular analysis of a gene from *B. fragilis* involved in metronidazole susceptibility in *E. coli*

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Chapter 3

Molecular analysis of a gene from *B. fragilis* involved in metronidazole susceptibility in *E. coli*

3.0 Abstract

The region of *B. fragilis* DNA on the recombinant plasmid pMT100 responsible for conferring metronidazole resistance in *E. coli* AB1157 was characterized. The gene on pMT100 was expressed from the λ promoter of the vector pEcoR251 in *E. coli* cells. An open reading frame (ORF1) of 195 bp encoded a protein of 64 amino acids with a predicted M_r of 7.3 kDa. Deletion analysis and protein gel analysis indicated that ORF1 conferred the metronidazole resistance phenotype and encoded a protein with an apparent M_r of approximately 8-10 kDa.

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3.1 Introduction

A recombinant plasmid, pMT100, containing *B. fragilis* pBFC1 insert DNA, conferred increased resistance to metronidazole and increased sensitivity to ultraviolet irradiation to *E. coli* AB1157 under aerobic conditions (Chapter 2). The region of pMT100 encoding increased resistance to metronidazole and increased sensitivity to UV in *E. coli* AB1157 was contained within a *Mlu*I-*Hind*III restriction endonuclease fragment.

The DNA fragment of pMT100 encoding increased metronidazole resistance was sequenced to identify the gene(s) responsible for the observed phenotypes and to understand the mechanisms controlling them. This chapter reports the DNA sequence of pMT100, the deduced amino acid sequence and the computer aided analysis of the sequences. To substantiate the sequencing data, the synthesis of proteins encoded by the pMT100 insert DNA was investigated. The molecular analysis of the gene from pMT100 described here has been reported (Wehnert *et al.*, 1992).

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3.2 Materials and Methods

Commonly used materials and methods are described in the Appendix.

3.2.1 Bacterial strains and plasmids

E. coli AB1157 (ATCC 29055) was used in the metronidazole susceptibility studies. *E. coli* LK111 *recA* (Zabeau and Stanley, 1982) was used as a recipient for the pMT100 derived shortenings. Plasmid pMT100 and the deletion plasmids, pMT101, pMT102, pMT103 and pMT104, were described previously (Fig. 2.1.). The plasmid pcI857 (Remaut *et al.*, 1983), used in the promoter studies, carries the gene encoding the λ repressor. Plasmids BlueScript SK (Stratagene, San Diego, CA) and pEcoR251 (Zabeau and Stanley, 1982) were the plasmid vectors used. The M13 derived BlueScript phagemid is ideal for generating nested deletions for DNA sequencing, since its polylinker contains 26 unique restriction endonuclease recognition sites designed to enable exonuclease III shortenings from both the 5' and 3' ends of the insert. Plasmid pMT115 contained the *Mlu*1/*Hind*III restriction endonuclease fragment from pMT100 cloned into BlueScript SK, and plasmids pMT116, pMT118, pMT119 and pMT121 contained

exonuclease-III-shortened insert DNA fragments from pMT115. Plasmids pMT106 - pMT111 were derived by subcloning insert DNA fragments of plasmids pMT116 - pMT121 into pEcoR251.

3.2.2 Media and metronidazole susceptibility assays

E. coli strains were grown aerobically at 37°C in YT media supplemented with ampicillin (100 µg/ml) and metronidazole (0-1000 µg/ml) as described in the text. The determination of the MIC for metronidazole was done as described in Chapter 2. The cultures used in the λ promoter studies were grown at 30°C, since the plasmid pcI857 contains the temperature sensitive mutant *c*I gene that encodes the λ repressor.

3.2.3 Cell-free transcription and translation

The prokaryotic transcription/translation kit (no. N380; Amersham International, Amersham, England) was used, according to the manufacturer's instructions, in an attempt to produce proteins from the cloned *Bacteroides* insert DNA on pMT100.

3.2.4 T7 promoter-directed expression system

The method was adapted from Tabor and Richardson (1985) and Scholz *et al* (1989). Synthesis of proteins *in vivo* from the *Bacteroides* DNA insert in *E. coli* was investigated to associate a protein with the observed phenotype. *E. coli* AB1157 cells were transformed with both pGP1-2 (Tabor and Richardson, 1985), a plasmid carrying the T7 RNA polymerase structural gene under the control of the temperature-inducible bacteriophage λ expression system, and a selected BlueScript SK plasmid derivative, with the gene of interest under the control of the T7 promoter. The *E. coli* AB1157 transformants were maintained on media containing kanamycin (50 µg/ml) and ampicillin (100 µg/ml). The *E. coli* strain needs to be *rpo*$^+$, since *rpo* mutants are resistant to rifampicin. *E. coli* AB1157 is *rpo*$^+$. Stationary-phase cultures were diluted 1/50 into supplemented M9 medium (Appendix) and grown at 30°C to an early logarithmic phase (A_{600} = 0.2-0.4). Cultures were then heated to 42°C for 20 min to induce the production of the rifampicin resistant T7 RNA polymerase. Rifampicin (200 µg/ml) was added, and after an additional 15 min at 42°C the cells were grown for 20 min at 30°C. A 5 ml sample of each culture was then pulse-labeled with 1 µl [^{35}S] methionine (10 µCi) for 5 min at 30°C. The labeled cells were collected by centrifugation,

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resuspended in 30 µl of sample treatment buffer (Appendix), heated to 95°C for 3 min and loaded onto a 15% SDS-PAGE gel.

3.2.5 Exonuclease shortening

To facilitate nucleotide sequencing, the *Mlu*I-*Hind*III restriction endonuclease fragment of pMT100 was subcloned into the *EcoR*V-*Hind*III sites of BlueScript SK to produce pMT115. Exonuclease III deletions were generated by using a modified method of Henikoff (1984) as described in the Appendix. Progressive deletions from the 5' and 3' ends of the insert DNA were generated by unidirectionally digesting *BstX*1-*Xba*1 and *Apa*1-*Xho*1 restriction endonuclease fragments of pMT115 with endonuclease III. A range of nested deletions spanning the entire insert was chosen for nucleotide sequencing.

3.2.6 Nucleotide sequencing

The nucleotide sequence of both strands of the pMT115 insert DNA was determined using the dideoxynucleotide triphosphate chain termination method of Sanger *et al.* (1977), according to the protocol of Tabor and Richardson (1987), using the Sequenase DNA Sequencing kit (US Biochemical Corporation, Cleveland, OH) (Appendix).

The DNA and deduced amino acid sequences were analyzed on a VAX 6000-330 computer using the Genetics Computer Group Inc. suite of sequence analysis programs (Devereux *et al.*, 1984).

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3.3 Results

3.3.1 Analysis of the pMT100 promoter

Studies were carried out to determine whether expression of the gene(s) encoding increased metronidazole resistance and UV sensitivity in *E. coli* AB1157 was controlled from a promoter within the insert DNA fragment or by the λ rightward promoter of the cloning vector pEcoR251. Attempts to subclone the insert DNA fragment of pMT100 in the opposite orientation with respect to the λ rightward promoter in pEcoR251 were unsuccessful. The metronidazole resistance of *E. coli* AB1157 (pMT100), and *E. coli* AB1157 transformed with the deletion plasmids pMT101, pMT102, pMT103 and pMT104 (Fig. 2.1.), and the UV sensitivity of *E. coli* AB1157 (pMT100) and *E. coli* AB1157 (pMT104), were

therefore investigated in the presence and absence of the plasmid pcI857. Plasmid pcI857 carries the gene encoding the λ repressor (Remaut *et al.*, 1983). The presence of pcI857 repressed the increased metronidazole resistance phenotype in *E. coli* AB1157 (pMT100) and *E. coli* AB1157 transformed with the deletion plasmids pMT101 and pMT102, and the increased UV sensitivity phenotype in *E. coli* AB1157 (pMT100) (Table 3.1., Fig. 3.1.). The phenotypes of *E. coli* AB1157 transformed with pMT103 and pMT104 were not affected by the presence of pcI857, since the plasmids did not contain the loci conferring metronidazole resistance and UV sensitivity (Table 3.1., Fig. 3.1.). It was concluded that the gene(s) on pMT100 was expressed from the λ promoter of pEcoR251 in *E. coli* AB1157 (pMT100) cells.

3.3.2 Nucleotide sequencing and functional analysis of ORF1

A range of nested deletions, generated from the plasmid pMT115, was chosen for nucleotide sequencing from exonuclease-III derived shortenings spanning the entire insert (Fig. 3.2.). The 1.6 kb nucleotide sequence contained several small open reading frames (ORF). A smaller DNA region encoding metronidazole resistance, therefore, had to be defined. The exonuclease-III-shortened insert DNA fragments (pMT116 to pMT121) were subcloned back into pEcoR251 to make use of the λ promoter for expression studies (Fig. 3.2.). The resulting plasmids (pMT106 to pMT111) were transformed into *E. coli* AB1157 and the transformants were tested for their MIC to metronidazole under aerobic conditions. As controls *E. coli* AB1157 transformed with pMT100 and the deletion plasmid pMT104, where most of the insert was deleted (Fig. 2.1.), were used. Only *E. coli* AB1157 (pMT108) and *E. coli* AB1157 (pMT111) showed increased resistance to metronidazole similar to that of *E. coli* AB1157 (pMT100), whereas *E. coli* AB1157 transformed with the other subclones showed levels of metronidazole resistance similar to that of *E. coli* AB1157 (pMT104). This indicated that metronidazole resistance was encoded by gene(s) contained within a 440 bp DNA region. This region contained only one complete open reading frame (ORF1)(Fig. 3.2.).

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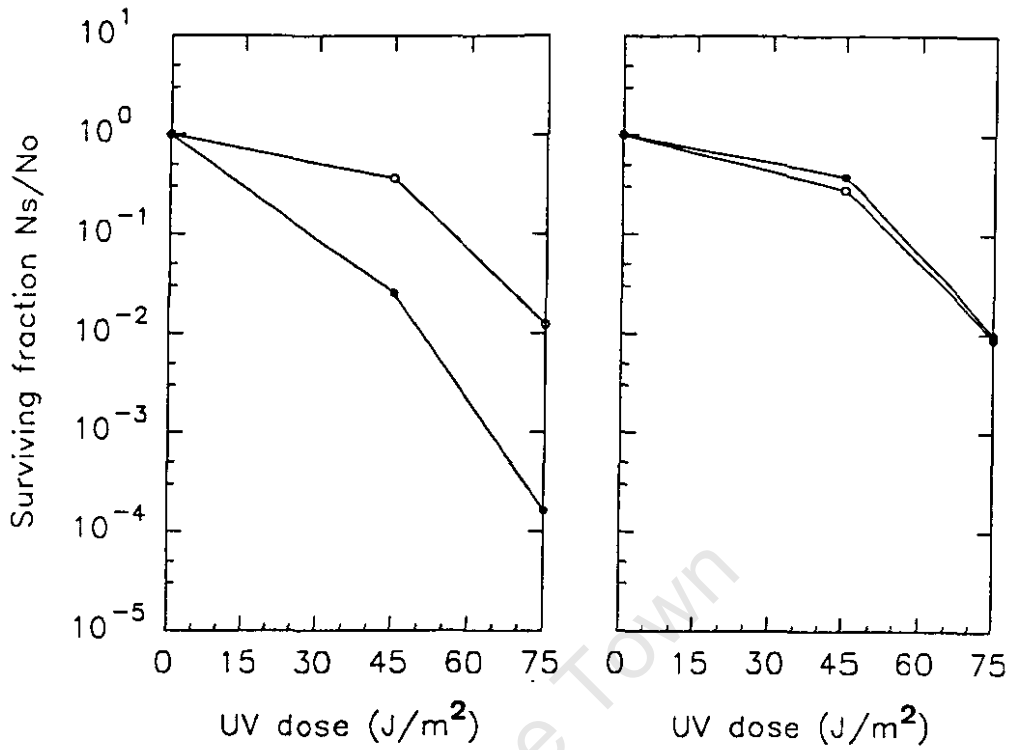

Fig. 3.1. Aerobic UV survival curves of *E. coli* AB1157 transformed with pMT100 (●) or the control plasmid pMT104 (○) with or without the λ repressor plasmid pcI857. The UV survival curve of *E. coli* AB1157 without pcI857 is shown on the left, and *E. coli* AB1157 (pcI857) is shown on the right.

Table 3.1. Susceptibility to metronidazole of *E. coli* AB1157 transformed with pMT100 and its deletion plasmids with or without plasmid pcI857.

E. coli strain	MIC (μg/ml)[a]
AB1157 (pMT100)	800
AB1157 (pMT100+pcI857)	500
AB1157 (pMT101)	800
AB1157 (pMT101+pcI857)	500
AB1157 (pMT102)	800
AB1157 (pMT102+pcI857)	500
AB1157 (pMT103)	500
AB1157 (pMT103+pcI857)	500
AB1157 (pMT104)	500
AB1157 (pMT104+pcI857)	500

[a] The minimal inhibitory concentration (MIC) to metronidazole was determined on LB agar under aerobic conditions.

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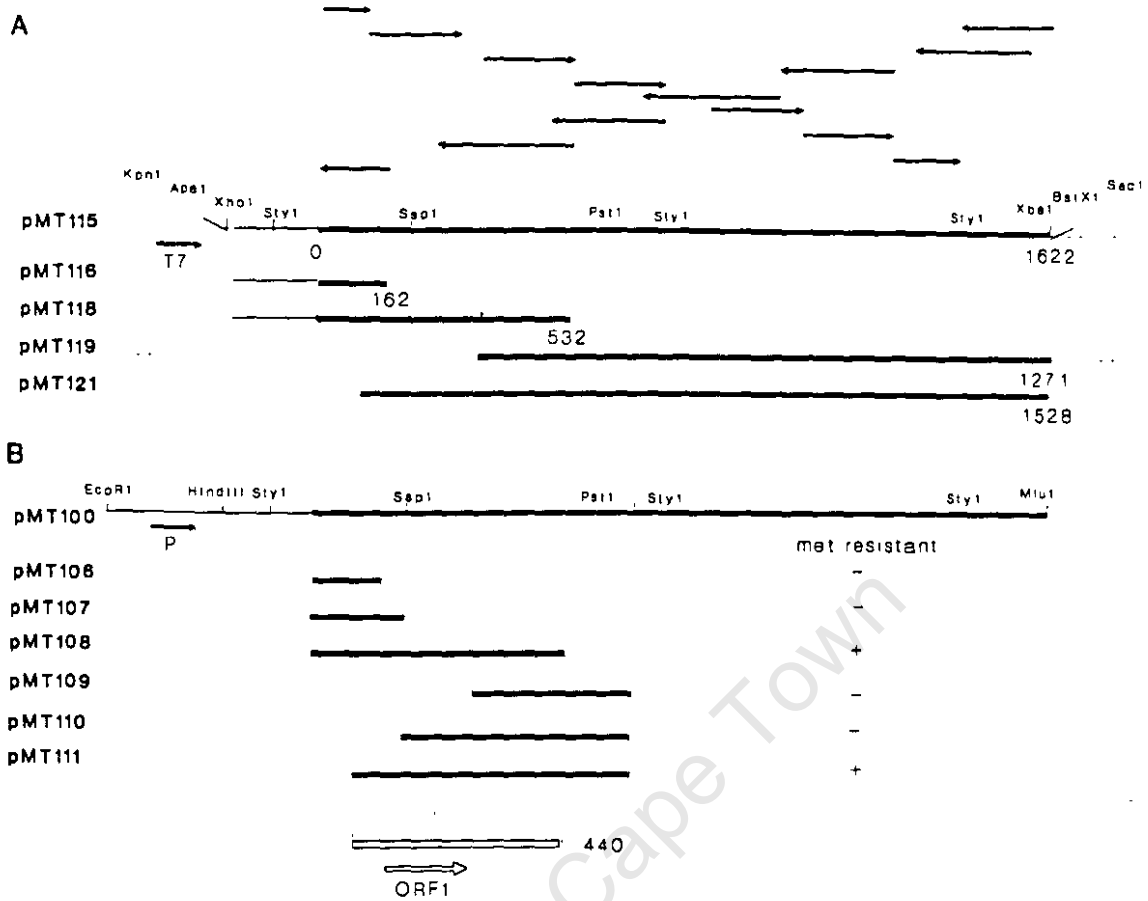

Fig. 3.2. (A) Plasmid pMT115 contained the 1.622 kb metronidazole resitance-encoding *B. fragilis* fragment from pMT100 subcloned into Bluescript SK. The exonuclease III derived shortenings are shown as arrows above the restriction map of pMT115. The arrows indicate the direction and extent of sequence derived from the different nested deletions. Plasmids pMT116, pMT118, pMT119 and pMT121 contain exonuclease-III-shortened DNA fragments of pMT115. The lengths of the *B.fragilis* inserts in nucleotide bases is indicated. The location and direction of transcription of the T7 promoter is indicated ($\overrightarrow{T7}$).
(B) Plasmids pMT106 to pMT111 were derived by subcloning Bluescript SK exonucleaseIII-shortened insert DNA fragments (pMT116 to pMT121) into pEcoR251 adjacent to the lambda rightward promoter ($\overrightarrow{P}$). At the top of the figure the *EcoR*1-*Mlu*1 DNA fragment of pMT100 is shown for comparison. The metronidazole resistance phenotypes conferred in *E. coli* AB1157 by pMT106 to pMT111 are indicated: +, metronidazole resistance; -, parental level of metronidazole sensitivity. The 440 bp region containing ORF1 (□) is shown. Bluescript SK DNA (....); pEcoR251 DNA (—); *B. fragilis* DNA (■).

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3.3.3 Sequence analysis

Translation of ORF1 was initiated by a potential ATG start codon and terminated by a TAG stop codon. The open reading frame was comprised of 195 bp which can encode a protein of 64 amino acids (Fig. 3.3.). The 440-bp DNA sequence encoding metronidazole resistance has been assigned the GenBank DNA sequence database accession number M76551.

```
                                GGGTTTACTTGGTCGATGTTTACGATAAGGCGGACTATTCTACTG

TAGATGTGTCAGCCGTTAAGAAGATGATAGCAGGGTTGGATAACTATGATTAAATCGTT

ATG GTA GAA TAT TGT GTT TAC TGG TTA GAG GAC GGA GAG CCC GTG
 M   V   E   Y   C   V   Y   W   L   E   D   G   E   P   V

CAC GAG GTG TTT TCT TCT CTT GCC GCC GCC GAG ATG TTC TCA TGT
 H   E   V   F   S   S   L   A   A   A   E   M   F   S   C

GCG ATA AGA GGG AAA GAA AAC GTT GAA TGG GTG GAA GTG TCC GAA
 A   I   R   G   K   E   N   V   E   W   V   E   V   S   E

GAA GAA GCC ATT GAC CTT GAC GAA CTG GAA GCC ATG TTT CCC GGT
 E   E   A   I   D   L   D   E   L   E   A   M   F   P   G

GAT TTC AAG CTG TAG  ATTTTCTCTTTTCCGGTAGCAAAGATAACTCCATACT
 D   F   K   L   *

CTGGAACCGGACTTGCGAAGCCCTTGACGGTTCCGGAGTATGGAGTTGCGAGGAAGGC

ATGGAAAAGAGAAAATCAGTGTGTGCCTATGCAGCGTGTTACCGTG
```

Fig. 3.3. Nucleotide sequence of the 440 bp DNA region of pMT115 containing ORF1. The deduced amino acid sequence for ORF1 is given in single letter code below the nucleotide sequence. The inverted repeat sequences are underlined.

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In *E. coli* the ribosome binding site consists of a start codon and a purine rich sequence 4-15 bases upstream of the start (Shine and Dalgarno, 1974). The start codon is usually ATG, but other start codons, GTG or TTG, are occasionally identified (Kozak, 1983). No typical ribosome binding site could be detected upstream of ORF1. A 5'-ATGA-3' sequence was located 10 bases upstream of the ATG start. An alternative GTG start, located 42 bp downstream of the ORF1 ATG start codon, has a potential ribosome binding site (5'-GGAG-3') located 9 bp upstream. The resulting ORF would encode a protein smaller than that detected by SDS PAGE after T7 promoter-directed expression. In addition, computer analysis using TestCode predicted a coding region the size of ORF1 (Fig. 3.4.).

Ribosome binding sites in *Bacteroides*, based on 16S rRNA sequences (5'-AGGAAAG-3'), have been identified and compared to those of *E. coli* (5'-GGAGGAA-3') (Weisburg *et al.*, 1985). *E. coli* consensus ribosome binding sites have not been detected in the following *B. fragilis* genes: *recA*, 5'-ACAGG-3' 10 bases upstream (Goodman and Woods, 1990); *glnA*, 5'-AAAAGAGA-3' 15 bases upstream (Hill *et al.*, 1989); *scrL*, 5'-CCGG-3' 5 bases upstream (Blatch and Woods, Dept. of Microbiology, U.C.T., personal communication) of the start codon. It was perhaps not surprising that no typical ribosome binding site was found upstream of ORF1.

The termination of transcription in *E. coli* can be either factor dependent or factor independent. The factor dependent terminators require a factor, such as the Rho-protein (Roberts, 1969), and are not readily identified by a consensus sequence. The Rho-independent terminators are characterized by a G + C-rich region of dyad symmetry followed by a T-rich sequence. A typical hairpin stem is 6-10 base pairs long with a loop of 4-6 bases (Brendel and Trifonov, 1984). No typical factor independent terminator was identified downstream of ORF1.

Further analysis of the sequence containing ORF1 showed a potential secondary structure downstream of ORF1, starting at the last nucleotide of the ORF1 stop codon. It consisted of a 47 bp inverted repeat, containing a 14 bp perfect repeat, a 12 bp intervening region followed by a 20 bp near perfect repeat (19/20), and a 18 base open loop (Fig. 3.3.). An analysis of plasmid features, such as the origins of replication (*oriV*) or the incompatibility loci (*inc*), was undertaken since the insert DNA on pMT100 originated from a *Bacteroides* plasmid. Plasmid *oriV*'s characteristically contain more than one repeated sequence, e.g. 13 bp repeated

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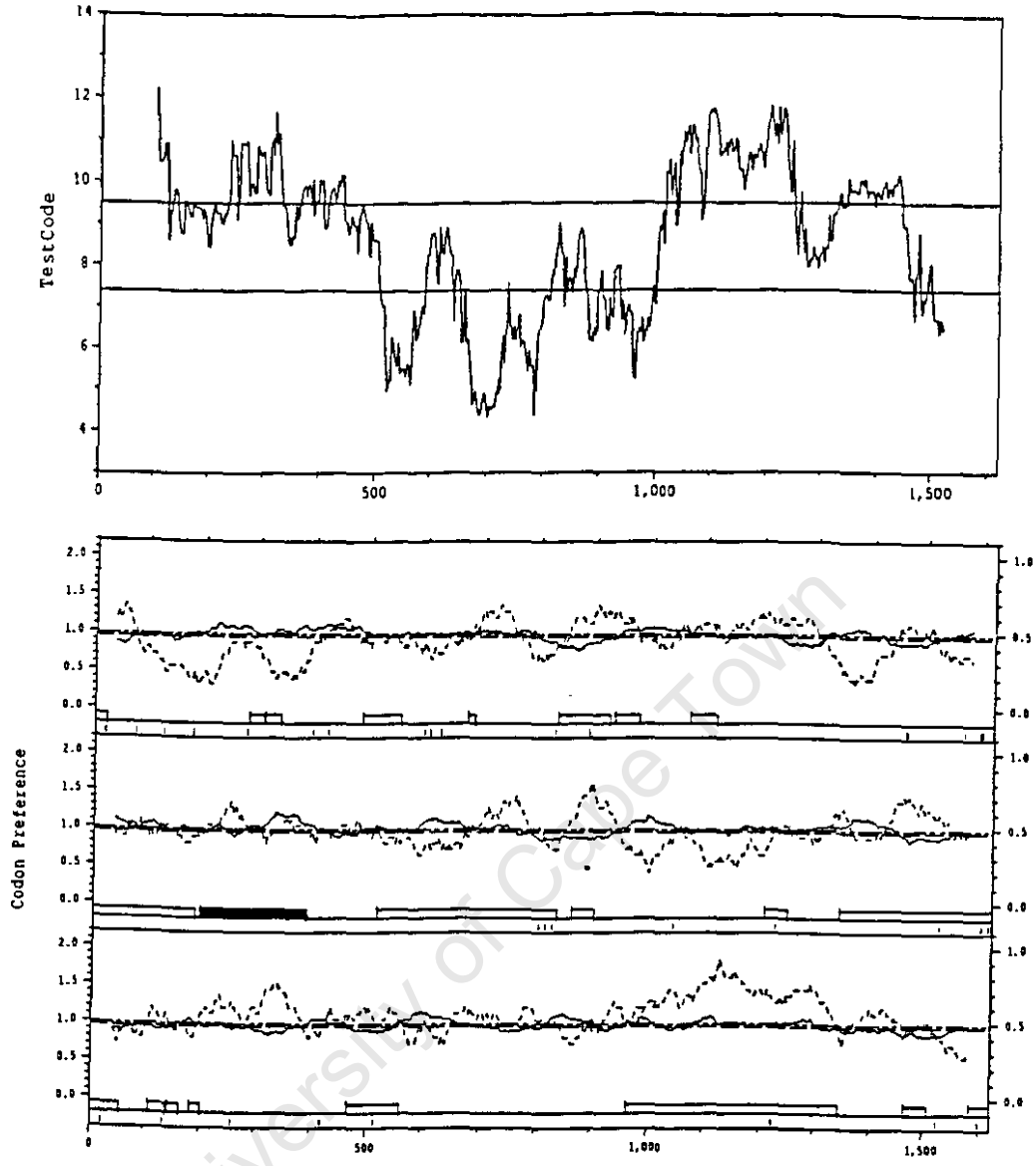

Fig. 3.4. The results from the computer programs TestCode and CodonPreference for the three forward reading frames of pMT100. All possible open reading frames are shown as boxes, with the start codons shown as short lines that extend above the height of the box, and stop codons are marked by lines that extend below the bottom of the box. The box representing ORF1 is filled in.

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twice in plasmid pSC101 (Yamaguchi and Yamaguchi, 1984) and five 17 bp repeats separated by 22-23 bp spacer sequences in plasmid RK2 (Stalker *et al.*, 1981). The *inc* locus of pSC101 contained three 18 bp repeats believed to be involved in the binding of the RepA protein (Vocke and Bastia, 1983). No sequence similarity to other plasmid *oriV* or *inc* regions was found in the pMT100 insert DNA repeats.

Insertion (IS) elements and transposons are generally characterized by a pair of inverted repeats of 16-41 base pairs flanking a coding region for one or more proteins (Freifelder, 1985). The *Bacteroides* R plasmid pBF4 contained the gene encoding resistance to macrolide, lincosamide and streptogramin B flanked by two copies of the IS*4351* element (Rasmussen *et al.*, 1986). Another *Bacteroides* R plasmid, pBI136, containes the clindamycin resistance transposon Tn*4551* (Smith and Spiegel, 1987). A single copy of the 1.2 kb repeat sequence, which flanks the transposon Tn*4551*, as well as the entire transposon, can insert in the *E. coli* chromosome (Smith and Spiegel, 1987). There was no functional evidence that pMT100 contained an insertion element, except that pMT100 showed some instability in *E. coli* cells (Chapter 2).

A *B. fragilis* gene which is controlled by repeated sequences has been described. Two direct repeats of 45 and 46 bp, which are involved in the regulation of transcription of the *glnA* gene, have been detected upstream of the *B. fragilis glnA* start codon (Hill *et al.*, 1989; Abratt *et al.*, 1992). The significance of the repeat sequence in the pMT100 insert DNA is, however, not known.

3.3.4 Computer analysis of the DNA sequence

The programs TestCode and CodonPreference (Devereux *et al.*, 1984) were used to obtain further evidence for the presence of a functional ORF1. The codon frequency table used in CodonPreference was compiled from the sequence data of the following twelve *B. fragilis* genes: conjugal transfer genes *btgA* and *btgB* (Hecht *et al.*, 1991), β-lactamase *ccrA* (Rasmussen *et al.*, 1990), methyltransferase *ermFU* (Halula *et al.*, 1991), ORF of IS*942* (Rasmussen and Kovacs, 1991), neuraminidase *nanH* (Russo *et al.*, 1990), levanase *scrL* (Blatch and Woods, Dept. of Microbiology, U.C.T., personal communication), *ermF* on Tn*4351* (Rasmussen *et al.*, 1987), ATP synthase β-subunit (Amann *et al.*, 1988), *cfiA* (Thompson and Malamy, 1990), glutamine synthetase *glnA* (Hill *et al.*, 1989) and *recA* (Goodman and Woods, 1990). The output from both programs suggest the presence of a coding region in the area of ORF1 (Fig. 3.4.).

The deduced amino acid sequence (64 aa) of ORF1 was used to search the GenBank, EMBL, SWISS-Protein, NBRF-nucleic and NBRF-Protein databases for related amino acid sequences using the FastA and TFastA computer programs.

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Only limited similarity to short regions of a number of proteins was found (Table 3.2.).

Table 3.2. Similarities of the ORF1 gene product to sequences in the database (the sequences were aligned using the program BestFit).

Gene product/reference	% Identity[a]	% Similarity[b]
E. coli nupG gene (Westh Hansen *et al.*, 1987)	31	56
Saccharomyces cerevisiae phe-tRNA synthetase (Sanni *et al.*, 1988)	24	56
Drosophila melanogaster Adh-s gene (Benyajati *et al.*, 1981)	21	44
IS*986* hypothetical 8.2 kD protein (McAdam *et al.*, 1990)	19	31

[a] Percentage identity and similarity to the entire ORF1 protein.

[b] The percentage similar amino acids were obtained by conservative substitutions.

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The analysis of similar proteins was of limited significance since the ORF1 gene product was considerably smaller than the proteins to which it was compared. The only exception was the small IS*986* hypothetical 8.2 kD protein, but the percentage similarity to ORF1 was low.

The homologous regions identified above were analyzed for the presence of a consensus sequence with associated functionality, such as a helix-turn-helix DNA binding domain (Dodd and Egan, 1987). No such consensus sequences could be identified in ORF1.

3.3.5 Production of proteins from the insert DNA of pMT100

The synthesis of proteins from the insert in pMT100 was investigated using a cell-free prokaryotic transcription and translation system, and the T7 promoter directed expression system of Tabor and Richardson (1985). The *in vitro* system

produced non-specific protein bands that could not be associated with the increased resistance to metronidazole of *E. coli* AB1157 (results not shown). The T7 promoter directed expression system, which is an *in vivo* system designed to express only plasmid-born genes, was therefore employed.

The following constructs were transformed into *E. coli* AB1157 and used for the T7 promoter directed expression system: Bluescript SK, pMT116 and pMT119 both deleted for ORF1, and pMT118 and pMT121 both containing ORF1 (Fig. 3.2.). Under aerobic conditions *E. coli* AB1157 (pMT118) and *E. coli* AB1157 (pMT121) produced a pronounced protein band with an apparent M_r of approximately 8 kDa, and a less intense protein band with an apparent M_r of approximately 10 kDa (Fig. 3.5.). These proteins were not produced by *E. coli* AB1157 (pMT116) or *E. coli* AB1157 (pMT119). Although the insert DNA of pMT118 has the coding capacity for only ORF1 it produced two proteins with apparent M_r of 8 and 10 kDa. It is possible that in *E. coli* AB1157 the 8 kDa protein is a processed or degradation product of the 10 kDa protein. The predicted M_r of the protein produced by ORF1 is 7.3 kDa. Although this is less than the 10 kDa protein observed on the SDS-PAGE gels it is within the range of accuracy of the gel techniques. These results indicated that ORF1 encoded a protein in *E. coli* cells. This protein with an apparent M_r of approximately 10 kDa appeared to be responsible for increased resistance to metronidazole in *E. coli* AB1157.

The results from the TestCode analysis predicted a second ORF downstream of ORF1 (Fig. 3.4.) which encoded a protein with a predicted M_r of 14.7 kDa. A protein with an approximate M_r of 16 kDa was produced in *E. coli* AB1157 (pMT119) and *E. coli* AB1157 (pMT121), but not in *E. coli* AB1157 (pMT116) or *E. coli* AB1157 (pMT118) (Fig. 3.5.). The presence or absence of ORF2 had no effect on the metronidazole phenotype of the cells. *E. coli* AB1157 transformed with Bluescript SK produced neither the 8-10 kDa nor the 16 kDa proteins.

3.4 Discussion

The region of *B. fragilis* Bf-2 DNA on the recombinant plasmid pMT100 encoding increased metronidazole resistance in *E. coli* AB1157 was sequenced. An open reading frame of 195 bp encoded a 64-residue polypeptide. The DNA locus,

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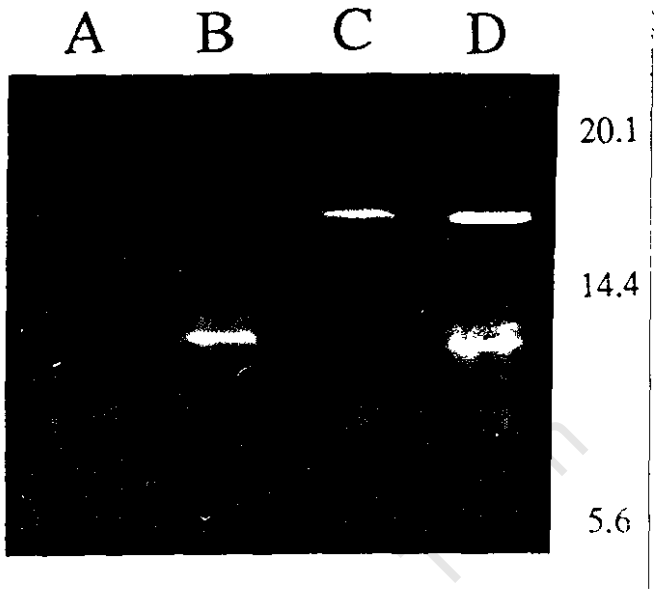

Fig. 3.5. T7 promoter directed expression of the ORF1 protein. The lanes A to D on the radiograph of the polyacrylamide gel contained cell extracts of the following: (A) *E. coli* AB1157 (pMT116), (B) *E. coli* AB1157 (pMT118), (C) *E. coli* AB1157 (pMT119) and (D) *E. coli* AB1157 (pMT121). The positions of the proteins encoded by ORF1 and ORF2 are indicated by arrows. Approximate M_r are indicated in kDa.

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contained within 440 nucleotides, conferred the metronidazole resistance phenotype and expressed a protein from the λ promoter of pEcoR251 with an apparent M_r of 8-10 kDa.

A search through the nucleic acid and protein sequence databases revealed sequences with limited similarity to those of ORF1. These were analyzed further. It needs to be emphasised that the ORF1 gene product is much smaller than most of the proteins to which it was compared. No known functional domains in the sequence containing ORF1 could be identified by comparison with consensus sequences. It is possible, however, that consensus sequences for the functional domains in the published sequence data have not been identified.

The *nupG* encoded protein, to which the ORF1 protein showed 31% identity over the length of ORF1, is part of the *E. coli* nucleoside transport system (Westh

Hansen *et al.*, 1987). The similarity was found towards the amino terminal region of the 418 aa *nupG* protein. The *nupG* system is known to transport all nucleosides and to be regulated by the *cytR* and *deoR* genes (Westh Hansen *et al.*, 1987). Another member of the nucleoside transport system and the CytR/DeoR regulon is the *tsx* gene (Bremer *et al.*, 1988). The *tsx* gene encodes a channel-forming outer-membrane-protein, which is required for the transport of nucleosides when only low concentrations of nucleosides are available (Bremer *et al.*, 1990; Hantke, 1976). The *E. coli* AB1157 strain, which showed an increase in resistance to metronidazole when transformed with the ORF1-containing plasmid pMT100, was mutated for *tsx* (Chapter 2). It is possible that the ORF1-encoded protein functionally complemented this mutation in *E. coli* AB1157. The increased availability of nucleosides could then facilitate the more efficient repair of DNA lesions caused by metronidazole. This theory fails to explain the increase in sensitivity to another DNA damaging agent, far UV irradiation, in *E. coli* AB1157 (pMT100) (Chapter 2).

The ORF1 gene product showed 24% identity to the yeast phenylalanyl-tRNA synthetase α subunit (Sanni *et al.*, 1988). The similarity was found towards the carboxy terminus of the 595 aa synthetase protein, which included the proposed ATP binding motif 'QIGH' (Sanni *et al.*, 1988). The homology of the ORF1 sequence to this motif, however, was low (only two amino acids were similar over the four amino acid sequence).

A 21% identity was shown between the ORF1 protein and the alcohol dehydrogenase (Adh) of *Drosophila* (Benyajati *et al.*, 1981). The similarity was found towards the carboxy terminus of the 256 aa dehydrogenase, which contained the putative catalytic domain of the enzyme. Alcohol dehydrogenases catalyze the reversible oxidation of alcohol to the corresponding aldehyde or ketone with the reduction of a pyridine nucleotide. Some *adh* genes were found to be induced by anaerobiosis (Dolferus *et al.*, 1985). It is theoretically possible that alcohol dehydrogenase could reduce metronidazole to its toxic intermediate. It has been shown that a range of different enzymes are capable of reducing metronidazole to the active compound, for example: hydrogenase (Church *et al.*, 1988), pyruvate-ferredoxin oxidoreductase (Narikawa, 1986), nitrate reductase (Yeung *et al.*, 1984) and flavodoxin (Santangelo *et al.*, 1991). If the ORF1 encoded protein had a reducing potential, it would explain the increase in sensitivity to metronidazole of the nitrate reductase deficient *E. coli* F19 when transformed

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with pMT100 (Chapter 2). This theory does not explain the increase in resistance to metronidazole in *E. coli* AB1157 when transformed with pMT100.

The ORF1 protein showed a 19% identity to the IS*986* hypothetical protein (McAdam *et al.*, 1990). Even though the similarity was very low, it was significant because the IS*986* protein (8.2 kD) was the only one of similar size to the ORF1 protein (7.4 kD). The IS*986* element was isolated from *Mycobacterium tuberculosis* and found to be related to insertion elements of the IS3 family from the Enterobacteriaceae (McAdam *et al.*, 1990). No attempt was made by the authors to assign a function to the hypothetical protein. Some indications of the presence of an insertion element in pMT100 exist, even though no functional evidence exists. A 46 bp inverted repeat region was detected downstream of ORF1, pMT100 was unstable in *E. coli* cells and pBFC1, the origin of pMT100, was demonstrated to exist in two forms in *B. fragilis* (Chapter 2).

This analysis of possible functional domains in proteins similar to the ORF1 gene product gave no definite answer as to the function of the ORF1 protein, although it gave rise to several theories that could explain some of the phenotypes observed in *E. coli* cells transformed with pMT100.

Using the T7 promoter directed expression system, pronounced protein bands that could be associated with the increased resistance to metronidazole in *E. coli* AB1157 were produced. In contrast, the cell-free transcription/translation system did not produce specific proteins. An *in vitro* system lacks the regulatory mechanisms of a living cell, and it is therefore possible that spurious promoters and start codons were recognized, which would explain the non-specific protein bands observed. The prokaryotic transcription and translation kit also utilizes the cell-free extract of a specific *E. coli* strain (MRE600). The gene on pMT100 had different effects on different *E. coli* strains depending on their genotypes (Chapter 2) and it was therefore conceivable that the regulation of the production of proteins from pMT100 was also affected by the genotype of the cell.

It is difficult to believe that several different functions, such as increasing or decreasing the cell's tolerance to metronidazole and decreasing the cell's tolerance to UV irradiation, can be attributed to only one small polypeptide coded for by ORF1. It was therefore proposed that the pMT100 gene product functioned in conjunction with the cell's DNA repair machinery. *E. coli* strains

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with mutations of different DNA repair enzymes were therefore transformed with pMT100 to elucidate the effect of pMT100 on DNA repair (Chapter 4).

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Chapter 4
Effect of the cloned *B. fragilis* plasmid gene on metronidazole susceptibility and UV sensitivity in *E. coli* DNA repair mutants and plasmid-free *Bacteroides* strains

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Chapter 4

Effect of the cloned *B. fragilis* plasmid gene on metronidazole susceptibility and UV sensitivity in *E. coli* DNA repair mutants and plasmid-free *Bacteroides* strains

4.0 Abstract

A cloned gene from the naturally occuring plasmid pBFC1 from *B. fragilis* Bf-2 affected the DNA repair systems of *E. coli* strains. Susceptibility to metronidazole, an antibiotic agent which causes DNA damage when it is activated by reduction, was used as an indication of the repair capacity of the *E. coli* cells. The gene on the recombinant plasmid pMT100 encoded increased resistance to metronidazole in *E. coli* strains with intact *recA* and *recBC* systems under aerobic and anaerobic conditions. It conferred increased sensitivity to metronidazole in *E. coli* strains with mutations in either the *recA* or the *recBC* genes only under anaerobic conditions. In combination with the functional RecE pathway, the recombinant plasmid conferred increased sensitivity to metronidazole in *E. coli* strains under both aerobic and anaerobic conditions.

The gene on pMT100 also encoded increased sensitivity under aerobic and anaerobic conditions to another DNA damaging agent, far UV irradiation, in *E. coli* strains which were either repair proficient, *recE*$^+$ (*sbcA*) strains, or *uvrA*, *uvrB* or *uvrC* mutants.

The pBFC1 gene, under the control of the λ promoter, was subcloned into the *Bacteroides-E. coli* shuttle vector, pVAL-1, and introduced into plasmid-free *Bacteroides* strains. This construct did not affect the susceptibility of the *Bacteroides* strains to metronidazole.

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4.1 Introduction

E. coli strains transformed with the recombinant plasmid pMT100 showed either an increase in resistance or an increase in sensitivity to metronidazole, depending on the *E. coli* strain used (Chapter 2). These results suggested that the observed phenotype may depend on the genotype of the *E. coli* strain, and that the pMT100 gene product may act in conjunction with the DNA repair machinery of the cell. A selection of *E. coli* strains, mutated in different DNA repair genes, was therefore transformed with pMT100 and tested for susceptibility to metronidazole. The DNA repair genes analyzed were part of the excision repair system (*uvrA*, *uvrB*, *uvrC*) or part of the recombinational repair system (*recA*, *recBC*, *recE*). The metronidazole assays were performed under both aerobic and anaerobic conditions, because the insert DNA of pMT100 originated from an obligate anaerobic bacterium (Chapter 2) and metronidazole needs reducing conditions to be activated (Mueller, 1983).

The *E. coli* AB1157 and *E. coli* AB1886 *uvrA* strains also showed an increase in sensitivity to another DNA damaging agent, far ultraviolet irradiation, after transformation with pMT100 (Chapter 2). *E. coli uvrB* and *uvrC* mutant strains were therefore used in UV survival assays to examine the effect of the pMT100 encoded gene product on the excision repair system. In an attempt to study the effect of the pMT100 gene product on *E. coli recE*+ strains, the survival of an *E. coli sbcA* (pMT100) strain after far UV irradiation was determined.

The *B. fragilis* Bf-2 strain carrying pBFC1 was shown to be more resistant to metronidazole than two plasmid-free *Bacteroides* strains (Chapter 2). This suggests a possible role for plasmid-borne genes in metronidazole susceptibility. Since pBFC1 was the origin of the pMT100 insert DNA, the effect of the insert DNA fragment of pMT100 on two plasmid-free *Bacteroides* strains was investigated. This study was considered important, because the function of the pMT100 insert DNA encoded gene had only been assessed in a heterologous host.

4.2 Materials and methods

Commonly used materials and methods are described in the Appendix.

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4.2.1 Bacterial strains

The *B. fragilis* Bf-2 (Mossie *et al.*, 1979), *B. fragilis* 638 (Privitera *et al.*, 1979) and *B. thetaiotaomicron* (ATCC 29148) strains were used in the metronidazole assays and in the mating experiments. The following *E. coli* strains were used: *E. coli* TK603 (Kato and Shinoura, 1977), *E. coli* AB1157 (ATCC 29055), *E. coli* AB1886/5/4 (Howard-Flanders *et al.*, 1966), *E. coli* JC5519 (A.J. Clark), *E. coli* JC8679 (ATCC 47001), *E. coli* CC118 (Manoil and Beckwith, 1985), *E. coli* F19/F19* (Santangelo *et al.*, 1991), *E. coli* HB101 (Boyer and Roulland-Dussoix, 1969), *E. coli* DK1 (ATCC 35691) and *E. coli* C600 (Appleyard, 1954). Full genotypes are listed in the Appendix and the genotypes relevant to this chapter are shown in Table 4.1.

4.2.2 Media and growth conditions

Bacteroides strains were grown anaerobically at 37°C in supplemented brain heart infusion broth (BHI) (Holdeman and Moore, 1972), or on trypticase yeast glucose (TYG) medium. *E. coli* strains were grown at 37°C aerobically on YT medium and anaerobically on prereduced YT+g+n medium (Chapter 2). Agar was added to these media at a concentration of 1.5 % (w/v) when necessary. A solution of sterile NaCl (0.03 M) was used as a dilution buffer. Anaerobic experiments were carried out under stringent anaerobic conditions as described in Chapter 2.

4.2.3 Plasmids

The plasmid pMT100, containing the *B. fragilis* insert DNA, and its deletion derivative pMT104 were described in Chapter 2. The low-copy-number vector pACYC184 (Chang and Cohen, 1978), which carries the genes encoding chloramphenicol and tetracycline resistance, is compatible with ColE1 derived plasmids, such as pEcoR251 (Zabeau and Stanley, 1982). The plasmid pRAC3, encoding a functional *recE* gene, was a gift from A.J. Clark (Willis *et al.*, 1985; Luisi-DeLuca *et al.*, 1988). The *E. coli-Bacteroides* shuttle vector pVAL-1 contains the tetracycline and ampicillin resistance genes for selection in *E. coli*, the erythromycin/clindamycin resistance gene for selection in *Bacteroides*, the origin of replication of the *E. coli* vector pBR328, and the rep (replication) and mob (mobilizable) regions from the cryptic *Bacteroides* plasmid, pB8-51, (Valentine *et al.*, 1988). The shuttle vector was mobilized into *Bacteroides* by tra (transfer) functions encoded by the broad-host-range self-mobilizing incP plasmid R751 (Shoemaker *et al.*, 1986a). The vector pEcoR252 is identical to pEcoR251 (Zabeau

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and Stanley, 1982) except that it contained only the one *Pst*1 restriction endonuclease site in the *EcoR1* gene.

4.2.4 Construction of pM-RAC3

The 5.7 kb *Hind*III restriction endonuclease fragment from the plasmid, pRAC3, encoding a truncated, active exo VIII of *E. coli* (Chu *et al.*, 1989), was subcloned into the *Hind*III restriction endonuclease site in the tetracycline resistance gene of vector pACYC184. The resulting plasmid was transformed into *E. coli* JC5519 *recBC* and selected on chloramphenicol (20 µg/ml) and metronidazole (400 µg/ml). The plasmid construct was designated pM-RAC3.

4.2.5 Construction of pM-VAL

The 1.3 kb *EcoR*1-*Pst*1 restriction endonuclease fragment from pMT100, which contained the ORF1 encoding region and the λ promoter from pEcoR251, was subcloned into the *EcoR*1-*Pst*1 restriction endonuclease-digested pVAL-1. The resulting construct, pM-VAL, was selected for in *E. coli* by tetracycline resistance (10 µg/ml) and ampicillin sensitivity, since the ampicillin gene was disrupted by the cloning event. The plasmid pM-VAL was constructed to include the λ promoter of pEcoR251, since it was shown that in *E. coli* the gene on pMT100 was expressed from the λ promoter (Chapter 3).

4.2.6 Construction of pMT130

A pMT100 derivative, pMT130, was constructed in an attempt to reduce the instability of pMT100. pMT130 contained the 0.8 kb *Sty*1/*Pst*1 restriction endonuclease fragment from pMT100 including ORF1, subcloned into pEcoR252 in the same orientation with respect to the λ promoter as pMT100.

4.2.7 Metronidazole assays

The MIC of the *E. coli* strains to metronidazole was determined on YT (aerobic) and YT+g+n (anaerobic) agar plates as described in Chapter 2. *Bacteroides* strains were grown on BHI for the metronidazole assays (Chapter 2).

4.2.8 Far UV irradiation

UV irradiation of *E. coli* cells was carried out as previously described (Jones and Woods, 1981). Irradiation was performed under aerobic or strict anaerobic conditions, and the irradiated *E. coli* cells were plated onto YT (aerobic) and YT+g+n (anaerobic) agar plates supplemented with ampicillin (100 µg/ml).

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4.2.9 Conjugation of *Bacteroides*

The conjugation method was adapted from Valentine *et al.* (1988). The donor *E. coli* HB101 (pM-VAL), cotransformed with plasmid R751, was grown aerobically to early logarithmic phase (A_{600} = 0.2) in YT broth. *E. coli* HB101 (pVAL-1) was used as a positive control, and individual donor and recipients as negative controls. The recipients, *B. fragilis* 638 and *B. thetaiotaomicron*, were grown synchronously to early logarithmic phase in TYG broth under anaerobic conditions. The bacterial cultures were mixed aerobically at a ratio of 5:1 (1.0 ml recipient to 0.2 ml donor), collected by centrifugation and resuspended in 0.2 ml TYG broth. The concentrated cell mixture was added dropwise onto a 25 mm Millipore HAWP filter disk that had been placed on a TYG agar plate, and incubated aerobically for 16 h. Filters containing the mating mix were washed with 3.0 ml TYG broth, and 0.3 ml aliquots were plated onto TYG agar plates containing erythromycin (10 µg/ml) for selection against the recipient, and gentamycin (200 µg/ml) for selection against the donor. The plates were incubated anaerobically for 2-3 days to obtain transconjugants, which were chosen for further study. A small scale plasmid extraction was carried out on the *Bacteroides* transconjugants. Since the amount of plasmid obtained was too small to analyze directly, this DNA was used to retransform plasmid-free *E. coli* strains. Plasmid DNA extracted from these transformants was analyzed using routine molecular biological techniques.

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4.3 Results and discussion

4.3.1 Metronidazole susceptibility of DNA repair deficient *E. coli* strains transformed with pMT100

The recombinant plasmid pMT100 conferred metronidazole resistance to an *E. coli uvrA* mutant and a *uvr*$^+$ strain under aerobic conditions (Chapter 2). Furthermore, pMT100 also conferred metronidazole sensitivity to *E. coli recA* mutants under anaerobic conditions (Chapter 2). The observation of these conflicting results initiated the study of the effect of pMT100 on the susceptibility to metronidazole of *E. coli* strains mutated in different DNA repair genes. The study of the effect of pMT100 on the repair capabilities of the *E. coli* strains was approached in two ways: analysis was carried out using either a family of *E. coli* strains with identical genotypes except for the mutation of one DNA repair gene,

or using *E. coli* strains that were less closely related but with a similar DNA repair mutation. The results of this study are shown in Table 4.1. The metronidazole assays for every strain were repeated at least three times with similar results.

The results of this analysis will be discussed in four sections: the effect of metronidazole treatment on the survival of different repair deficient *E. coli* strains under aerobic (1) and anaerobic conditions (2), and the effect of metronidazole treatment on the survival of the same repair deficient *E. coli* strains transformed with pMT100 under aerobic (3) and anaerobic conditions (4).

(1) The effect of metronidazole treatment on the survival of *E. coli* strains under aerobic conditions

Several general conclusions about the effect of metronidazole on *E. coli* could be drawn by analyzing the MIC results of *E. coli* (pMT104), since these strains showed the same susceptibility to metronidazole as plasmid-free strains (results not shown).

The *E. coli* strains grown under aerobic conditions were moderately sensitive to metronidazole, even though it had been suggested that aerobically grown cultures were not sensitive to metronidazole (Edwards, 1980). Aerated liquid cultures were shown to be far less sensitive to metronidazole (results not shown), but the plate assay used for the aerobic experiments possibly provided a constant microaerophillic environment within each colony, which enabled the reduction of metronidazole to its active derivative.

E. coli strains with defects in either their *uvrA,B* or C genes (eg. *E. coli* AB1886, MIC 300 µg/ml), the *recA* gene (eg. *E. coli* CC118, MIC 50 µg/ml) or the *recBC* genes (eg. *E. coli* JC5519, MIC 400 µg/ml) were more sensitive to the DNA damage caused by metronidazole than DNA repair wild type strains (eg. *E. coli* AB1157, MIC 750 µg/ml). This supports the conclusions of Yeung *et al.* (1984) and Jackson *et al.* (1984) who showed that *E. coli recA* and *uvr* mutants were more sensitive to DNA damage caused by metronidazole than the DNA repair wild type strains.

Metronidazole must therefore cause DNA damage that is recognized and repaired by the excision repair system, as well as by the recombinational repair

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Table 4.1. Susceptibility of *E. coli* strains to metronidazole under aerobic and anaerobic conditions.

E. coli strain	Relevant genotype[a]							O_2MIC[b]		AnO_2MIC[b]	
	uvrA	*uvrB*	*uvrC*	*recA*	*recBC*	*recE*	*ntr*	pMT104	pMT100	pMT104	pMT100
TK603	-	+	+	+	+	-[c]	+	300	500	140	200
AB1157	+	+	+	+	+	del[c]	+	750	1000	600	800
AB1886	-	+	+	+	+	del	+	300	500	120	120
AB1885	+	-	+	+	+	del	+	300	500	nd[d]	nd
AB1884	+	+	-	+	+	del	+	300	500	nd	nd
JC5519	+	+	+	+	-	del	+	400	400	120	60
JC8679	+	+	+	+	-	+	+	700	500	400	200
CC118	+	+	+	-	+	-	+	50	50	20	5
F19	+	+	+	-	+	-	-	400	400	30	5
HB101	+	+	+	-	+	-	+	25	25	15	5
DK1	+	+	+	-	+	-	+	nd	nd	15	5
F19*	+	+	+	+	+	-	-	>1000	>1000	>800	>800

[a] In addition to the mutations, the relevant functional genes of the *E. coli* strains are listed.

[b] The MIC to metronidazole (μg/ml) of the *E. coli* strains was determined under aerobic conditions (O_2) and anaerobic conditions (AnO_2). The metronidazole assays for every strain were repeated at least three times with similar results. *E. coli* strains transformed with the control deletion plasmid pMT104, which showed metronidazole susceptibility similar to those of the plasmid free *E. coli* strains, were used as controls (Chapter 2).

[c] The *rac* prophage which contains the *recE* gene is deleted in *E. coli* AB1157 and its derivatives. The *recE* gene in other *E. coli* strains is non-functional due to a point mutation (Chu *et al.*, 1989).

[d] not determined; The *E. coli uvrB/C* mutants grew slowly and as irregular sized colonies under anaerobic conditions, making the determination of a MIC difficult.

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system, of which the *uvrA,B,C*, *recA* and *recBC* genes of *E. coli* are a part (Friedberg, 1985). The UvrABC excision repair system recognizes helix distortions, such as those caused by bulky modifications of the bases (Van Houten, 1990). The documented substrates of the enzyme complex do not include strandbreaks (Van Houten, 1990), which are the lesions induced *in vitro* by activated metronidazole (Edwards, 1977). The UvrABC enzyme itself induces incisions as part of its excision repair, and it therefore seems unlikely that it would recognize single strand breaks as substrates. It is conceivable, therefore, that metronidazole induces DNA lesions other than strand breaks *in vivo*.

The UvrABC enzyme complex of *E. coli* has however been implicated, in the repair of hydrogen peroxide lesions (Hagensee and Moses, 1986). Although most of the peroxide induced lesions consist of modified bases, DNA strand breaks were also induced. It is therefore possible that the UvrABC excision repair system of *E. coli* can recognize and repair DNA strand breaks. It can not be excluded that metronidazole induces strandbreaks *in vivo*.

The sensitivity to metronidazole of the *recBC* mutant strain was suppressed by the presence of a functional *recE* gene (compare *E. coli* JC5519, MIC 400 µg/ml, to *E. coli* JC8679, MIC 700 µg/ml). The *E. coli* JC8679 *recE*$^+$ strain, which was constructed by the conjugation of an *E. coli sbcA* strain with *E. coli* JC5519 *recBC*, showed restored recombination proficiency and resistance to UV irradiation as compared to the *recBC* mutant strain (Gillen *et al.*, 1981). The *recE* gene product of *E. coli* JC8679 mediated the efficient repair of DNA damage caused by metronidazole.

The *E. coli recA* mutant strains were up to 30 fold more sensitive to metronidazole than *recA*$^+$ strains (compare *E. coli* HB101, MIC 25 µg/ml, to *E. coli* AB1157, MIC 750 µg/ml). Therefore, the RecA protein appears to be the key regulator of the repair of metronidazole-induced DNA damage. *RecA* mutant strains were shown to spontaneously degrade DNA (Clark, 1973), which is possibly due to the lack of control of the other repair enzymes. The RecA protein regulates the exonuclease activity of the RecBCD repair enzyme (Pollard and Randall, 1973; Brcic-Kostic *et al.*, 1991). The RecA protein also acts at the site of damage by binding directly to the DNA as a long filament, which prevents other enzymes from accessing the site of damage (Cox, 1991).

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(2) The effect of metronidazole treatment on the survival of *E. coli* strains under anaerobic conditions

All the *E. coli* strains were more sensitive to metronidazole under anaerobic conditions than under aerobic conditions. This was expected, since the reduced toxic intermediate of metronidazole is produced more readily under anaerobic conditions (Edwards, 1980).

As was observed under aerobic conditions, the repair mutations reduced the survival of *E. coli* after metronidazole treatment as follows: *recA*> *uvrA*> *recBC*> wild type. The presence of a functional *recE* gene, again, resulted in increased resistance to metronidazole of the *recBC* mutant strain (compare *E. coli* JC5519, MIC 120 μg/ml, to *E. coli* JC8679, MIC 400 μg/ml). *E. coli* wild type strains usually contain a point mutation which disrupts *recE* (Chu *et al.*, 1989).

It was observed that the decrease in survival after metronidazole treatment in the repair mutants compared to the wild type strains was disproportionally high under anaerobic conditions as compared to aerobic conditions. This will be analyzed in detail in Chapter 6 (General conclusion).

(3) The effect of metronidazole treatment on the survival of *E. coli* strains transformed with pMT100 under aerobic conditions

The presence of pMT100 increased resistance to metronidazole in *E. coli* strains that contained both functional *recA* and *recBC* systems (eg. *E. coli* AB1157, MIC_{pMT104} 750 μg/ml; MIC_{pMT100} 1000 μg/ml).

The *E. coli* strains that were mutated in either the *recA* gene (eg. *E. coli* CC118, MIC_{pMT104} 50 μg/ml; MIC_{pMT100} 50 μg/ml) or the *recBC* genes (*E. coli* JC5519, MIC_{pMT104} 400 μg/ml; MIC_{pMT100} 400 μg/ml) showed no change in the metronidazole susceptibility under aerobic conditions after transformation with pMT100. The presence of pMT100 in *E. coli recBC* strains containing a functional *recE* gene decreased survival after metronidazole treatment (*E. coli* JC8679, MIC_{pMT104} 700 μg/ml; MIC_{pMT100} 500 μg/ml). The effect of pMT100 on *recE*+ *E. coli* strains is discussed below (4.3.2).

The MIC results from the *E. coli recBC* and *recA* mutant strains indicate that the pMT100 encoded metronidazole resistance phenotype required both a functional *recA* and a functional *recBC* system under aerobic conditions. These results

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suggested that the pMT100 gene product enhanced the way in which the RecA protein together with the RecBC protein repaired metronidazole-induced DNA damage. If either of the two enzymes were absent, the pMT100 gene product did not affect survival of the *E. coli* strains after metronidazole damage under aerobic conditions.

(4) The effect of metronidazole treatment on the survival of *E. coli* strains transformed with pMT100 under anaerobic conditions

Under anaerobic conditions, *E. coli* strains that contained both functional *recA* and *recBC* systems were more resistant to metronidazole in the presence of pMT100 (eg. *E. coli* AB1157, MIC_{pMT104} 600 µg/ml; MIC_{pMT100} 800 µg/ml). This effect was also observed under aerobic conditons in these *E. coli* strains.

The presence of pMT100 further increased the sensitivity of *recA* (eg. *E. coli* CC118, MIC_{pMT104} 20 µg/ml; MIC_{pMT100} 5 µg/ml) and *recBC* (*E. coli* JC5519, MIC_{pMT104} 120 µg/ml; MIC_{pMT100} 60 µg/ml) repair mutant strains to metronidazole under anaerobic conditions. It is therefore possible that, under anaerobic conditions, the pMT100 gene product interfered with the remaining repair gene functions when either RecA or RecBC were absent. The effect of pMT100 on the *recBC recE*+ strain is discussed below (4.3.2).

The presence of pMT100 did not affect the repair capacity of *recA* or *recBC* mutant strains under aerobic conditions, whereas pMT100 had a marked effect on these strains under anaerobic conditions. It is possible either that the repair genes functioned differently, that the pMT100 gene product functioned differently, or that the metronidazole caused a different type or extent of DNA damage under aerobic versus anaerobic conditions. These possibilities will be discussed in Chapter 6.

No DNA binding motif was detected in ORF1 (Chapter 3) and it is therefore unlikely that the pMT100 gene product bound directly to the damaged DNA. It is unlikely that the small ORF1 encoded protein (Chapter 3) interacted with different proteins, except if there was a common motif among the proteins. The pMT100 gene product possibly interacted with a repair enzyme, which functioned in conjunction with RecA and RecBC, such as the single stranded binding protein or RecF (Weinstock, 1987; Madiraju *et al.*, 1988). The RecA and

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RecBC proteins are excluded since the pMT100 encoded phenotypes were also observed in *recA* and *recBC* mutants.

The *uvrA,B,C* mutations were not affected by the pMT100 encoded gene product, since *E. coli* AB1886 *uvrA*, *E. coli* AB1885 *uvrB*, *E. coli* AB1884 *uvrC* and *E. coli* AB1157 *uvr*+ (parental strain) all showed increased resistance to metronidazole as well as sensitivity to far UV irradiation under aerobic conditions (Chapter 2, and section 4.3.3). The *E. coli* TK603 *uvrA* mutant strain, which is less closely related to *E. coli* AB1157 also showed increased resistance to metronidazole after transformation with pMT100 under aerobic conditions.

E. coli AB1157 and *E. coli* TK603 *uvrA* both showed increased resistance to metronidazole under anaerobic conditions. It is unclear why, under anaerobic conditions, *E. coli* AB1886 *uvrA* (pMT100) showed no increase in resistance to metronidazole.

The plasmid pMT130, containing less than 0.8 kb of insert DNA including ORF1, was transformed into the *E. coli* repair proficient and deficient strains. The plasmid encoded the same phenotype and levels of metronidazole susceptibility as pMT100 and was slightly more stable than pMT100. This showed that the gene product responsible for conferring the different phenotypes to the *E. coli* strains was encoded for by ORF1.

E. coli AB1157 (pMT104) and *E. coli* AB1157 (pMT100) had aerobic MIC's to metronidazole of 750 µg/ml and 1000 µg/ml on YT agar plates, respectively. This is higher than the MIC to metronidazole obtained on LB agar (*E. coli* AB1157, MIC_{pMT104} 500 µg/ml; MIC_{pMT100} 750 µg/ml, (Chapter 2)). The growth media dependent difference in the response of *E. coli* strains to metronidazole has been reported previously (Jackson *et al.*, 1984). The metronidazole MIC of *E. coli* AB1157 was 1000 µg/ml on tryptone soya agar, and 500 µg/ml on cooked meat agar (Jackson *et al.*, 1984). *E. coli* strains grown under anaerobic respiration were shown to be more resistant to metronidazole than those grown under fermentative conditions (Santangelo *et al.*, 1991).

The MIC of metronidazole of *E. coli* F19* with or without pMT100 could not be assessed, because the strain was very resistant to metronidazole. The increased

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resistance of the *E. coli* F19* strain was due to both an efficient DNA repair system (*recA*+) and the reduced ability to activate metronidazole (*ntr*-).

The *E. coli* JC8679 *recBC recE*+ strain showed increased sensitivity to metronidazole under both aerobic and anaerobic conditions after transformation with pMT100. *E. coli* JC8679 was derived from *E. coli* JC5519 *recBC*, which showed increased sensitivity to metronidazole only under anaerobic conditions after transformation with pMT100. This suggested that a component of the RecE pathway interacted with the pMT100 encoded protein. A plasmid containing the cloned *recE* gene of *E. coli* (Willis *et al.*, 1985) was used to further investigate the effect of pMT100 on the RecE pathway (4.3.2).

4.3.2 Metronidazole susceptibility of *E. coli* strains transformed with both pMT100 and a plasmid containing the *recE* gene of *E. coli*

The DNA endonuclease restriction fragment from the plasmid pRAC3, containing the functional, truncated *E. coli recE* gene, was subcloned onto a low-copy-number plasmid, pACYC184, which is compatible with pMT100. The resulting construct, pM-RAC3 was cotransformed with pMT100 into *E. coli* strains in order to study the effect of the pMT100 gene product on the cloned *recE* gene product. The results of this study are shown in Table 4.2. and will be analyzed in two sections: the effect of metronidazole treatment on the survival of an *E. coli recBC* mutant strain (**1**) and an *E. coli* repair wild type strain (**2**) transformed with both pM-RAC3 and pMT100.

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(1) The effect of metronidazole treatment on the survival of an *E. coli recBC* mutant strain transformed with both pM-RAC3 and pMT100

The *E. coli* JC5519 *recBC* strain showed an increase in resistance to metronidazole under both aerobic and anaerobic conditions when transformed with a plasmid containing the functional *recE* gene (compare *E. coli* JC5519 (pMT104-pACYC184), MIC 400 & 120 µg/ml, to *E. coli* JC5519 (pMT104-pM-RAC3), MIC 700 & 400 µg/ml; MIC aerobic & anaerobic). The functional *recE* gene is known to suppress mutations of the *recBC* DNA repair system (Barbour *et al.*, 1970). The suppression of the *recBC* mutation by the functional *recE* gene was more marked under anaerobic conditions.

As reported previously (sections 4.3.1), the pMT100 gene product only affected the metronidazole susceptibility of anaerobically grown *recBC* mutant strains,

Table 4.2. Susceptibility of *E. coli recE*+ to metronidazole

E. coli strain	Relevant genotype			O_2 MIC[a]	AnO_2 MIC[a]
	recE	recA	recBC		
JC5519(pMT104+pACYC184)[b]	-	+	-	400	120
JC5519(pMT104+pM-RAC3)	+	+	-	700	400
JC5519(pMT100+pACYC184)	-	+	-	400	60
JC5519(pMT100+pM-RAC3)	+	+	-	400	200
AB1157(pMT104+pACYC184)	-	+	+	750	400
AB1157(pMT104+pM-RAC3)	+	+	+	750	400
AB1157(pMT100+pACYC184)	-	+	+	1000	600
AB1157(pMT100+pM-RAC3)	+	+	+	500	300

[a] The MIC of metronidazole (μg/ml) was determined under aerobic (O_2) and anaerobic (AnO_2) conditions. The metronidazole assays of each strain were repeated at least three times with identical results.

[b] The deletion plasmid, pMT104, and the vector used to construct pM-RAC3, pACYC184, were used as controls against any variations due to the selection on ampicillin (100 μg/ml) and chloramphenicol (20 μg/ml).

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and not the sensitivity of aerobically grown *recBC* strains (compare *E. coli* JC5519 (pMT104+pACYC184), MIC 400 & 120 μg/ml, to *E. coli* JC5519 (pMT100+pACYC184), MIC 400 & 60 μg/ml, aerobic & anaerobic growth, respectively).

Under aerobic and anaerobic conditions the addition of pMT100 to the cloned *recE* present in the *recBC* strain decreased the tolerance of the strain to metronidazole (compare *E. coli* JC5519 (pMT104+pM-RAC3), MIC 700 & 400 μg/ml, to *E. coli* JC5519 (pMT100+pM-RAC3), MIC 400 & 200 μg/ml). The presence of pMT100, therefore, appeared to inhibit the suppression of the *recBC*

mutation by the cloned *recE* gene, or, in other words, the pMT100-encoded gene product appeared to inhibit exo VIII. In the *recBC* mutant strain, the *recE* gene product in combination with the pMT100-encoded gene product reduced the tolerance of the cells to metronidazole, even though the *recE* gene product on its own increased the cells tolerance to metronidazole.

(2) The effect of metronidazole treatment on the survival of an *E. coli* repair proficient strain transformed with both pM-RAC3 and pMT100

The *E. coli* AB1157 *recBC*$^+$ strain is proficient in DNA repair, although it is deleted for the *rac* prophage including *recE* (Bachman, 1972). The introduction of the cloned *recE* gene did not affect the metronidazole sensitivity of the strain (compare *E. coli* AB1157 (pMT104+pACYC184), MIC 750 & 400 µg/ml, to *E. coli* AB1157 (pMT104+pM-RAC3), MIC 750 & 400 µg/ml; MIC aerobic & anaerobic). As reported previously, the gene product encoded by pMT100 increased the resistance to metronidazole of *E. coli* AB1157 under both aerobic and anaerobic conditions (compare *E. coli* AB1157 (pMT104+pACYC184), MIC 750 & 400 µg/ml, to *E. coli* AB1157 (pMT100+pACYC184), MIC 1000 & 600 µg/ml).

The introduction of the cloned *recE* gene into the pMT100-containing *E. coli* AB1157 strain reduced the tolerance of the strain to metronidazole under both aerobic and anaerobic conditions (compare *E. coli* AB1157 (pMT100+pACYC184), MIC 1000 & 600 µg/ml, to *E. coli* AB1157 (pMT100+pM-RAC3), MIC 500 & 300 µg/ml). Conversely, the introduction of the pMT100-encoded gene into *E. coli* AB1157 *recE*$^+$ cells also decreased the tolerance of the strain to metronidazole (compare *E. coli* AB1157 (pMT104+pM-RAC3), MIC 750 & 400 µg/ml, to *E. coli* (pMT100+pM-RAC3), MIC 500 & 300 µg/ml). It was concluded from these findings that in the *E. coli recBC*$^+$ strain, the RecE system and the pMT100 gene product acted together to reduce the cells survival in the presence of metronidazole.

The addition of the cloned *recE* gene, together with the pMT100 gene, into different genetic backgrounds (repair wild type and *recBC* mutant) resulted in a significant decrease in cell survival following metronidazole treatment. It is possible that the pMT100 gene product enhanced the exo VIII activity which resulted in unspecific degradation of metronidazole-damaged DNA. As stated before, it is unlikely that the small ORF1 encoded protein interacted with more than one protein. The pMT100 encoded gene product conferred increased

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resistance or increased sensitivity to *E. coli* strains with non-functional *recE* genes. It is therefore doubtful that the pMT100 encoded protein interacted directly with RecE.

Alternatively, it is possible that the pMT100 encoded protein interfered with an enzyme that is required in the RecE pathway, such as RecF (Gillen *et al.*, 1981), RecO or RecJ (Lovett and Clark, 1984). The RecF protein has a single stranded DNA binding activity (Griffin and Kolodner, 1990) and is believed to enhance RecA activation (Sassanfar and Roberts, 1991). The function of the RecO protein is unclear at this stage. The RecJ protein has single stranded exonuclease activity (Lovett and Kolodner, 1989). In the *E. coli* strains used, neither the *recF*, *recO* nor the *recJ* genes were mutated. It is possible that the pMT100 protein interacted with one of these gene products to decrease survival in an exo VIII containing strain. In the *recA* or *recBC* mutant strains, or in the repair wild type strain, the interaction between the pMT100 encoded gene product and one of these enzymes could result in increased or decreased survival following metronidazole treatment, depending on the residual repair capacity of the cell.

The susceptibility to metronidazole of *E. coli* JC5519 (pMT104+pM-RAC3)(MIC 700 & 400 µg/ml) and *E. coli* JC8679 (pMT104)(MIC 700 & 400 µg/ml) was the same. This confirmed that the truncated product of the cloned *recE* gene was as active in repairing metronidazole lesions as the product of the functional chromosomal *recE* gene of *E. coli* JC8679. It also showed that there was no effect on metronidazole susceptibility due to the increased copy number of the cloned *recE* gene.

E. coli strains transformed with pM-RAC3 and pMT100 exhibited increased plasmid instability. It was therefore necessary to retransform the strains prior to every experiment. Increased plasmid instability by *E. coli recBC sbcA* strains has been reported previously (Biek and Cohen, 1986).

The *E. coli* strains used in this study were cotransformed with two plasmids, one encoding resistance to ampicillin (pMT104 or pMT100), the other encoding resistance to chloramphenicol (pACYC184 or pM-RAC3), to determine whether there was any variation due to the simultaneous selection of the *E. coli* strain on three antibiotics. No synergistic effect between the antibiotics was observed. The

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presence of the vector pACYC184 or the control plasmid pMT104 did not influence the susceptibility of the strains to metronidazole.

4.3.3 Ultraviolet survival of DNA repair deficient *E. coli* strains transformed with pMT100

To further investigate the effect of the pMT100 gene product on DNA repair mechanisms, the response of *E. coli* wild type and DNA repair deficient strains to far UV irradiation was investigated in the presence and absence of pMT100.

The strains *E. coli* AB1157 and *E. coli* C600 were tested under both aerobic (Chapter 2) and anaerobic conditions, whereas *E. coli* AB1885 *uvrA* (Chapter 2), *E. coli* AB1885 *uvrB*, *E. coli* AB1884 *uvrC* and *E. coli* JC8679 *recBC recE*$^+$ were only assayed under aerobic conditions. The *uvr* mutants grew as irregular sized colonies under anaerobic conditions making it impossible to enumerate the colonies for accurate UV survival curves.

The plasmid pMT100 encoded increased sensitivity to far UV irradiation in all *E. coli* strains tested under aerobic and anaerobic conditions, irrespective of their repair genotype (Fig. 4.1.). The difference in survival after UV irradiation between the strains transformed with pMT100 and those transformed with the control plasmid pMT104 was about 100-fold at the maximum UV dosage used, except for *E. coli* C600 and *E. coli* AB1884 *uvrC*, which were affected less by the presence of pMT100.

The shapes of the UV survival curves were analyzed in an attempt to understand the regulatory mechanisms involved. The shoulder region of a survival curve has been interpreted as the competition between the fixation of DNA damage and its repair (Ward *et al.*, 1991). The exponential drop in survival following the shoulder can be understood as the saturation of the repair process and the progressive inactivation of DNA repair enzymes with increased UV dose (Ward *et al.*, 1991). A reduced shoulder region of a UV survival curve is indicative of an altered DNA repair system. This could be demonstrated by the comparison of the survival curves of the *uvr*$^+$ strains and the *uvr* mutant strains (Fig. 4.1.).

A slight decrease of the shoulder region of the survival curves of the strains *E. coli* C600 (under anaerobic conditions), *E. coli* AB1157 and *E. coli* AB1886 after

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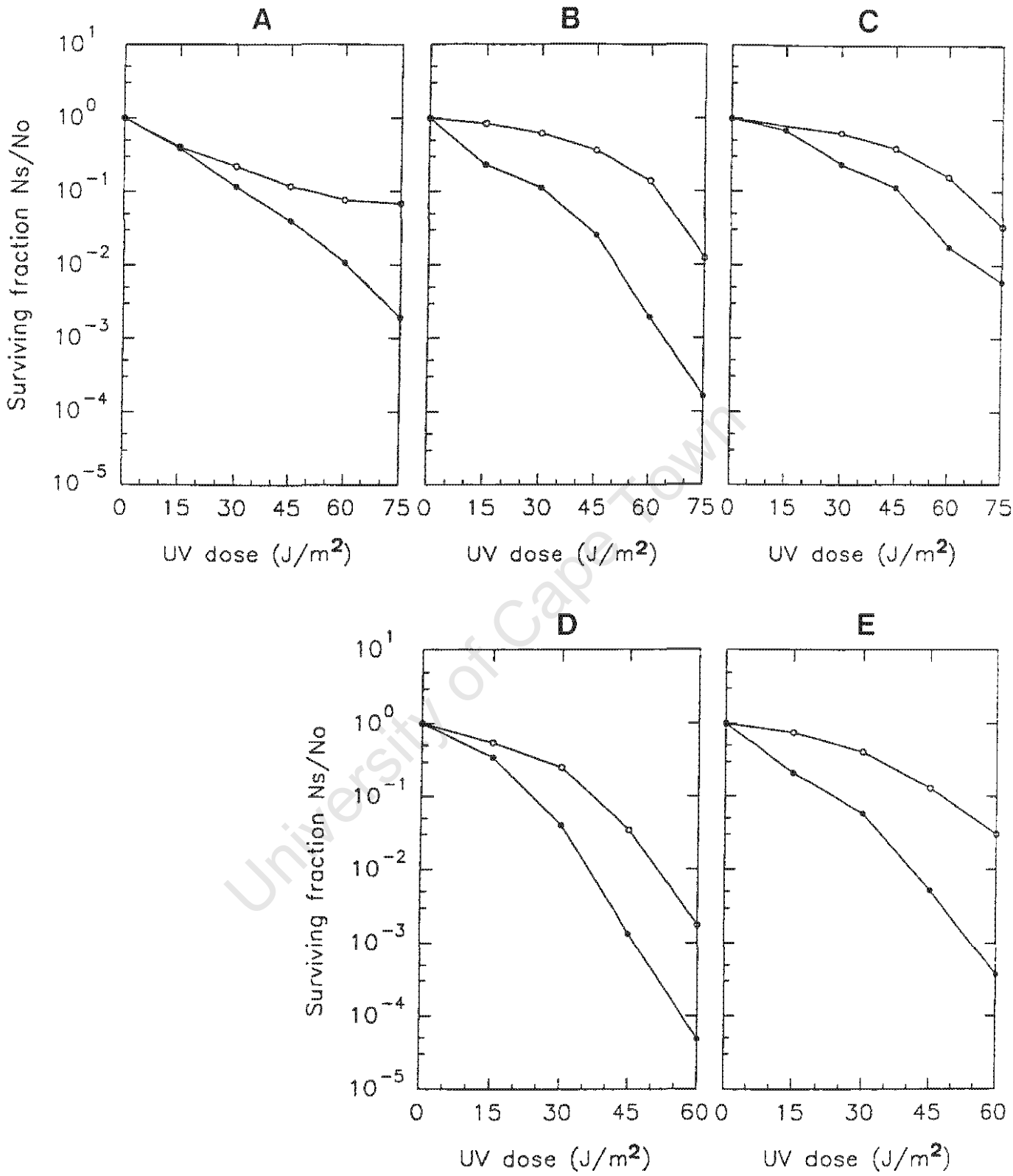

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Fig. 4.1.a. UV survival curves of *E. coli* strains transformed with pMT100 (●) or pMT104 (○) under aerobic (**A, B, C**) and anaerobic (**D, E**) conditions. (**A**) *E. coli* JC8679 *recBC sbcA*; (**B, D**) *E. coli* AB1157; (**C, E**) *E. coli* C600.

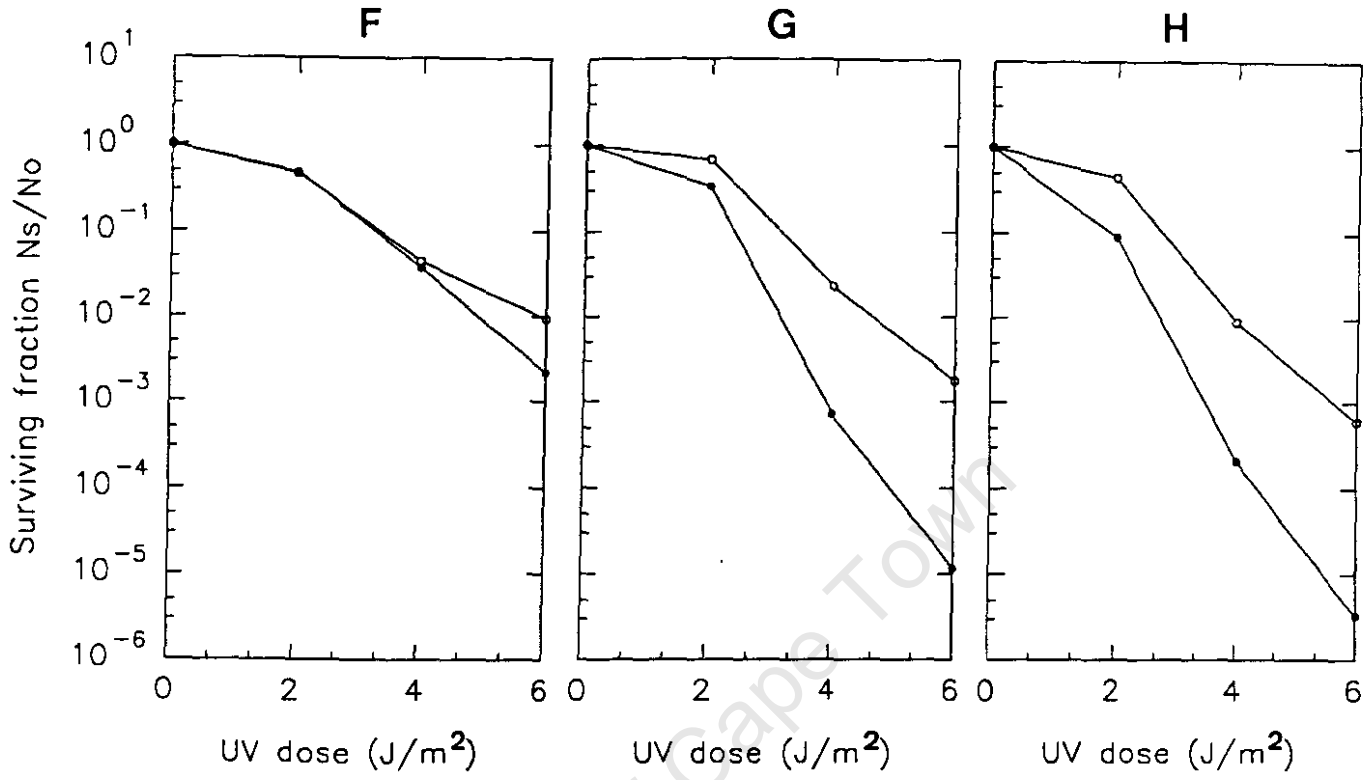

Fig. 4.1.b. UV survival curves of *E. coli uvr* mutant strains transformed with pMT100 (●) or the control plasmid pMT104 (○) under aerobic conditions. (**F**) *E. coli* AB1884 *uvrC*; (**G**) *E. coli* AB1885 *uvrB*; (**H**) *E. coli* AB1886 *uvrA*.

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transformation with pMT100 indicated an interference by the pMT100 gene product in the repair machinery of the cell.

In *E. coli*, the mutation of either *uvrA* or *uvrB* results in the loss of the entire excision repair system (Van Houten, 1990). In *uvr* mutants the UV induced lesions are repaired by the recombinational repair system. During excision repair, in the UvrB-DNA complex, the major grove is open to solution so that the damaged site is accessible to other repair enzymes. *UvrC* mutants exhibit an increased residual activity as compared to *uvrA* or *uvrB* mutants. Possibly the UvrAB complex stabilizes the damaged DNA sufficiently to increase the efficiency of the other repair systems in a *uvrC* mutant. The pMT100 gene

product did not interact with the excision repair system, since the *uvrA* and *uvrB* mutants showed the same increased UV sensitivity phenotype as the excision repair proficient strain after transformation with pMT100.

In the absence of a functional UvrC subunit, the pMT100 encoded gene product affected the UV survival of the cell to a lesser degree. UvrC is the subunit of the UvrABC excision repair complex that is responsible for the incision function (Van Houten, 1990). UvrC also binds preferentially to single stranded DNA (Sancar *et al.*, 1981). It is possible that the pMT100 encoded protein interacted with the UvrC protein in *uvrC*$^+$ strains to decrease the survival of the cells following UV irradiation. This possibility is discussed further in Chapter 6.

The strain *E. coli* JC8679 *recBC recE*$^+$ (pMT104), although relatively resistant to UV irradiation due to the function of the *recE* protein, displayed an almost shoulder-less survival curve which leveled out. The shape of the graph resembled that of a *recA* mutant, although it leveled out at a much higher UV dose (Friedberg, 1985). This implies that alternative repair systems are induced at a later stage. The survival curve of *E. coli* JC8679 (pMT100) did not level out, which would indicate the continued interference of the pMT100 gene product with the induced DNA repair gene(s). It was shown in the previous section, that the pMT100 gene product in combination with the RecE pathway caused increased sensitivity to metronidazole. It is therefore possible that the combination of the pMT100 gene product and a component of the RecE pathway also reduced the repair capability of the cell after UV irradiation.

All strains, except *E. coli* C600, showed an increase in sensitivity to UV irradiation after transformation with pMT130. Plasmid pMT130 contained less than 0.8 kb of the *Bacteroides* DNA insert including ORF1. This supported the findings that ORF1 was responsible for both the metronidazole susceptibility and the UV sensitivity phenotypes in the *E. coli* strains. In the construction of pMT130 a DNA region required for expression in *E. coli* C600 might have been deleted (Chapter 2).

4.3.4 Metronidazole susceptibility of *Bacteroides* strains containing the pMT100-derived plasmid pM-VAL

A 1.3 kb restriction endonuclease fragment containing the λ promoter from pEcoR251 and ORF1 from pMT100 was subcloned into the *Bacteroides* shuttle

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vector, pVAL-1, and transferred into plasmid-free *Bacteroides* strains. The presence of this construct, pM-VAL, in *Bacteroides* was confirmed by plasmid extraction. Transformation with pM-VAL increased the resistance of *E. coli* AB1157 to metronidazole, which proved that a construct functional in *E. coli* was transferred into the *Bacteroides* strains. The results of the metronidazole susceptibility assays of the *Bacteroides* transconjugants are shown in Table 4.3. The results of the *B. fragilis* Bf-2 (pBFC1) strain were incorporated for comparative purposes.

Table 4.3. Metronidazole susceptibility of *Bacteroides* transconjugants

Bacteroides strain	MIC (μg/ml)[a]
B. fragilis 638 (pVAL-1)[b]	0.3
B. fragilis 638 (pM-VAL)	0.3
B. thetaiotaomicron (pVAL-1)[b]	0.3
B. thetaiotaomicron (pM-VAL)	0.3
B. fragilis Bf-2 (pBFC1)	1.0

[a] The MIC of metronidazole was determined on BHI agar under anaerobic conditions. The metronidazole assays were repeated at least three times with every strain with similar results.

[b] The shuttle vector pVAL-1 was used as a control.

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The construct pM-VAL did not affect the MIC for metronidazole of the two plasmid-free *Bacteroides* strains. Several explanations for this result were considered. One possible reason is that the *B. fragilis* plasmid pBFC1 contained a non-functional gene that was only expressed in *E. coli* due to the strong λ promoter of the vector. A gene that was non-functional in *Bacteroides* but encoded a phenotype in aerobically grown *E. coli* strains has been reported (Guiney *et al.*, 1984a). Although the pMT100 gene product affected *E. coli* under both aerobic and anaerobic conditions, it could be influenced by other factors prevailing in *E. coli* but not in *Bacteroides*.

Alternatively, the gene on pBFC1 was functional in *Bacteroides*, but the shuttle vector construct pM-VAL did not contain the *Bacteroides* promoter region. In the cloning of pMT100 less than 200 bp upstream of ORF1 of the *Bacteroides* plasmid were cloned into pEcoR251. The sequencing studies of the pMT100 insert DNA did not reveal a promoter region upstream of ORF1, and in *E. coli* strains the gene was expressed from the λ promoter (Chapter 3).

It is possible that the λ promoter of the *E. coli* vector in pM-VAL was not recognized by *Bacteroides*. Little is known about *Bacteroides* promoters and only recently have promoter probe vectors been constructed to study *Bacteroides* promoter regions (Abratt *et al.*, 1992; Feldhaus *et al.*, 1991). Feldhaus *et al.* (1991) could not demonstrate expression of the GUS reporter gene from the *E. coli* lac promoter in *Bacteroides*. A comprehensive study by Smith *et al.* (1992) demonstrated that none of the *E. coli* promotors tested were recognized in *Bacteroides*.

In future studies on the effect of the pMT100 gene product on *Bacteroides* strains, the upstream region of ORF1 on pBFC1 would need to be incorporated on the shuttle vector, in an attempt to include the promotor region of the gene. Alternatively, a known *Bacteroides* promotor could be fused to ORF1 prior to the transfer of the construct into *Bacteroides*. Only then could one possibly ascribe a function to the pMT100 encoded gene product in the host organism.

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4.4 Conclusion

In order to investigate the effect of metronidazole on bacterial cells, the metronidazole susceptibility of DNA repair proficient and deficient *E. coli* strains was analyzed. Due to the reductive activation characteristic of metronidazole, anaerobically grown *E. coli* cells showed increased sensitivity to metronidazole as compared to aerobically grown cells. *E. coli* strains mutated in either the excision repair or the recombination repair systems exhibited increased sensitivity to metronidazole as compared to repair proficient strains. Of the *E. coli* strains tested, the *recA* mutant strains were most sensitive to metronidazole under both aerobic and anaerobic conditions. A functional RecA protein therefore appears to be vital for the effective repair of metronidazole-

induced lesions. The metronidazole MIC results showed that, although, the RecE pathway is not required, it can aid in the repair of metronidazole-induced lesions.

Since the excision repair system is believed to detect helix distortions, it was proposed that metronidazole may cause lesions other than strand breaks, which cause a distortion of the DNA helix. This question will be addressed further in Chapter 6.

To study the role played by *B. fragilis* genes in influencing the potency of metronidazole, the effect of the cloned *B. fragilis* gene on the susceptibility to metronidazole of *E. coli* DNA repair proficient and deficient strains was tested. Independent of the oxygen conditions, the pMT100 encoded gene product conferred increased resistance to metronidazole to *E. coli recA*$^+$ *recBC*$^+$ strains. Under anaerobic conditions, it conferred increased sensitivity to metronidazole to *E. coli recA* and *E. coli recBC* mutant strains, whereas under aerobic conditions it did not affect the susceptibility of these strains. In an *E. coli* strain carrying a functional *recE* gene either on the chromosome or on a plasmid, pMT100 conferred increased sensitivity to metronidazole independent of the oxygen conditions. It is feasible that the pMT100 encoded gene product interfered with a controlling repair enzyme to induce the observed phenotypes. The possible function of the pMT100 encoded gene product is studied in Chapter 5.

The effect of the cloned gene on the susceptibility to far UV irradiation of repair proficient and deficient *E. coli* strains was also tested. The pMT100 gene product conferred increased sensitivity to UV irradiation in all *E. coli* strains tested. The enhanced UV sensitivity phenotype was independent of the oxygen conditions.

To study the effect of the *B. fragilis* gene on the homologous host, the DNA insert fragment containing ORF1 under the control of the λ promoter was subcloned into the *Bacteroides-E. coli* shuttle vector, pVAL-1. The resulting construct, pM-VAL1, did not affect the susceptibility of plasmid free *Bacteroides* strains to metronidazole. It is not known whether the gene is expressed in these strains, and it is possible that the gene requires the regulation of a *Bacteroides* promoter in the original host. The mode of action of metronidazole on *E. coli* cells was investigated to illucidate the function of the *B. fragilis* gene product in *E. coli* cells (Chapter 5).

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Chapter 5
Functional analysis of the plasmid gene from *B. fragilis* that affects DNA repair in *E. coli*

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Chapter 5

Functional analysis of the plasmid gene from *B. fragilis* that affects DNA repair in *E. coli*

5.0 Abstract

Treatment of *E. coli* cultures with high concentrations of metronidazole under aerobic and anaerobic conditions resulted in the induction of a significant amount of single strand, but not double strand DNA breaks. The DNA strand breaks appeared to be largely generated by the action of repair enzymes on metronidazole-induced DNA lesions, since the DNA degradation was observed mainly during the repair period after metronidazole treatment. The RecBCD enzyme of *E. coli* was possibly involved in the degradation, since *recBC* strains had less strand breaks than the repair wild type or the *recA* strains.

The function of the plasmid pMT100 encoded gene product was analyzed using a variety of different approaches. The gene on pMT100 encodes a protein which was unable to protect *E. coli* strains from metronidazole or alter the metronidazole concentration of the culture media. It also did not affect the metronidazole uptake ability of the strains, or the extent of cell lysis caused by metronidazole. The pMT100 encoded gene product did, however, reduce the amount of DNA strand breaks induced by metronidazole as detected by differential precipitation of total DNA.

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5.1 Introduction

A cloned gene from the *B. fragilis* Bf-2 plasmid pBFC1 encoded resistance to metronidazole under aerobic and anaerobic conditions in *E. coli recA*$^+$ *recBC*$^+$ strains, sensitivity to metronidazole in *E. coli recA* and *E. coli recBC* mutant strains only under anaerobic conditions, and sensitivity to metronidazole in *E. coli recE*$^+$ strains under aerobic and anaerobic conditions (Chapter 4). It also encoded increased sensitivity to far UV irradiation in *E. coli recA*$^+$ *recBC*$^+$, *E. coli uvr* mutant and *E. coli recBC recE*$^+$ strains (Chapter 4). An analysis was therefore conducted to ellucidate the mode of action of metronidazole on *E. coli* cells, and through that the possible function of the pMT100 encoded gene product in *E. coli* cells.

In order to analyze the mode of action of metronidazole, the different steps involved from metronidazole addition to cell death were studied. These included studies of: (1) the alteration of metronidazole, (2) the uptake of metronidazole, (3) DNA, the target molecule, and (4) other targets.

(1) Alteration of metronidazole

Metronidazole is an inert compound which needs activation to its toxic compound (Edwards, 1980). The resistance to metronidazole of *E. coli* AB1157 (pMT100) and *E. coli* AB1886 *uvrA* (pMT100) under aerobic conditions would not include detoxification of the inert starting material, but possibly differential processing of the drug. This possibility was studied by analyzing the effect of spent media obtained from *E. coli* (pMT100) strains on other *E. coli* strains.

(2) Uptake of metronidazole

To date only isolated cases of resistance to metronidazole in susceptible organisms have been reported (Ingham *et al.*, 1978; Breuil *et al.*, 1989). Ingham *et al.* (1978) isolated a metronidazole resistant *B. fragilis* strain from a patient who was on long term therapy for Crohn's disease. The biochemical basis for metronidazole resistance in this *B. fragilis* strain was studied and found to be due to the reduced ability of the strain to take up metronidazole combined with a reduced rate of reduction of metronidazole (Tally *et al.*, 1979). A study was therefore conducted to investigate whether the increased resistance to metronidazole of *E. coli* AB1157 (pMT100) was due to a decreased ability to take up metronidazole. The rate of disappearance of metronidazole from the growth

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medium, as well as the uptake and accumulation of radiolabeled metronidazole of *E. coli* AB1157 (pMT100) were investigated.

(3) DNA, the target molecule

The actual mechanism of the cytotoxic action of metronidazole has not yet been ellucidated, partly because the active unstable intermediate of the drug has not been isolated (Mueller, 1983). Of all the macromolecules within the cell, DNA is believed to be the main target of the reduced cytotoxic form of metronidazole (Mueller, 1983; Edwards, 1977). Studies have shown that DNA repair deficient *E. coli* strains were more sensitive to metronidazole (Yeung *et al.*, 1984; Jackson *et al.*, 1984). *In vitro* experiments demonstrated single and double strand DNA breaks caused by the reduced drug (Knight *et al.*, 1978; Edwards *et al.*, 1980). An investigation of the molecular effect of metronidazole on the DNA of growing *E. coli* cells was therefore carried out.

The measurement of DNA single and double strand breaks is important in the study of induction and repair of DNA damage (Ahnstroem, 1988). Several techniques have been developed to identify and quantify DNA damage. To detect single strand DNA breaks, the double stranded form of the DNA needs to be disrupted. The separation of total DNA on a denaturing gel (Maniatis *et al.*, 1982) was the qualitative method used in this study to observe the extent of single strand DNA breaks caused by metronidazole.

The most widely used technique to quantify DNA damage is alkaline sucrose sedimentation. However, a range of new techniques has been developed recently, which include alkaline filter elution, viscoelastrometry, nick translation and DNA precipitation (Ahnstroem, 1988). In this study the differential precipitation by trichloroacetic acid (TCA) of radiolabeled total DNA (Simic *et al.*, 1991) extracted from metronidazole treated *E. coli* cells was used to quantify the amount of DNA strand breaks caused by metronidazole.

(4) Other targets

Although interaction with DNA was the most likely cause of toxicity, other cellular targets were suggested (Ings *et al.*, 1974; Mueller and Lindmark, 1976; Mueller, 1983). A recent study proposed that metronidazole caused rapid death in *C. pasteurianum* due to cell lysis (Church *et al.*, 1991). This theory was tested in *E. coli* AB1157 under aerobic conditions by analyzing the amount of cytoplasmic

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leakage caused by, and the amount of viable cells remaining after treatment with metronidazole. The morphology of these metronidazole treated cells was also examined by phase contrast microscopy.

5.2 Materials and methods

Commonly used materials and methods are described in the Appendix.

5.2.1 Bacterial strains and plasmids

E. coli AB1157 (ATCC 29055), *E. coli* AB1886 (Howard-Flanders *et al.*, 1966), *E. coli* JC5519 (A.J. Clark), *E. coli* JC8679 (ATCC 47001) and *E. coli* F19 (Santangelo *et al.*, 1991) were used. The full genotypes of the strains are listed in the Appendix. The plasmid pMT100, containing *B. fragilis* insert DNA, and the control plasmid pMT104 have been described before (Chapter 2).

5.2.2 Growth conditions

The *E. coli* strains were grown at 37°C on LB or YT (aerobic conditions) or YT+g+n (anaerobic conditions) media (Chapter 2), except for the *E. coli* F19 strain which was grown in BHI broth (Appendix) under anaerobic conditions. The aerobic liquid cultures containing metronidazole were grown without shaking in shallow broth (20 ml in a 100 ml volumetric flask) to imitate as far as possible the microaerophillic conditions of the plate assay.

5.2.3 Spent media assay

The method was adapted from Speer and Salyers (1988). The effect of the pMT100 gene product on metronidazole under aerobic conditions was investigated using two closely related techniques involving the diffusion of culture supernatant from a well of an agar plate seeded with an indicator strain.

To investigate whether a resistance factor was produced by *E. coli* (pMT100) strains, the protection of *E. coli* (pMT104) by the spent medium from *E. coli* (pMT100) cultures was examined. Stationary phase aerobic *E. coli* AB1157 (pMT104) and *E. coli* AB1886 (pMT104) strains were seeded at 10^4-10^5 cells/ml into 0.7 % LB agar held at 50°C. This sloppy agar layer was poured onto LB agar plates supplemented with metronidazole (0-1000 µg/ml) and ampicillin (100 µg/ml). Wells in these agar plates were filled with spent media, which was made by filtering (through 0.22 µm Millipore filters) culture supernatants of

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E. coli strains grown in LB broth supplemented with ampicillin (100 µg/ml). The spent media were tested and shown to be sterile. Each agar plate, containing a different concentration of metronidazole and seeded with either *E. coli* AB1157 (pMT104) or *E. coli* AB1886 (pMT104), had wells each containing either sterile LB, or spent media from *E. coli* AB1157 (pMT104), *E. coli* AB1157 (pMT100), *E. coli* AB1886 (pMT104) or *E. coli* AB1886 (pMT100) cultures. The plates were incubated aerobically at 37°C and analyzed for increased growth of the indicator strain, *E. coli* (pMT104), around the wells due to the diffusion of a resistance factor.

To investigate whether the metronidazole concentration in the media was being altered by *E. coli* (pMT100) strains, the effect of the spent medium of *E. coli* (pMT100), grown in media containing metronidazole, on *E. coli* (pMT104) was examined. The agar plates were prepared as described above, except that they only contained ampicillin and no metronidazole. The wells in the agar plates were filled with a metronidazole solution or spent media, which was prepared from *E. coli* cultures grown in LB broth supplemented with metronidazole (0-1000 µg/ml) as described above. Each agar plate seeded with either *E. coli* AB1157 (pMT104) or *E. coli* AB1886 (pMT104), had four wells containing sterile LB, a metronidazole solution (0-1000 µg/ml) and spent media from *E. coli* AB1157 (pMT104) and *E. coli* AB1157 (pMT100), or *E. coli* AB1886 (pMT104) and *E. coli* AB1886 (pMT100), respectively. The plates were incubated aerobically at 37°C and analyzed for zones of inhibition of growth of the indicator strain, *E. coli* (pMT104), around the wells due to the diffusion of metronidazole from the wells.

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5.2.4 Detection of metronidazole in the growth medium

The method was adapted from Chen and Blanchard (1979). The optical density of different concentrations of metronidazole in LB broth diluted 1/100 into sterile water was used to construct a standard graph. Cultures of *E. coli* AB1157 (pMT100), *E. coli* AB1157 (pMT104), *E. coli* AB1886 (pMT100) and *E. coli* AB1886 (pMT104) were grown aerobically at 37°C without shaking in LB broth supplemented with metronidazole (0-800 µg/ml) and ampicillin (100 µg/ml), and samples were taken at 2 h intervals during the growth of the cultures from inoculation to stationary phase. The samples were centrifuged to pellet the cells and the absorbtion at 320 nm (the absorbtion maximum of metronidazole) of the supernatant diluted 1/100 into sterile water was determined. The absorption of sterile LB broth supplemented with metronidazole (0-800 µg/ml), which was

incubated in parallel with the *E. coli* cultures, was determined as a control to monitor metronidazole degradation.

5.2.5 Uptake of radioactive metronidazole

The method was adapted from Tally *et al.* (1978). [^{14}C] metronidazole (x µCi/ml) was added at subinhibitory concentrations (100 µg/ml) to aerobically grown logarithmic phase (A_{600} = 0.2 - 0.4) *E. coli* AB1157 (pMT100) and *E. coli* AB1157 (pMT104) cultures. Samples (50 µl) were taken at 1 h intervals during growth at 37°C, filtered onto 0.22 µm Millipore filters and washed with 2.0 ml LB broth to remove the radiolabeled metronidazole that had not been taken up. The amount of radioactivity retained on the filters was determined by using a scintillation counter.

5.2.6 Detection of single strand DNA breaks using denaturing gel conditions

The method was adapted from Abratt *et al.* (1986) and Maniatis *et al.*, (1982). *E. coli* cells were grown aerobically or anaerobically to an early logarithmic phase (A_{600} = 0.2 - 0.4). Metronidazole (2000 µg/ml) was added and the cultures were incubated without shaking for 1 h. The concentration of metronidazole was chosen from pilot experiments since it was the lowest concentration that produced visible DNA strand breaks on denaturing gels. A 10 ml sample was removed prior to centrifugation to represent time zero, followed by centrifugation and resuspension of the pelleted cells in fresh medium without metronidazole. Samples (10 ml) were removed at time intervals during further incubation. The samples were processed as described below.

Cells from the 10 ml samples were harvested by centrifugation, resuspended in 0.5 ml cold lysis buffer (Appendix) with 1.0 % SDS and lyzed on ice for 15 min. Total DNA was extracted with 1 volume phenol and 2 volumes water-saturated diethyl ether. The DNA was handled with great care to prevent shearing. RNA was removed by incubating the solution containing DNA for 1 h with DNase-free RNase (0.1 mg/ml), followed by a 1/10 phenol extraction (Appendix). The DNA was precipitated at -70°C for 1 h with 1 volume of 100 % EtOH. The precipitate was pelleted by centrifugation and resuspended in 50 µl of TE. Equal amounts of total DNA, as determined by optical density scanning, were loaded on a horizontal agarose gel or alkaline agarose gel for electrophoresis (Appendix).

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5.2.7 Detection of DNA strand breaks by precipitation of radiolabeled DNA

The method was adapted from Simic *et al.* (1991). The experiment was done both under aerobic and under strict anaerobic conditions. Stationary phase *E. coli* cells were inoculated 1/100 into broth containing deoxyadenosine (100 µg/ml) and [^{14}C]thymidine (0.5 µCi/ml) and grown to early logarithmic phase (A_{600} = 0.2 - 0.4) at 37°C. The cultures were then pelleted, washed twice with equal volumes of H_2O to remove unincorporated [^{14}C]thymidine, and resuspended in fresh broth. The cultures were split and reincubated after the addition of metronidazole (2000 µg/ml) or an equivalent amount of H_2O. The concentration of metronidazole used was chosen to be the same as that used in the denaturing gel experiment for comparative purposes. Samples of 0.1 ml were taken immediately for time zero and at 20 min intervals over a 60 min metronidazole treatment period and processed as described below. After the treatment period the cultures were pelleted, resuspended in fresh broth without metronidazole and left to grow for 2 h (repair period). Samples were taken at intervals during the repair period and processed as described below.

The 0.1 ml samples were transferred to 0.22 µm Millipore GS filters, washed with 0.1 ml of 0.3 M NaOH to lyze the cells, then washed with 1.0 ml of an ice cold 10 % TCA solution, followed by 2.0 ml of a 5 % TCA solution. The filters were dried with a mixture of ethanol/ether (1:1). The amount of TCA-precipitable radioactivity was determined by using a scintillation counter.

The fraction of TCA-precipitable DNA remaining after metronidazole treatment was calculated as a percentage of the TCA-precipitable DNA of the water-treated samples at the equivalent time point. This took growth conditions and other losses of radioactivity, such as loss through centrifugation, into account.

5.2.8 Cell lysis assay

The method was adapted from Church *et al.* (1991) and the β-galactosidase activity was determined by the method of Pardee *et al.* (1959). Metronidazole (2000 µg/ml) or sterile water, as a control, was added to logarithmic phase (A_{600} = 0.2 - 0.4) *E. coli* AB1157 (pMT100) and *E. coli* AB1157 (pMT104) cultures grown aerobically in YT broth supplemented with ampicillin (100 µg/ml) and IPTG (2 mM), to induce the β-galactosidase activity of the cell. The concentration of

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metronidazole used was chosen to be the same as that used in the DNA strand break experiments, for comparative purposes. Samples were taken at the end of the 60 min metronidazole treatment period, followed by pelleting the cells and resuspending the pellet in fresh YT broth without metronidazole. Samples were taken during the 2 h repair period. The samples were split and treated as described below.

Cells from 0.1 ml of the sample were diluted and plated onto YT agar supplemented with ampicillin (100 µg/ml) for a viable cell count. The cells in the remainder of the sample were pelleted and the supernatant was assayed for β-galactosidase activity (Appendix) to determine the amount of cell leakage caused by metronidazole. The pelleted cells were lyzed by toluene treatment and assayed for cell-internal β-galactosidase activity.

5.2.9 Microscopic examination

Using phase contrast microscopy, the *E. coli* AB1157 cells were photographed after metronidazole treatment using a Zeiss photomicroscope fitted with phase- and interference-contrast optics.

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5.3 Results

The different responses of *E. coli* (pMT104) and *E. coli* (pMT100) to metronidazole were analyzed in an attempt to determine the *in vivo* effect of metronidazole on *E. coli* cells, and to investigate the molecular function of the pMT100 encoded gene product. This study included an analysis of:

- spent media of pMT100 containing cells, to investigate the effect of a putative resistance factor encoded by pMT100 on other *E. coli* cells, and the differential processing of metronidazole by *E. coli* cells (**5.3.1.**);
- the uptake of metronidazole, by investigating the rate of disappearance of metronidazole from the growth medium, and the accumulation of radiolabeled metronidazole in *E. coli* cells (**5.3.2.**);
- DNA strand breaks, by investigating the extent of single strand DNA breaks caused by metronidazole, and the amount of DNA strand breaks caused by metronidazole *in vivo* (**5.3.3.**); and
- the degree of cell lysis caused by metronidazole (**5.3.4.**).

The studies were performed on actively growing cells (logarythmic phase cells), since metronidazole requires metabolic activation.

5.3.1 Alteration of metronidazole: analysis of spent media from *E. coli* (pMT100) strains

The cell-free supernatants of *E. coli* (pMT100) cultures were analyzed to determine whether it either contained a factor which could protect other *E. coli* strains from the effects of metronidazole, or whether it contained an altered concentration of metronidazole.

A ring of enhanced bacterial growth around the wells containing the supernatant of *E. coli* (pMT100) cultures was expected if the pMT100 gene product was found in the supernatant of the *E. coli* (pMT100) cultures, and if this product could protect the more sensitive indicator strain, *E. coli* (pMT104), from the effect of metronidazole. No increase in growth of the indicator cultures around the wells was observed (results not shown).

Different size zones of inhibition of growth of the indicator cultures around the wells were expected if the *E. coli* (pMT100) strains altered the concentration of metronidazole in the growth medium. The *E. coli* AB1886 (pMT104) strain was shown to be a suitable indicator strain. Zones of inhibition of growth of *E. coli* AB1886 (pMT104) were detected around wells containing the supernatant of all *E. coli* strains grown in LB broth supplemented with 900-1000 µg/ml metronidazole, and around wells containing sterile LB broth containing 800-1000 µg/ml metronidazole (results not shown). The zones around the wells containing supernatants of *E. coli* cultures were slightly smaller than the zones around wells containing an equivalent concentration of metronidazole in LB. No difference in the size of zones was observed between wells containing supernatants of *E. coli* (pMT100) or *E. coli* (pMT104).

The results showed that *E. coli* AB1157 decreased the amount of metronidazole of the culture supernatant, since the minimal initial concentration of metronidazole required to cause a zone of inhibition was 900 µg/ml in the presence of *E. coli* cells, and only 800 µg/ml in sterile medium. Also the zone size differed, indicating that more metronidazole was available in the sterile medium. Aerobically grown *E. coli* cells were therefore able to alter the effective concentration of metronidazole, possibly by reduction.

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5.3.2 Uptake of metronidazole

The uptake of metronidazole by *E. coli* cells was investigated both by the disappearance of metronidazole from the growth medium, and by the accumulation of radioactive metronidazole in the cells.

A scan of the optical absorption of metronidazole in LB broth diluted 1/100 in water peaked at 320 nm. A graph, constructed from the absorption of different concentrations of the metronidazole solution at 320 nm, showed a linear relationship between OD_{320} and metronidazole concentration (0-1000 µg/ml).

The concentration of metronidazole in the medium decreased during growth of the *E. coli* strains (Fig. 5.1.). Little difference in the disappearance of metronidazole from the growth media between the *E. coli* AB1886 (pMT100) and *E. coli* AB1886 (pMT104) strains was observed (Fig. 5.1.). There was some loss of metronidazole in the sterile control solution due to prolonged incubation at 37°C (Fig. 5.1.). Although this method was possibly not sensitive enough to detect small variations in metronidazole concentration, it did show that aerobically grown *E. coli* strains did decrease the concentration of metronidazole in the medium.

The uptake of radiolabeled metronidazole over 4 h by logarithmic phase *E. coli* AB1157 (pMT100), *E. coli* AB1157 (pMT104), *E. coli* F19 (pMT100) and *E. coli* F19 (pMT104) cultures was studied. No significant difference between strains containing pMT100 and strains containing the control plasmid was shown (results not shown). The method produced no clear result, since the filters retained some radiolabeled metronidazole even from sterile broth.

5.3.3 The target molecule: Detection of DNA strand breaks

Two different techniques were used to identify and to quantify DNA damage caused by metronidazole. Denaturing conditions were used to visualize single strand DNA breaks on agarose gels (5.3.3.1), and differential precipitation of total DNA was employed to measure the amount of DNA damage (5.3.3.2). The results of the two experiments will be discussed in two sections: (**1**) the effect of metronidazole on the DNA of *E. coli* strains with different repair deficiencies, and (**2**) the effect of the pMT100 encoded product on the response of these strains to metronidazole treatment.

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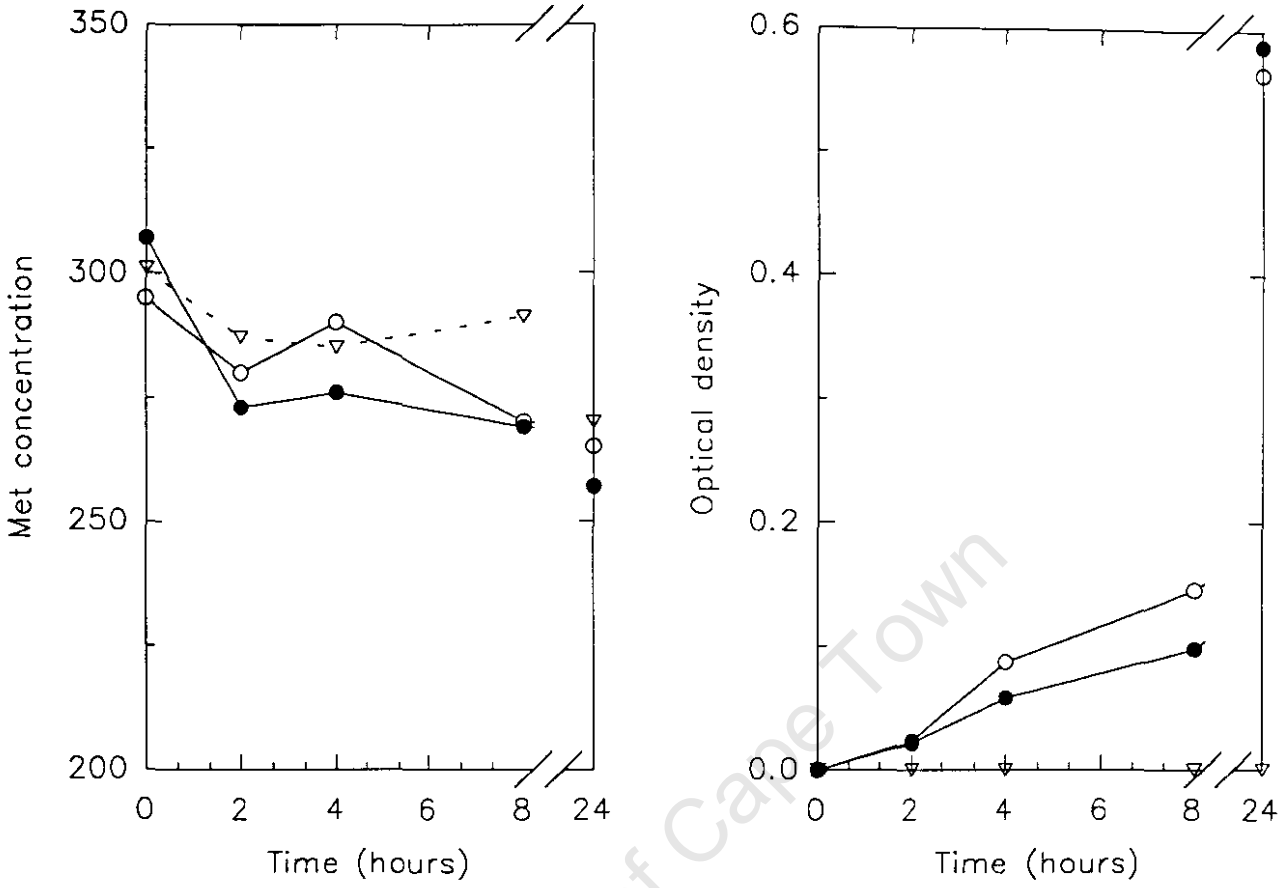

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Fig. 5.1. Residual metronidazole concentration (μg/ml) of the culture supernatants and cell density (OD_{600}) of metronidazole treated *E. coli* AB1886 transformed with pMT100 (●) or the control plasmid pMT104 (○). The metronidazole concentration and optical density of sterile medium is shown as comparison (▽).

5.3.3.1 Identification of DNA strand breaks

The denaturing gel experiment was conducted under aerobic conditions using the *E. coli* AB1157 and *E. coli* JC8679 strains, and under anaerobic conditions using the *E. coli* F19 strain, since the effect of pMT100 on the susceptibility to metronidazole of the various strains was most marked under these conditions (Chapter 4). In an initial experiment, total DNA of *E. coli* AB1157, grown for one hour in different concentrations of metronidazole, was loaded on a denaturing gel to determine the optimum metronidazole concentration that caused DNA damage. The lowest concentration of metronidazole that produced easily visible

DNA degradation on agarose gels was 2000 µg/ml. This concentration of metronidazole was used for all *E coli* strains in the following experiments.

(1) Metronidazole and *E. coli*

The addition of metronidazole (2000 µg/ml) to logarithmic phase *E. coli* AB1157, *E. coli* JC8679 and *E. coli* F19 cultures resulted in a decrease in high molecular weight chromosomal DNA, as well as the disappearance of the DNA bands corresponding to the plasmids (pMT100 or pMT104), as detected on alkaline gels (Fig. 5.2.). This indicated that metronidazole mediated DNA degradation and single stranded DNA breaks in both the chromosomal and the plasmid DNA of the cell. It was observed that the high molecular DNA of the *E. coli* JC8679 strain did not completely disappear during metronidazole treatment, indicating that the degree of DNA degradation was less than that observed in *E. coli* AB1157 (Fig. 5.2.). *E. coli* F19 exhibited increased DNA degradation as compared to *E. coli* AB1157.

During the 2 h repair period following the exposure to metronidazole, the smear of lower molecular weight DNA faded and heigh molecular weight chromosomal DNA together with discrete plasmid bands reappeared (Fig. 5.2.). This indicated that either the damaged DNA was repaired or that new DNA was synthesized in intact cells. It is not possible to distinguish these two possibilities, because, with the method used, one could not differentiate newly synthesized from parental DNA. The differential precipitation method could distinguish these possibilities (5.4).

Denaturing gel conditions separated the DNA helix into two single stranded DNA molecules, whereas on non-denaturing gels the double stranded form of the DNA helix was visualized. The damage of the *E. coli* F19 total DNA, as observed on the alkaline gels, was mainly due to single strand breaks, since hardly any DNA degradation of the same DNA samples could be seen on non-denaturing agarose gels (Fig. 5.3.).

(2) Metronidazole, *E. coli* and pMT100

No obvious differences between the *E. coli* (pMT100) and *E. coli* (pMT104) strains could be detected using the denaturing gel technique. Since the visualization of DNA damage was possibly not sensitive enough to see small variations, the

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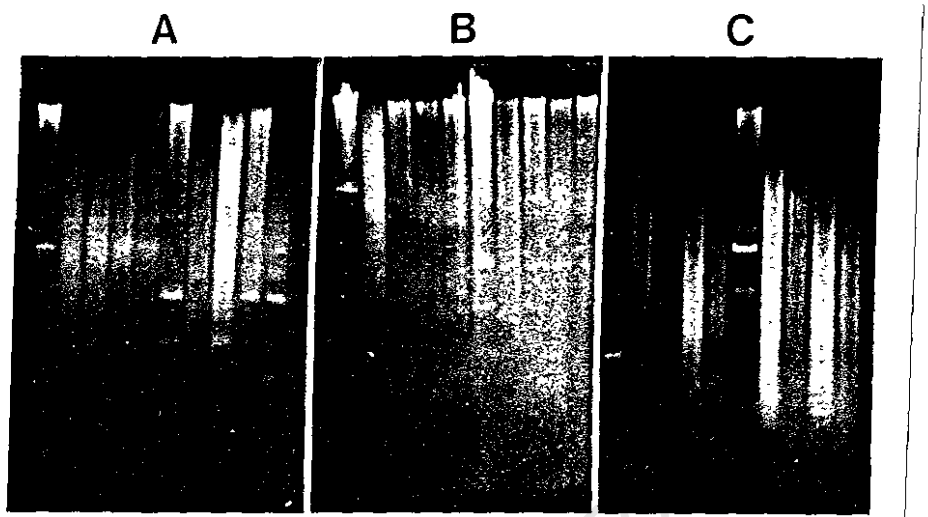

Fig. 5.2. Detection of metronidazole-induced DNA strand breaks. Total DNA extracted from metronidazole treated *E. coli* AB1157 (**A**), *E. coli* JC8679 *recBC sbcA* (**B**), and *E. coli* F19 *recA* (**C**) was separated by electrophoresis on alkaline gels. Lanes one to five of each gel contained DNA extracted from *E. coli* (pMT104) and lanes six to ten contained DNA extracetd from *E. coli* (pMT100). Lanes 1 and 6 of each gel contained DNA extracted from untreated cells; lanes 2 and 7 contained DNA extracted from cells treated for one hour with metronidazole (2000 μg/ml); lanes 3-5 and 8-10 contained DNA extracted from treated cells grown in the absence of metronidazole for 30 min, one hour and two hours, respectively. The experiment was performed under aerobic conditions using the *E. coli* strains AB1157 and JC8679, and under anaerobic conditions, using *E. coli* F19.

amount of DNA damage caused by metronidazole was quantified by differential precipitation, as described below.

5.3.3.2 Quantification of DNA strand breaks

TCA precipitates only large nucleic acid molecules, therefore, when using a 5% TCA solution, only DNA molecules more than 50 nucleotides in length will be precipitated onto the surface of the filter (Sambrook *et al.*, 1989). Therefore, the less DNA that is precipitated, the greater the DNA damage. To trace the precipitated DNA, the cells were grown in medium supplemented with

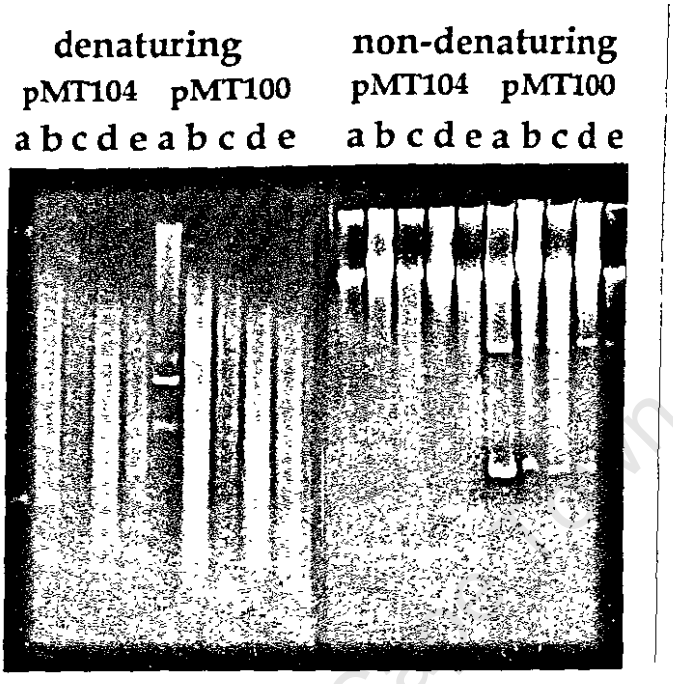

Fig. 5.3. Detection of single stranded breaks in total DNA extracted from *E. coli* F19 *recA* cells treated with metronidazole under anaerobic conditions. The alkaline gel, shown on the left, and the agarose gel on the right contained aliquots of the same samples of total DNA extracted from *E. coli* F19. Lane (a) contained DNA extracted from untreated cells; lane (b) contained DNA extracted from cells treated for one hour with metronidazole (2000 μg/ml); and lanes c-e contained DNA extracted from treated cells grown in the absence of metronidazole for 30 min, one hour and two hours, respectively.

[^{14}C]thymidine. The use of radiolabeled thymidine ensured the exclusive labelling of DNA.

Logarithmic phase *E. coli* AB1157, *E. coli* JC5519 *recBC*, *E. coli* JC8679 *recBC recE*$^+$ and *E. coli* F19 *recA* strains transformed with pMT100, or the control plasmid pMT104, were used under aerobic and anaerobic conditions for the TCA precipitation experiment. The experiment was repeated at least three times with every *E. coli* strain with similar results; the average of three results is depicted in Fig. 5.4.

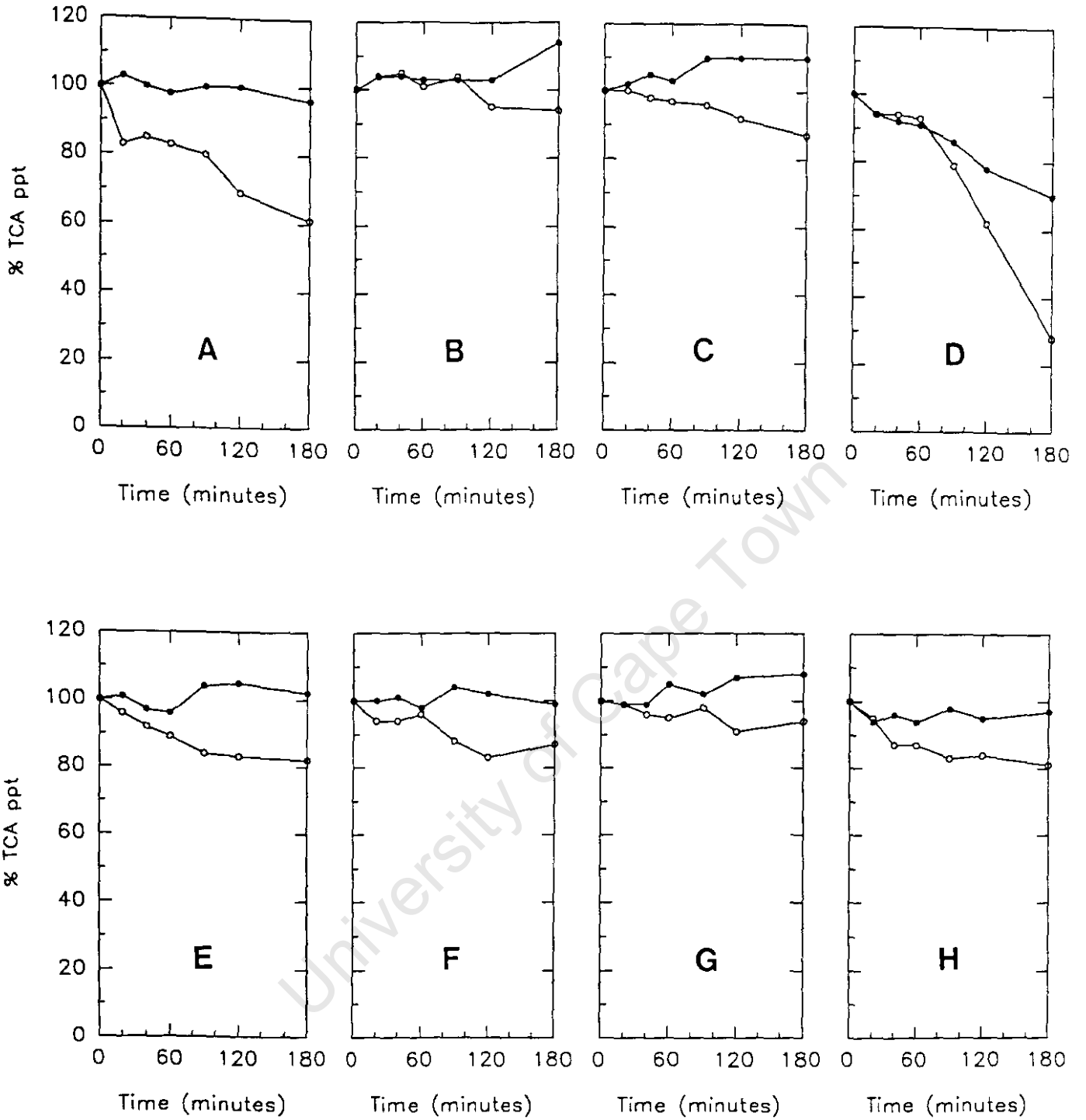

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Fig. 5.4. Quantification of DNA strand breaks induced by metronidazole. The percentage of TCA-precipitable DNA of *E. coli* strains transformed with pMT100 (●) or with the control plasmid pMT104 (○) under aerobic (**A-D**) and under anaerobic (**E-H**) conditions is shown. Samples were taken during a one hour growth period in the presence of metronidazole (2000 µg/ml), and during a two hour growth period without metronidazole. Graphs **A** and **E** show the results obtained from the treatment of *E. coli* AB1157 with metronidazole; graphs **B** and **F** of *E. coli* JC5519 *recBC*; graphs **C** and **G** of *E. coli* JC8679 *recBC sbcA*; and graphs **D** and **H** of *E. coli* F19 *recA*.

The concentration of metronidazole (2000 µg/ml) was chosen to be the same as that used in the denaturing gel experiments for comparative purposes. Raising the concentration to 4000 µg/ml did not alter the amount of TCA-precipitable DNA of the *E. coli* JC8679 strain (result not shown).

In the method of Simic *et al.* (1991) the cells are radiolabeled during overnight growth, followed by a centrifugation step and a wash, prior to exponential growth. This part of the method was modified, since the labelling was shown to be insufficient. The poor labelling was possibly due to the dilution of the label through cell division and DNA replication during logarithmic growth. The cells were therefore labeled during the exponential growth phase preceding the metronidazole treatment.

The results will be discussed in two sections: (1) the effect of metronidazole on the DNA of *E. coli*, and (2) the effect of metronidazole on the DNA of *E. coli* transformed with pMT100.

(1) The effect of metronidazole on the DNA of *E. coli*

Treatment of the *E. coli* (pMT104) strains with metronidazole caused the percentage of DNA precipitated by TCA to decrease, indicating that the DNA damage was due to strand breaks (Fig. 5.4.). The fraction of degraded DNA from the *E. coli* (pMT104) strains varied from only a few percent in the *E. coli* JC8679 strain to more than fifty percent in the *E. coli* F19 strain (Fig. 5.4.). Since all strains were treated with the same amount of metronidazole for the same amount of time under given oxygen conditions, these differences were possibly due to the different genotypes of the strains.

The amount of DNA strand breaks caused by metronidazole did not solely depend on the repair capacity of the cell, since under anaerobic conditions the *E. coli* AB1157 *recA*$^+$ *recBC*$^+$ strain had approximately the same amount of TCA-precipitable DNA as the *E. coli* F19 *recA* mutant strain (Fig. 5.4.). It did not depend solely on their metronidazole MIC either, since the *E. coli* F19 strain (MIC 400 µg/ml) had far less TCA-precipitable DNA than the *E. coli* JC5519 strain (MIC 400 µg/ml) under aerobic conditions (Fig. 5.4.). Also, even though anaerobically grown *E. coli* strains were more sensitive to metronidazole than

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aerobically grown strains (Chapter 4), the fraction of TCA-precipitable DNA was generally higher under anaerobic conditions (Fig. 5.4.).

It was observed that most of the DNA degredation occured during the repair period. Also, the *recBC* mutants contained less strand breaks than the other strains. It is therefore possible that most of the DNA strand breaks were not induced directly by the action of metronidazole, but were due to the enzymatic action of the repair enzymes on metronidazole-induced lesions. This possibility is discussed further in section 5.4.

(2) The effect of metronidazole on the DNA of *E. coli* transformed with pMT100

All *E. coli* (pMT100) strains had a higher percentage TCA-precipitable DNA as compared to *E. coli* (pMT104) strains, irrespective of the genotype of the strains or their metronidazole susceptibility phenotype (Fig. 5.4.). The pMT100 encoded gene product therefore appeared to reduce the amount of DNA strand breaks.

The pMT100 gene product could interact either directly with the damaged DNA or with the other repair enzymes to lower the amount of strand breaks after metronidazole treatment. The first option was unlikely since no DNA binding motif was detected in the ORF1 sequence (Chapter 3). It is therefore possible that the pMT100 encoded product interacted with the repair enzymes to reduce the amount of metronidazole mediated strand breaks. This interference could then lead to either an increased resistance (in *recA*$^+$ *recBC*$^+$ strains) or an increased sensitivity to metronidazole (in *recA* or *recBC* mutants), depending on the available repair enzymes (Chapter 4).

The amount of radioactivity precipitated from some of the *E. coli* (pMT100) strains after metronidazole treatment as compared to the water-treated samples was more than 100% (Fig. 5.4.). This was also detected by comparing the amount of TCA precipitable radioactivity at the end of the repair period to that of time zero of the metronidazole treated culture of some strains (results not shown). No unincorporated radiolabeled thymidine should have been present in the medium after the washes, since none was added after the start of the metronidazole treatment period, and therefore the total amount of radioactivity in the system should have been static.

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It is possible that in the metronidazole treated samples, more radioactive DNA per unit volume was added onto the filter due to filamentation of the cells as observed by microscopic examination (see section 5.3.4). This would account for a greater than 100% fraction of TCA precipitated DNA.

5.3.4 Other targets: cell lysis

Recently a mode of killing by metronidazole, which was due to rapid lysis of the cells, was proposed (Church *et al.*, 1991). This theory was tested on aerobically grown *E. coli* AB1157 (pMT100) and *E. coli* AB1157 (pMT104) strains, by determining the viable cell count and the amount of cell leakage caused by metronidazole. It was assumed that if a high rate of cell death was accompanied by a significant increase in cell leakage, the propable cause of cell death was cell lysis.

In the method by Church *et al.* (1991), the protein concentration of the minimal medium supernatant was determined as a measure of the amount of cell leakage. In order to do comparative studies, it was necessary to grow the *E. coli* strains for this experiment under the same growth conditions as those utilized for the strand break studies. The YT growth medium contains yeast extract and tryptone, which would interfere with the determination of the protein concentration. Since the *E. coli* β-galactosidase is a well studied cytoplasmic enzyme, the β-galactosidase activity in the supernatant was determined as a measure of cell leakage (Pardee *et al.*, 1959).

Under aerobic conditions, metronidazole (2000 µg/ml) had a bacteriostatic effect on the *E. coli* AB1157 strains (Fig. 5.5.). The difference in the viable cell count between *E. coli* AB1157 transformed with pMT100 or the control plasmid pMT104 was less than two-fold. *E. coli* AB1157 (pMT104) showed a slight decrease in survival during metronidazole treatment, followed by a slow but steady increase in survival during the repair period. *E. coli* AB1157 (pMT100) showed a slight increase during treatment, followed by a decrease in survival in the first hour of the repair period (Fig. 5.5.). Cultures treated with sterile water showed an exponential increase in viable cells over the entire period.

The degree of cell leakage caused by metronidazole was insignificant in aerobic *E. coli* AB1157 cells (results not shown). The percentage of the β-galactosidase activity that was present in the culture supernatant of cells treated with

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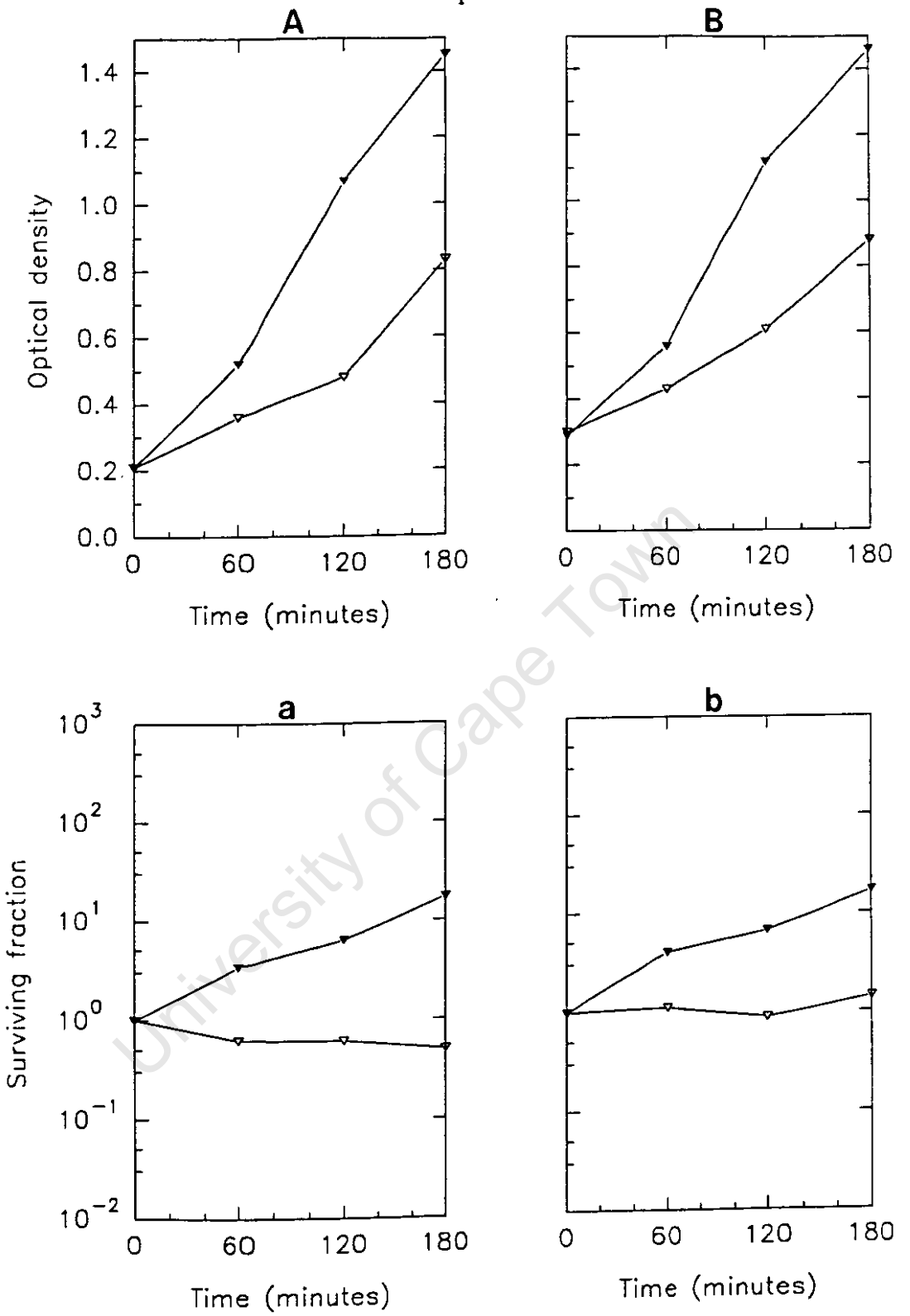

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Fig. 5.5. The effect of metronidazole treatment on the optical density (OD_{600}) and the viability of *E. coli* AB1157 transformed with the control plasmid pMT104 (**A** and **a**) or pMT100 (**B** and **b**). Samples were taken during a one hour growth period in the presence of metronidazole (2000 µg/ml), followed by a two hour growth period without metronidazole under aerobic conditions. Metronidazole treated (▽) and water treated cultures (▼).

metronidazole was not markedly different to that of cell cultures treated with water. Also, no significant difference in the amount of cell leakage was observed in cells with or without pMT100. It appeared, therefore, that metronidazole did not induce cell lysis in aerobically grown *E. coli* cells.

The optical density (OD_{600}) of the metronidazole treated cultures was followed throughout the experiments (Fig. 5.5.). It was noticed that the cell density increased during the metronidazole treatment and repair period, even though the viable cell count remained static or even decreased slightly. Microscopic analysis showed that the metronidazole treated *E. coli* AB1157 cells exhibited a filamentous morphology (Fig. 5.6.). This morphological response to metronidazole has been described before (Jackson *et al.*, 1984). The water treated cultures contained normal rod-shaped bacterial cells. The increase in cell density of the metronidazole treated cultures was therefore due to the lengthening of the cells instead of an increase in cell numbers.

The filamentation of the *E. coli* cells was an indication of the induction of the SOS response by metronidazole treatment (Kushner, 1987). The inhibition of cell devision is due to the function of the SOS inducible *sulA* gene (Cole, 1983).

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5.4 Discussion

The analysis of the *in vivo* responses of *E. coli* cells to metronidazole treatment is complex. Unlike *in vitro* studies, the living systems are affected by a wide variety of factors which influence the final result. The effect of metronidazole on the *E. coli* cells as a whole, and on the genetic material specifically, is influenced inter alia by oxygen and growth conditions, reducing enzymes, and a selection of DNA repair enzymes, which can act alone or in conjunction with other enzymes.

The study of the response of *E. coli* strains to metronidazole treatment showed that *E. coli* could reduce metronidazole to its active compound and thereby decrease the concentration of metronidazole in the medium even under aerobic conditions.

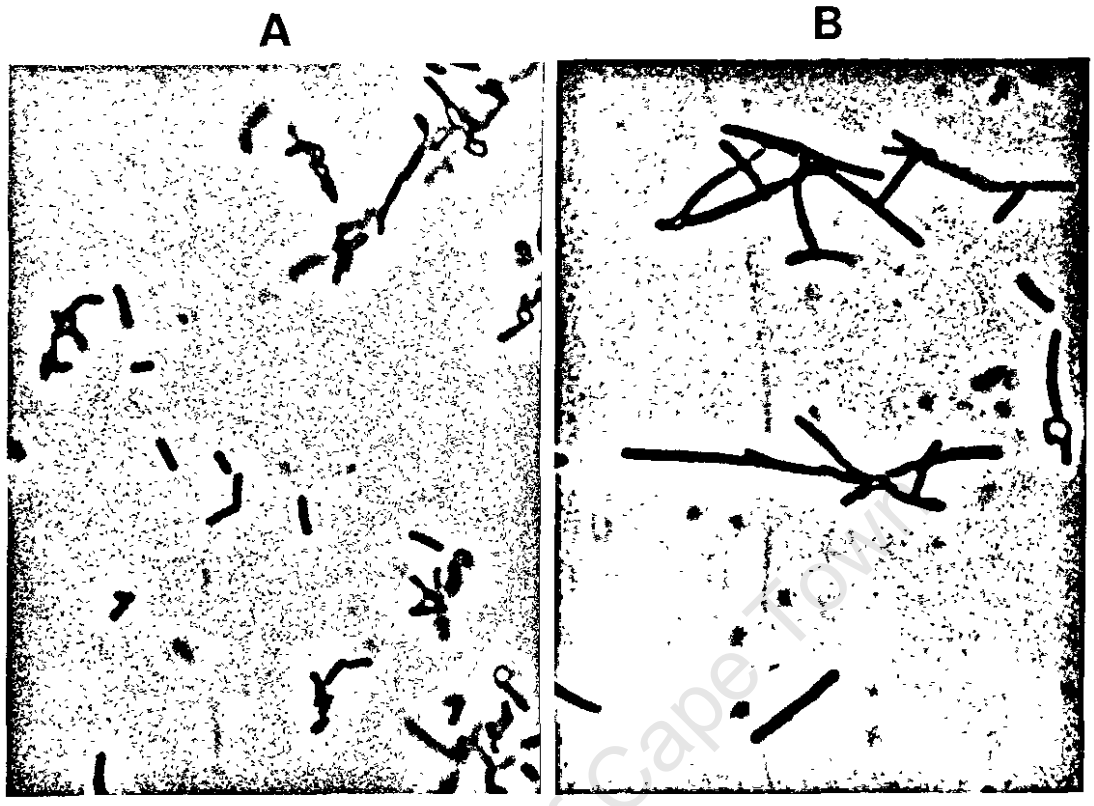

Fig. 5.6. Morphological changes of *E. coli* AB1157 cells treated with metronidazole. (A) Water treated culture; (B) metronidazole treated culture; magnification x400.

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Since the metronidazole susceptibility assays had indicated that the pMT100 product was possibly involved in DNA repair mechanisms, only preliminary uptake studies were done. In order to accurately assay the uptake of radioactive metronidazole the protocol would need to be revised. The problems could possibly be overcome by reducing the background of unspecific radioactivity with extra washes. Also the optimum growth conditions and the optimum concentration of metronidazole required to assay uptake would need to be established.

The analysis of the alkaline gel versus agarose gel results, with respect to the effect of metronidazole treatment on the DNA of *E. coli* F19 *recA* cells, showed that metronidazole mediated mainly single strand DNA breaks. Only insignificant amounts of double strand breaks could be detected on non-denaturing agarose gels. Although metronidazole-induced both single and

double stranded DNA breaks *in vitro* (Edwards, 1980), metronidazole-induced mainly single and not double stranded breaks *in vivo*.

An increase in intact, high-molecular weight DNA was observed towards the end of the repair period on the denaturing gels. The differential precipitation experiment showed no such recovery of the damaged, labeled DNA. It has therefore been suggested, that the increase of intact chromosomal and plasmid DNA detected on the alkaline gels was due to newly synthesized DNA strands, and not due to the repair of the damaged DNA strands. It therefore appears that metronidazole damage was removed by degrading the strand containing the lesion, followed by the resynthesis of the strand.

It needs to be pointed out, though, that alkaline conditions themselves could mediate DNA strand breaks at weakened points of the DNA backbone (Friedberg, 1985). It was unlikely, though, that the alkaline conditions played a principal role in causing DNA strand breaks, since DNA extracted from the control untreated cells did not show the degredation of the treated samples. Also, the *recBC* mutants showed less DNA degradation on alkaline gels than the DNA from the other two strains, although it was subjected to the same alkaline conditions.

The extent of DNA degradation could be due to the direct attack of reduced metronidazole on the DNA backbone, or due to the cellular response to metronidazole-induced lesions, or due to the combination of both, resulting in DNA strand breaks. The TCA precipitation results indicated that most of the strand breaks were enzymatically induced.

Simic *et al.* (1991) demonstrated DNA degradation caused by bleomycin, a potent anti-cancer drug, in different *E. coli* repair mutants using the TCA precipitation method. Bleomycin introduces breaks in the DNA by catalyzing the attack of free-radicals on the bases (Takeshita *et al.*, 1978). Strains lacking the RecBCD enzyme showed less DNA degradation than wild type strains following bleomycin treatment. The authors concluded that the RecBCD enzyme was essential for the processing of bleomycin lesions.

The RecBCD enzyme is believed to unwind the damaged DNA molecule providing a substrate for the RecA-mediated recombination repair (Weinstock,

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1987). The RecBCD enzyme exhibits exonuclease activity on double stranded DNA, and both exo- and endonuclease activity on single stranded DNA molecules *in vitro* (Taylor and Smith, 1980; Telender-Muskavitz and Linn, 1982). The RecBCD enzyme has also been shown to digest circular double stranded DNA as long as it contained single stranded gaps of at least five nucleotides in length (Telender-Muskavitz and Linn, 1982).

It is possible that the nuclease activities of the RecBCD enzyme on metronidazole-induced lesions were responsible for the observed DNA degradation, since the *recBC* strains contained significantly less DNA damage following metronidazole treatment. This suggested that the RecBCD enzyme digested DNA containing single strand breaks induced by metronidazole. The degradation of the damaged DNA appeared to be necessary prior to repair, since the repair wild type (*E. coli* AB1157) showed a significant amount of DNA degradation. This strain was more resistant to metronidazole than the mutant strains used in this study (Chapter 4).

The *E. coli* F19 *recA* strain showed a linear, rapid increase in strand breaks during the repair period. The degradation of DNA could be due to the uncontrolled exonuclease activity of the other repair enzymes in the absence of RecA, which has been observed previously in *recA* mutants (Clark, 1973). The *recBC* strains were shown to exhibit a 'cautious degradation' phenotype, *recA* strains showed a 'reckless degradation' phenotype, and *recA recBC* strains showed the same phenotype as the *recBC* strains, indicating that the RecBCD enzyme was under RecA control (Willetts and Clark, 1969). Brcic-Kostic *et al.* (1991) recently showed that the RecBCD enzyme is removed from the damaged site on the DNA by RecA.

Although pMT100 conferred different phenotypes to different *E. coli* repair mutants (Chapter 4), at the molecular level, the pMT100 encoded gene product affected the different *E. coli* DNA repair mutants in the same way. The pMT100 gene product decreased the amount of DNA degradation following metronidazole treatment in all strains tested. This suggested that the different metronidazole susceptibility phenotypes conferred by pMT100 are due to the different cellular responses to the interference of DNA degradation by the pMT100 gene product.

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Chapter 6
General Conclusions

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Chapter 6

General Conclusions

The antibiotic metronidazole is widely used in the clinical treatment of anaerobic infections, yet comparatively little is known about the mode of action of this drug *in vivo*, or the potential for bacterial cells to develop resistance mechanisms to it. The aim of the work reported in this thesis was to analyze aspects of the ways in which metronidazole may cause cell death, as well as the possible role played by *B. fragilis* genes in influencing the effectiveness of metronidazole in the treatment of infections of this important pathogen.

The facultative anaerobic bacterium *E. coli* provided a useful *in vivo*-system for this analysis for several reasons. *E. coli* can be cultivated under both aerobic and anaerobic conditions, its DNA repair systems are relatively well characterized, and a wide range of *E. coli* repair mutants is available. With the aid of these *E. coli* mutants, it was possible to screen for *B. fragilis* genes involved in the repair of metronidazole-induced DNA lesions. The mutants also enabled the *in vivo* study of the nature of metronidazole-induced DNA lesions in *E. coli*, and the investigation of the influence of the *B. fragilis* genes on the repair of these lesions. Several novel features emerged from these studies which require further examination.

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6.1 Metronidazole damage and repair in *E. coli*

Much of the published work on the nature of metronidazole damage to the DNA has involved *in vitro* analysis leading to the conclusion that DNA strand breaks are the major cause of cell death. The *in vivo* work reported in this thesis raises the possibility that this may not be the only mode of action of this antibiotic.

An analysis of the effect of metronidazole on different repair proficient and deficient *E. coli* strains (Chapter 4, Table 4.1.) showed a decrease in tolerance to metronidazole of repair deficient strains correlating with the loss of activity of the mutated repair enzyme. By calculating the residual repair capacity of a mutant with respect to the wild type strain (Table 6.1.), it was shown that the effect of a repair mutation on the susceptibility to metronidazole of the *E. coli*

strain was approximately twice as severe under anaerobic conditions as under aerobic conditions.

Table 6.1. Residual repair of metronidazole damage in DNA repair deficient *E. coli* strains under aerobic and anaerobic conditions.

Strains compared	Relevant genotype	O_2[a]	AnO_2[a]
AB1886/AB1157	*uvrA*	40%	20%
TK603/AB1157	*uvrA*	40%	23%
JC5519/AB1157	*recBC*	52%	20%
CC118/AB1157	*recA*	6.6%	3.3%
HB101/AB1157	*recA*	3.3%	2.5%

[a] The percentage of aerobic (O_2) and anaerobic (AnO_2) residual repair of the mutant relative to the wild type *E. coli* strain was calculated as follows: (MIC of mutated strain strain/MIC of wild type) times 100. The MIC values were obtained from Table 4.1.

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It was expected that the metronidazole MIC of any *E. coli* strain would be lower under anaerobic conditions as compared to aerobic conditions due to the increased reductive activation of metronidazole (Edwards, 1980). It was surprising, however, that the relative increase in sensitivity due to a mutation of a repair gene with respect to the wild type would differ between aerobic and anaerobic conditions. In other words, it was not expected that the residual repair capacity of a mutant should vary depending on the oxygen conditions. The consistent two fold difference in susceptibility under anaerobic compared to aerobic conditions could be occuring at three possible levels. (1) Under anaerobic conditions, metronidazole is taken up or activated more efficiently in the repair deficient mutants as compared to the repair wild type strain, (2) the repair enzymes are less efficient or not fully induced under anaerobic as compared to aerobic conditions, or (3) the lesions induced by metronidazole are different under aerobic and anaerobic conditions and cannot be recognized by these repair enzymes. To possibly distinguish these options a model is presented in

Figure 6.1. which illustrates the steps involved from the addition of metronidazole to the growth medium until cell death.

The model depicts a situation of minimum lethal dose, which is specific for each *E. coli* strain under specific oxygen conditions, and not a situation where the bacteria have been subjected to a constant amount of metronidazole. It is therefore possible to compare directly the steps from the addition of metronidazole until cell death of the two strains, because the effect, ie cell death, is the same.

The *uvrA* mutant strain is a direct derivative of the *uvrA*+ strain, and therefore the only genetic difference between the two strains is the loss of activity of the UvrA subunit, and with it the loss of the functional UvrABC exision repair complex. Under a given set of oxygen conditions (compare step A to step A', and step α to step α') a certain amount of metronidazole (M) is reduced to a certain amount of activated metronidazole (M*). The two strains have the same reducing abilities and grow under the same oxygen conditions, therefore step A is equivalent to step A' and step α is equivalent to step α'.

The *uvrA*+ strain can tolerate more metronidazole (750 µg/ml and 600 µg/ml, under aerobic and anaerobic conditions, respectively) than the *uvrA* mutant (300 µg/ml and 120 µg/ml). A given amount of activated metronidazole (M*) will cause a given amount of DNA damage (step B = step B'), which is in direct proportion to the amount of activated metronidazole (M*) in the cell. This is irrespective of the oxygen conditions of the media or the reducing abilities of the strain.

The obvious difference between the two strains in this model is their ability to repair the DNA lesions caused by the activated metronidazole (step C and step C'). The *uvrA*+ strain can repair more lesions than the *uvrA* mutant under both aerobic and anaerobic conditions.

A certain amount of unrepaired DNA lesions will cause cell death (step D = step D'). This is fixed for any *E. coli* cell and does not depend on the growth conditions or the genotype of the cell.

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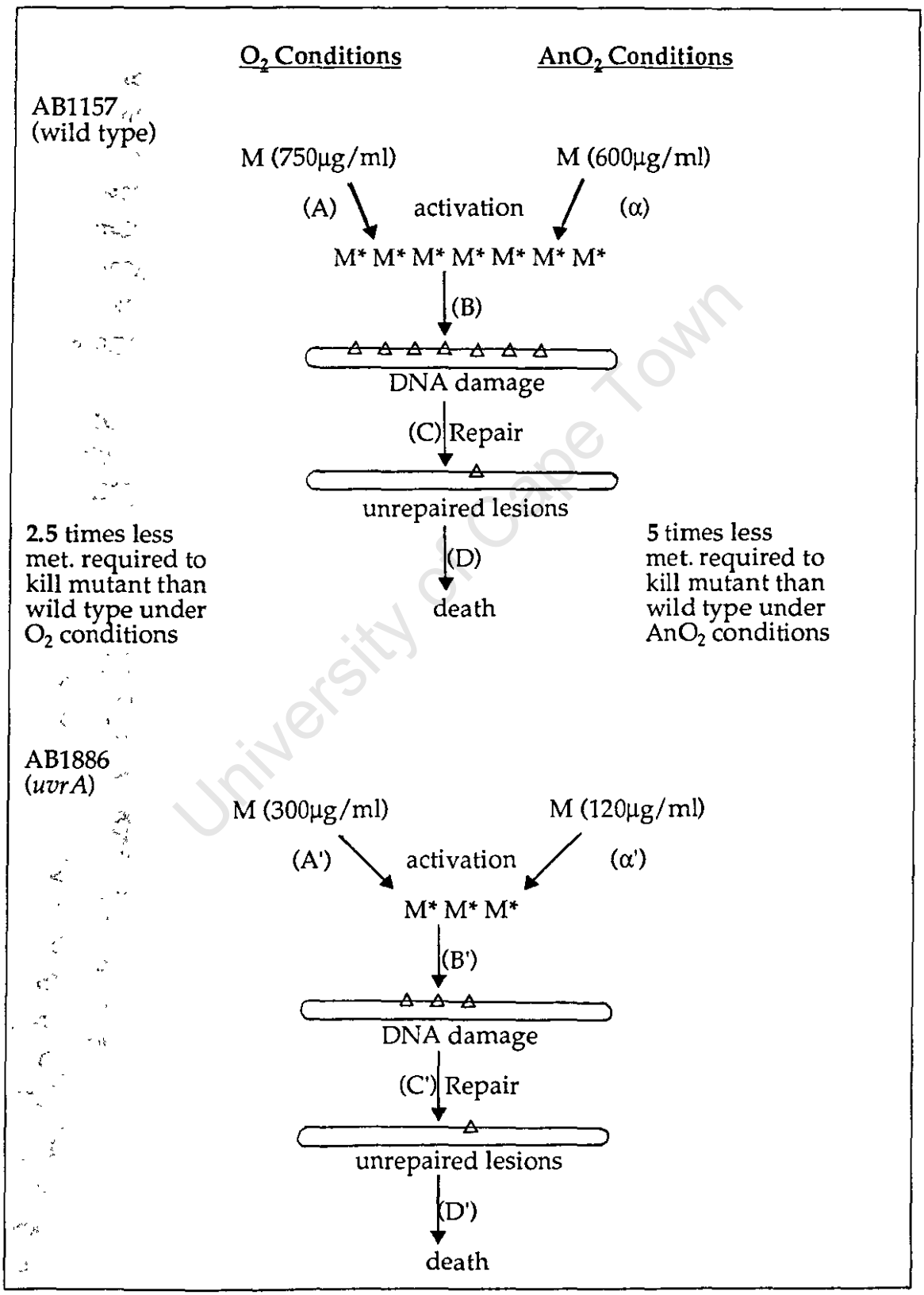
Fig. 6.1. Model of the mode of action of metronidazole in *E. coli*, using *E. coli* AB1157 and *E. coli* AB1886 *uvrA* as examples.



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The amount of metronidazole (M) needed to kill the *uvrA* mutant under aerobic conditions was 2.5 times less than that required to kill the wild type strain. However, under anaerobic conditions, five times less metronidazole was required to kill the mutant as compared to the wild type strain. All other steps, when comparing the two strains, are equal (A=A' and α=α';B=B'; D=D'). And, in a given strain at the minimal lethal dose, the same amount of activated metronidazole is present within the cell under aerobic and anaerobic conditions, which induces the same amount of DNA damage under aerobic and anaerobic conditions, and the same amount of unrepaired lesions will cause cell death of a given strain under aerobic and anaerobic conditions. From these observations the following conclusions can be drawn.

(1) Metronidazole uptake and activation

The only genetic difference between *E. coli* AB1157 and the *E. coli* strains AB1886 *uvrA*, TK603 *uvrA* and JC5519 *recBC*, is the loss of activity of a DNA repair enzyme, since the mutant strains are direct derivatives of *E. coli* AB1157. Although the *recA* strains are not directly related to *E. coli* AB1157, they contain no known mutations in genes that encode possible metronidazole activating enzymes. The relative increase in sensitivity of the repair mutant strains with respect to the wild type strain under anaerobic conditions is therefore probably not due to the enhanced uptake or activation of metronidazole by the repair deficient strains. Detailed studies of the reducing capabilities of the strains would need to be performed to establish this experimentally.

(2) Difference in efficiency or induction of repair enzymes

A repair proficient strain can repair more metronidazole-induced lesions than repair deficient mutants under both aerobic and anaerobic conditions. A repair wild type strain is therefore more resistant to metronidazole than a repair deficient mutant. It is conceivable that the two-fold relative increase in sensitivity to metronidazole of the repair deficient mutants as compared to the wild type strain could be due to a difference in the functioning of the repair enzymes.

The enzymes involved in metronidazole repair are also involved in the repair of UV lesions (Yeung *et al.*, 1984; Jackson *et al.*, 1984). These repair systems have been analyzed under aerobic and anaerobic conditions in the repair of UV induced DNA damage. *E. coli* strains irradiated with far UV irradiation did not

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show a significant difference in survival whether irradiated under aerobic or anaerobic conditions (Webb, 1977). This indicates that the repair enzymes function as well under aerobic as under anaerobic conditions, and that the disproportional increase in sensitivity of the repair deficient mutants under anaerobic conditions may not be due to the difference in efficiency or induction of the repair enzymes.

In order to monitor the levels of the repair enzymes produced under aerobic and anaerobic conditions, an analysis of the damage-induced proteins of the metronidazole treated cells would need to be performed. The analysis would include a comparative study of the pattern of total proteins extracted from treated and untreated cells, as well as studies using Western blots with antibodies made against specific DNA repair enzymes.

(3) Different lesions induced by metronidazole under aerobic and anaerobic conditions

It is possible that the observed relative increase in sensitivity to metronidazole under anaerobic conditions was caused by a type of DNA lesion being formed that required the repair function of a specific repair enzyme, and that the loss of functionality this enzyme caused the observed increase in sensitivity. A disproportionate increase in metronidazole sensitivity was, however, observed in strains mutated in either the *uvrA*, *recA* or *recBC* genes. It is possible that metronidazole may have caused lesions which require the combined repair function of all these repair enzymes.

The reactive intermediate of metronidazole has not yet been identified (Tocher and Edwards, 1992). It is possible that different intermediates are formed via the proposed one-electron reduction steps from the inert metronidazole molecule (Mueller, 1983). The ratio of the different derivatives of metronidazole may vary under aerobic versus anaerobic conditions, thereby causing a different ratio of types of DNA lesions.

The effect of metronidazole on *E. coli* strains at the molecular level was investigated in Chapter 5. The amount of DNA strand breaks detected by differential precipitation was generally less under anaerobic conditions as compared to aerobic conditions, although the sensitivity of the cells to metronidazole was increased by anaerobic conditions. It has been suggested that

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the DNA degradation was due to enzyme action on metronidazole-induced DNA lesions (Chapter 5). Either the repair enzymes functioned less efficiently under anaerobic conditions, or the type of lesion that was targeted by the repair enzymes, RecA, RecBCD or RecE, was present at reduced amounts under anaerobic conditions. As stated before, it is unlikely that the repair enzymes were affected by anaerobiosis. The results obtained from the DNA strand break assays therefore support the suggestion that metronidazole may induce different types of DNA lesions under different oxygen conditions. It appears, therefore, that metronidazole may not induce DNA strand breaks as the only mode of killing under anaerobic conditions in these *E. coli* strains.

To further investigate the induction of strandbreaks by metronidazole *in vivo* and to separate the metronidazole-induced lesions from the enzyme mediated DNA degradation, the parent and daughter strands need to be distinguished. This can be accomplished by pulse labeling uniformly [^{14}C]-labeled DNA with [^{3}H]thymidine after the addition of metronidazole to the culture (Howard-Flanders and Rupp, 1981). The extracted double-labeled DNA is then analyzed using alkaline sucrose sedimentation. This method will show the extent of gap formation in the newly synthesized DNA strand. To analyze the way in which these gaps are repaired, the newly synthesized DNA is additionally labelled with the heavy isotope containing bromodeoxyuridine. By separating the heavy and light chains on a cesium chloride density gradient, it is possible to detect sister strand exchanges. Strands produced through recombinational repair will have an intermediate molecular weight.

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6.2 The mode of action of the pMT100 encoded gene product

Resistance and sensitivity to metronidazole were used as selection methods for the cloning of *B. fragilis* genes affecting DNA repair mechanisms and metronidazole activation in *E. coli*. Genes from the naturally occuring plasmid pBFC1 from *B. fragilis* Bf-2 were cloned on the recombinant plasmid pMT100, which conferred both increased resistance and increased sensitivity to metronidazole to different *E. coli* strains. The presence of pMT100 in *E. coli* strains reduced the amount of DNA strand breaks mediated by metronidazole.

The locus affecting metronidazole resistance, metronidazole sensitivity and UV sensitivity was located on a 1.6-kb DNA fragment which was sequenced. An open reading frame (ORF1) of 195 bp encoded a protein of 64 amino acids with a

predicted M_r of 7.3 kDa. No significant sequence similarity to genes or proteins of the database was detected. The pMT100 encoded gene product is small and therefore unlikely to interact with more than one protein. Since it conferred such diverse phenotypes on the different *E. coli* strains, it is possible that the pMT100 encoded protein interfered with the activity of an *E. coli* multifunctional repair regulatory protein.

In order to define a possible mode of action of the pMT100 encoded gene product in causing the various phenotypes, the effect on *E. coli* repair mutants treated with metronidazole in the presence of pMT100 was examined. Several general conclusions may be drawn from these studies.

Metronidazole treatment and far UV irradiation possibly require different repair pathways, since, in the case of metronidazole, growth occurs in the presence of the damaging agent, and in the case of far UV irradiation, growth occurs in the absence of the damaging agent. It is possible that the metronidazole-induced DNA damage may require mainly the recombinational repair pathway, whereas the UV induced damage may require mainly the excision repair pathway.

Far UV irradiation produces pyrimidine dimers, which can be repaired by either the excision repair or the recombinational repair pathway. Excision repair requires a double stranded template and can therefore not function in the replicating areas of the chromosome. Daughter strand gaps opposite a lesion are consequently repaired via the recombinational repair pathway during cell division. Metronidazole-induced lesions are possibly repaired via the excision repair pathway during stationary phase growth, and via recombinational repair during exponential growth.

The two pathways both require the RecA protein. The RecA protein acts in strand exchange in its unactivated form, and mediates repressor cleavage in its SOS activated form (Walker, 1984). In order to explain the increased resistance or sensitivity to metronidazole accompanied by the increased sensitivity to UV in the *E. coli* strains after transformation with pMT100, it may be proposed that the pMT100 encoded gene product interfered with the two RecA promoted functions. Since pMT100 conferred a phenotype on *recA* mutants, it is unlikely that the pMT100 encoded gene product interacted directly with RecA.

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A possible candidate would be RecF. The RecF protein has been implicated in the activation of RecA (Sassanfar and Roberts, 1991). The RecF protein is also known to bind to single stranded DNA and is believed to thereby provide a substrate for RecA mediated recombination (Madiraju *et al.*, 1988; Griffin and Kolodner, 1990). The RecF-produced substrate may be sensitive to exo 1 (Clark *et al.*, 1984). Mutants lacking the *recF* gene have been shown to be selectively sensitive to far UV irradiation, whereas they showed no increased sensitivity to agents causing DNA strand breaks (Horii and Clark, 1973; Armegod and Blanco, 1978).

The RecF protein has been shown to be required in the RecE pathway (Gillen *et al.*, 1981), which was shown in this study to interact with pMT100 (Chapter 4). The *E. coli* strains used in this study were all *recF*+. It is conceivable that the pMT100 encoded protein interacted with RecF, which would interfere in RecA activation. The interaction with RecF would also result in the observed decrease in strand breaks following metronidazole treatment, by interfering with the production of a substrate for exo I. This may lead to increased sensitivity to UV and, depending on the residual repair enzymes, possibly to an increased resistance or inreased sensitivity to metronidazole.

To prove this hypothesis, *E. coli* strains carrying different *recA* mutations, such as *recA441* (coprotease more easily activated) and *recA430* (defect in coprotease activity), and different *lexA* mutations, such as *lexA*(Ind-) (protease resistant LexA) and *lexA*(Def) (defective repressor) (Walker, 1984), and strains containing *recF* and *sbcBC* mutations would need to be assayed for their metronidazole susceptibility after transformation with pMT100. If the theory holds, it would be expected that *recF* mutant strains would exhibit a similar UV survival curve as strains containing pMT100.

The involvement of other genes which are required in the RecE pathway and that affect RecA, such as the *recJ* or *ruv* genes, can not be excluded, and would need to be tested.

The pMT100 encoded protein has been suggested to interact with the UvrC subunit to reduce cell survival after far UV irradiation (Chapter 4). *E. coli uvrC* (pMT100) exhibited similar UV sensitivity but increased metronidazole resistance as compared to *E. coli uvrC*. Although it is possible that the pMT100 encoded gene product interacted with UvrC to decrease cell survival in *uvrC*+

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strains after UV irradiation, it could not have interacted with UvrC to increase resistance to metronidazole in the *uvrC* mutant. It is therefore unlikely that the pMT100 encoded gene product interacted with UvrC.

The interaction of the excision repair pathway and the RecF pathway is not well understood. The theory of the interaction of the pMT100 encoded protein with RecF may therefore still hold.

A recent report on mutation repair may prove to be the key to understanding the functioning of pMT100 in *E. coli* (Little *et al.*, 1991). The plasmid pKM101, which carries the *muc* genes under the control of LexA, has been shown to protect *E. coli* strains against UV irradiation (Walker, 1984). However, a derivative of pKM101, pGW16, was shown to sensitize some strains of *E. coli* to far UV irradiation (Little *et al.*, 1991). pGW16 was shown to sensitize *E. coli* AB1157 *uvrB*$^+$*umuC*$^+$ to low doses of UV (up to 75 J/m^2), but protected the strain against high doses. It sensitized a UvrB deficient *E. coli* strain to all UV doses tested. These findings are similar to the results reported in this thesis using pMT100. *E. coli* strains transformed with pMT100 were, however, not tested at higher UV doses, and it is therefore not known whether pMT100 could protect *E. coli* strains against high doses of UV.

The *E. coli* strains used in this thesis are all *umu*$^+$. pMT100 increased the resistance to metronidazole in *E. coli recA*$^+$*recBC*$^-$ strains, increased sensitivity to metronidazole in *recA* and *recBC* mutant strains, and increased sensitivity to UV in all *E. coli* strains tested. The interaction between the Umu proteins and the recombination proteins, which are studied here, has not been elucidated. The effect of pMT100 on UmuDC deficient strains would therefore need to be investigated.

pGW16 was shown to increase the rate of uptake of radiolabeled thymidine following UV irradiation, and also increase post-UV DNA synthesis in *E. coli* cells. The presence of pMT100 in the *E. coli* strains tested in this work, decreased the amount of DNA strandbreaks following metronidazole treatment. It is possible that an increase in DNA synthesis could result in a decrease in the observed DNA strandbreaks, due to recombinational repair of the lesions.

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The effect of pGW16 on the survival of *S. typhimurium*, which exhibits reduced UmuD activity, was shown to be dependent on the type of damaging agent (Podger and Hall, 1984). pGW16 protected the *S. typhimurium* strain against UV irradiation, but sensitized it to gamma irradiation and bleomycin, both of which are DNA strand breaking agents. The molecular basis of these findings is not understood, although different repair pathways may be involved.

The *mucAB* genes on pKM101 are under tight LexA control, which prevents mutagenesis in uninduced cells (Walker, 1984). Plasmid pGW16 was shown to contain a mutation in one of the two LexA binding sites that both decreases LexA binding and increases transcription of the *mucAB* operon (Walker, 1984; McNally *et al.*, 1990). The overproduction of Muc or Umu proteins was shown to inhibit SOS induction possibly due to a competition between these proteins and LexA for activated RecA (Marsh and Walker, 1987; Blanco *et al.*, 1986). Plasmid pMT100 was shown to be regulated by the λ promoter of the *E. coli* vector, and appeared to lack its own *B. fragilis* promoter (Chapter 3). It is therefore possible that the uncontrolled expression of the pMT100 encoded gene product resulted in the observed phenotypes.

No sequence similarity was detected between ORF1 of pMT100 and the *mucAB* genes of pGW16 (Chapter 3). This may not exclude a functional similarity, since, although, the known mutagenic repair enzymes are related, their sequences are not homologous. The amino acid similarity between the chromosomal UmuDC proteins and their plasmid analogues varies from 42% to 83% (Woodgate and Sedgwick, 1992).

It was suggested that the overproduction of Muc proteins by pGW16 results in sensitization of *E. coli* strains to UV irradiation due to an 'over-involvement' of the Muc protein, which results in overactive, detrimental DNA repair (Little *et al.*, 1991). It is feasible that the pMT100 encoded gene product functions in an analogous way in sensitizing *E. coli* to UV and metronidazole. pMT100 may carry a gene which is part of the *B. fragilis* mutation repair system. To investigated this, the spontaneous and induced mutation rate of *E. coli* strains transformed with pMT100 would need to be studied.

The exact function of the gene which originated from the *B. fragilis* plasmid pBFC1 has not been established, although it was shown to encode a gene product

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that interfered with the *E. coli* DNA repair mechanisms by reducing the amount of metronidazole-induced DNA strand breaks. It is possible that the pBFC1 encoded gene product functionally resembled an *E. coli* enzyme and recognized the target of that enzyme, possibly a controlling protein. This would interfere with the normal functioning of the controlling protein.

A phenomenon called negative complementation by foreign genes that functionally resemble *E. coli* genes has been reported (Prudhomme *et al.*, 1991). The genes encoding the mismatch repair system of *S. pneumonia*, when introduced into wild type *E. coli*, inhibited the resident *E. coli* mismatch repair system. It was suggested that the *S. pneumonia* mismatch repair proteins recognized the same lesions and specifically interfered with the *E. coli* repair enzymes.

The *B. fragilis* strain containing pBFC1 showed increased resistance to metronidazole as compared to two plasmid-free *Bacteroides* strains. Future work would need to analyse the function of the pMT100 encoded gene product in its homologous host, *B. fragilis*. Also, the isolation of other *B. fragilis* DNA repair genes involved in the repair of metronidazole damage would aid in the understanding of the mode of action of this important antibiotic agent against the pathogen *B. fragilis*.

Woodgate and Sedgwick (1992):'...it is always possible that...genes have quite different functions to those studied in the laboratory'...'It would have been an extremely tortuous process to have reached our current understanding of...chaperone proteins from a starting point in mutagenic DNA repair'.

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Appendix

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Appendix

A1. Standard methods and techniques

A1.1 Small scale isolation of plasmid DNA (miniprep)

Plasmid was isolated from a 5 ml overnight culture in the presence of the appropriate antibiotic as described by Ish-Horowicz and Burke (1981). Cells from a 1.5 ml sample of the culture were harvested by centrifugation in an Eppendorf microfuge tube for 1 min. The pellet was resuspended in 200 µl Solution I (50 mM glucose; 25 mM Tris-HCl, pH 8.0), incubated for 5 min at room temperature, and then 400 µl of Solution II (0.2 M NaOH, 1% (w/v) SDS) was added. The sample was vortexed briefly and incubated for 5 min at room temperature, before the addition of 300 µl Solution III (5 M KOAc, pH 4.8). The sample was vortexed briefly, and, after 5 min, cellular debris and denatured chromosomal DNA were pelleted by centrifugation for 5 min. The supernatant (750 µl) was removed to a fresh tube, an equal volume of isopropanol was added and the DNA precipitated by centrifugation for 5 min. The DNA pellet was resuspended in TE (600 µl) (A2.) before adding $NaClO_4$ (60 µl, 5 M) and an equal volume of isopropanol, and precipitating the DNA by centrifugation for 15 min. The DNA pellet was washed with 70% ethanol, air dried and resuspended in 20 µl TE buffer.

A1.2 Large scale isolation of plasmid DNA (maxiprep)

A 200 ml culture was grown overnight at 37°C in the presence of the appropriate antibiotic. The cells were harvested by centrifugation at 5 000 rpm for 5 min and then resuspended in 4 ml Solution I. After 5 min at room temperature 8 ml Solution II was added, and the mixture was kept on ice for 5 min, before the addition of 6 ml ice cold Solution III. After a further 5 min on ice the cellular debris was removed by centrifugation at 10 000 rpm for 10 min. An equal volume of isopropanol was added to the supernatant and the DNA was precipitated by centrifugation at 15 000 rpm for 15 min. The pellet was washed with 70% ethanol and resuspended in 4.2 ml TE buffer, and purified by isopycnic CsCl-EtBr ultracentrifugation (Maniatis *et al.*, 1982). The plasmid preparation was prepared for ultracentrifugation by the addition of CsCl (1 mg/ml) and EtBr (0.5 ml of a 10 mg/ml stock). The solution was centrifuged at 15 000 rpm for 15 min to precipitate any remaining protein debris. The refractive index of the supernatant was adjusted to 1.396, the sample sealed in Beckman Quickseal ultracentrifuge tubes and centrifuged for 12 h at 55 000 rpm at 15°C in a Beckman Vti 65.2 rotor. The plasmid DNA band was visualized by long wave UV light (350 nm), and

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removed in the smallest volume possible. The EtBr was removed by extraction (3 times) with equal volumes of NaCl-saturated isopropanol. The DNA was precipitated from the CsCl solution by the addition of two volumes of water followed by an equal volume of isopropanol, and centrifugation in an Eppendorf microfuge for 15 min. The pellet was resuspended in 100 µl TE buffer and the concentration was determined spectrophotometrically by measuring the absorbance of a diluted sample between 220 and 310 nm. The concentration was determined by using the relationship $OD_{260} = 1$ for 50 µg/ml double-stranded DNA.

A1.3 Restriction endonuclease digestion

Restriction endonuclease digestion was carried out as described by Maniatis *et al.* (1982). Restriction endonuclease digestions were performed in a total volume of 20 µl in the presence of 1 unit of restriction endonuclease enzyme per 100 ng of DNA. Restriction endonuclease buffers obtained from the suppliers of the restriction endonucleases (Anglian, Boehringer Mannheim and Amersham), were used as recommended. Restriction endonuclease digestions were incubated at the recommended temperatures for 1-5 h.

For electrophoretic analysis, the digestions were terminated by the addition of 5 µl DNA loading solution (A2.) to the 20 µl digestions. If the digestion products were to be ligated, or filled in before ligation, they were purified by a 1/10 phenol-ether extraction. The digestion products were diluted with sterile distilled water (380 µl), and TE-saturated phenol was added (40 µl; A2.). After vortexing briefly, the phenol was completely removed by extracting several times with water-saturated ether. The DNA was precipitated from the aqueous phase by the addition of one-tenth volume of 3 M sodium acetate (pH 4.8), and 2 volumes of 95% ethanol, cooling to -70°C for 5 min, and centrifuging for 30 min in a microfuge at 4°C. After centrifugation the pellet was washed with 70% ethanol, dried and resuspended in TE buffer.

A1.4 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out using a horizontal submerged gel system. Tris-acetate EDTA (TAE) buffers (A2.) was used routinely. Sigma type II agarose was used at varying concentrations (0.8%-1.2%). The amount of DNA loaded per lane also varied with the sizes and number of fragments, but under normal circumstances about 300 ng of plasmid DNA was used. The gels were electrophoresed at 2 V per cm for 16 h. Gels were stained in electrophoresis buffer containing EtBr (0.5 µg/ml) for 15-30 min. DNA bands were visualized

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using a 254 nm transilluminator. A 310 nm transilluminator was used if the DNA was to be recovered from the gel.

Gels were photographed using a Polaroid CU-5 Land camera fitted with a red filter and a fixed focal length attachment. Polaroid type 667 film (ASA 3 000) was used with an exposure time of 1-2 sec at f4.7. If a negative was required then a Polaroid type 665 film (ASA 64) with an exposure of 120-140 sec at f4.7 was used.

DNA fragments were sized according to standard curves prepared by plotting the mobility against the log molecular mass of DNA fragments of known mass. Standard DNA fragments were obtained by the restriction endonuclease digestion of λ DNA with *Pst*I, *Hin*dIII or *Eco*R1.

A1.5 Alkaline agarose gel electrophoresis

The method was adapted from Maniatis *et al.* (1982). The agarose gel was prepared in a neutral, unbuffered solution and the solidified gel was equilibrated for 1 h in alkaline buffer before running, since NaOH hydrolyses agarose polymers and prevents them from solidifying. Before loading, 5 µl of alkaline tracking dye was added to 20 µl of sample. The samples were electrophoresed in a 0.5 % agarose gel for 20-24 h at 20 volts. After electrophoresis the gel was neutralized for at least 30 min in 0.1 M Tris buffer, pH 8.0, followed by staining with ethidium bromide (0.5 µg/ml) in fresh 0.1 M Tris buffer, pH 8.0, for 30 min. The gel was visualized on a short wave length viewing box.

A1.6 DNA ligation reactions

DNA ligation reactions were of two basic types: recircularization of plasmids for the isolation of deletion clones (use low DNA concentrations, 1 pmole DNA/ml) and recombination reactions when subcloning insert fragments into vectors (use 5-15 pmole DNA/ml). DNA concentration was calculated using the formula 1 pmole=(0.662 x kb)mg. Vector and insert DNA were added to the ligation reactions at a molar ratio of 1:2.

Ligation reactions containing DNA, ligation buffer (A2), ATP (A2.) and water to the required volume, were performed in sterile microfuge tubes. Sticky-end ligations were performed at room temperature for 3 h or at 15°C overnight using 0.1-0.25 units of ligase, whereas blunt-end ligations were performed at room temperature for 3-20 h using 20-100 x more ligase.

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A1.7 Rapid subcloning protocol using gel purification

The rapid subcloning protocol of Struhl (1985) was used. The DNA fragments were separated by electrophoresis through low melting point (LMP) agarose (0.8%) (Seaplaque^R) in Tris-acetate buffer (50 mM, pH 8.2, no EDTA, no EtBr). The gel was stained with EtBr after electrophoresis and the DNA bands were viewed under UV light (310 nm), as briefly as possible. The desired bands were excised using sterile scalpel blades, in as small a volume as possible. The gel slices were melted at 70°C for 5 min in a microfuge tube and the required amounts (2 µl vector DNA, 8 µl insert DNA) were added hot to the prepared ligation mixture containing ligation buffer, ATP, ligase and water (10 µl). The ligation was incubated at room temperature for 3 h. Before transformation of *E. coli* competent cells, the gelled ligation reactions were melted at 70°C for 5 min, and then diluted with 4 volumes of TSB solution (A2.).

A1.8 The preparation and transformation of competent *E. coli* cells

E. coli cells were made competent for DNA uptake according to the method of Chung and Miller (1988). A 1/100 dilution of an overnight *E. coli* culture in LB was inoculated into 25 ml prewarmed LB and incubated at 37°C, with shaking, until the culture had reached mid-exponential phase (A_{600}=0.6) (3 h). The cell culture was poured into a pre-cooled sterile SS34 tube and the cells were harvested at 5000 rpm for 5 min at 4°C. The cell pellet was resuspended in 2.5 ml (1/10 volume) ice-cold transformation and storage buffer (TSB) (A2.) and held on ice for 10 minutes. The *E. coli* cells (100 µl) were then mixed with DNA (routinely 50 ng) and held on ice for a further 30 min. TSB solution (0.9 ml) containing glucose (20 mM) was added to each transformation mixture and incubated at 37°C for 60 min, to allow expression of the plasmid borne antibiotic marker.

Unused cells could be stored at -70°C after rapid freezing in a dry ice/ethanol bath or liquid nitrogen and retained viability provided that the cells were thawed slowly on ice when needed.

A1.9 Exonuclease III shortening

Exonuclease III (exo III) digestion was carried out by a modification of the method of Henikoff (1984). Plasmid DNA (12 µg), previously double digested with appropriate restriction endonucleases, was resuspended in 100 µl Exo-buffer and equilibrated at 37°C for 5 min. Eleven microfuge tubes containing 25 µl of ice cold S1 nuclease mixture were prepared and kept on ice before the shortening reaction was started. At t=0 a 9 µl sample was removed before the addition of exo III to act as undigested control. The shortening reaction was

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carried out at 37°C and initiated by the addition of 300 units of exo III. Samples of 9 µl were removed at 20 second intervals and added to the microfuge tubes containing the S1 nuclease mixture. The microfuge tubes were then incubated at room temperature for 30 min for the S1 nuclease to digest single stranded DNA. The S1 nuclease reaction was stopped by the addition of 3.4 µl/tube of S1 stop solution, followed by incubation at 70°C for 10 min. The exo III-generated ends were filled in by the addition of 1 unit/tube of Klenow enzyme in 3.4 µl Klenow buffer, incubation at room temperature for 3 min, followed by further incubation of 5 min in the presence of a mixture of each dNTP (0.125 mM each). The shortened DNA was blunt end ligated by the addition of 120 µl of ligation mixture to each tube. Competent *E. coli* LK111 cells were transformed with the ligation mixtures and transformants were selected on YT (Ap 100 µg/ml) agar plates.

A1.10 Nucleotide sequencing

Primer annealing reaction: The supercoiled DNA (6-10 µg, in TE buffer) was diluted to a final volume of 20 µl in distilled water. Alkaline denaturation in 0.2 N NaOH (5 min at room temperature) was followed by the addition of 5 µl of 3 M sodium acetate (pH 5.2), 25 µml of distilled water and 150 µl of chilled ethanol. This mixture was chilled to -70°C, centrifuged at 4°C for 20 min in a microfuge and washed with 200 µl of ethanol (70%). The DNA pellet was dried and resuspended in a final volume of 10 µl of sequencing buffer (40 mM Tris-HCl, pH 7.5; 20 mM $MgCl_2$; 50 mM NaCl) and 12 ng of primer. This mixture was annealed for 30 min at 40°C immediately prior to sequencing. The forward sequencing primer as supplied in the Sequenase DNA sequencing kit (US Biochemical Corp., Cleveland, Ohio) and the M13 forward and reverse sequencing primers (Amersham) were used.

Sequencing reactions: DNA sequencing was done by the dideoxynucleotide triphosphate chain termination method of Sanger *et al.* (1977) according to the protocol of Tabor and Richardson (1987), using T7 DNA polymerase and a Sequenase sequencing kit supplied by the US Biochemical Corporation, Cleveland, Ohio. The DNA chain was radiolabelled with [^{35}S]dATP (1200 Ci/mmol; Amersham).

Gel electrophoresis and autoradiography: The sequencing reactions were analyzed on standard 6% denaturing acrylamide urea sequencing gels. The composition and running conditions of the gels were as described in the Amersham M13 Sequencing Handbook. After electrophoresis the gels (0.2 mm

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thick) were dried onto Whatman No. 3 filter paper using a Dual Temperature Slab Gel Dryer (Model 1125B; Hoefer Scientific Instruments, San Francisco). Gels containing ^{35}S-labelled DNA were placed under XAR-5 autoradiographic film and exposed for 1-2 days. The autoradiographs were developed using Kodak GBX X-ray developer and fixer.

A1.11 DNA alkali blotting procedure

DNA fragments resolved by agarose gel electrophoresis were transferred to a Hybond N+ hybridization membrane (Amersham) essentially by the protocol of Reed and Mann (1985). The use of a nylon transfer membrane allows the capillary transfer of DNA restriction fragments in alkali and eliminates the need for post-transfer fixation (Reed and Mann, 1985). After electrophoresis the gel was rinsed in 2 volumes of HCl (0.25 M) for 20 min at room temperature with gentle agitation, followed by a brief rinse in distilled water. The gel was then placed on top of 2 sheets of Whatman 3 MM filter paper (wetted with 0.4 N NaOH, and placed on top of an inverted gel-casting tray in a plastic box, such that the filter paper touched the base of the box, forming a wick), and was flooded with 50-100 ml of 0.4 N NaOH. A sheet of Hybond N+ (wetted by floating onto and then immersion in distilled water) was placed on top of the gel, and any air bubbles were removed. Three sheets of Whatman 3 MM filter paper, wetted in 0.4 N NaOH, were laid onto the membrane, followed by a 4 cm thick layer of absorbent paper. A light weight was placed on top of this, and transfer left to continue overnight. After transfer, the membrane was rinsed briefly with gentle agitation in 2 x SSC (A2.). The membrane was now ready for hybridization or could be wrapped in saran wrap and stored at 4°C.

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A1.12 β-galactosidase assay

The method was adapted from Pardee *et al.* (1959). *E. coli* cells are grown in the presence of 2 mM IPTG to induce the expression of the gene encoding β-galactosidase. A 1 ml sample of culture or culture supernatant was equilibrated at 28°C for 5-10 min before the addition of 200 µl of substrate (13 mM ONPG in 0.25 M sodium phosphate buffer). The reaction was allowed to continue for 5-30 min and stopped by the addition of 500 µl of Na_2Co_3 (14% w/v). The optical density at 420 nm of the solution was determined.

A1.13 Total DNA extraction from B. fragilis

The method was adapted from Cambell and Yasbin (1984). A 500-1000 ml culture of *B. fragilis* was grown for 48 h at 37°C in BHI broth. The cells were harvested by centrifugation at 3 000 rpm for 7 min, resuspended in 50 ml Ringers solution and

harvested again by centrifugation at 5000 rpm for 10 min. The pellet was resuspended in 25 ml lysis buffer (50 mM Tris HCl, 10 mM EDTA, 400 mM NaCl, pH 7.5) and left at room temperature for 5 min. 2.5 ml of a 10% SDS solution was added to the cell suspension, and the mixture was kept on ice for 15 min. 25 ml phenol was added and the mixture was slowely agitated at room temperature for at least 20 min. The phenol mixture was spun at 15 000 rpm for 15 min to separate the DNA-containing supernatant from the cell debris. The procedure was repeated by adding an equal volume of phenol to the supernatant, followed by centrifugation. The phenol was extracted three times with water-saturated ether. An equal volume of isopropanol was then added to the supernatant, followed by cooling at -70°C for 30 min and the DNA was precipitated by centrifugation at 10 000 rpm for 15 min at 4°C. The pellet was washed with 70% ethanol and resuspended in 0.5 ml TE buffer. The *B. fragilis* plasmid pBFC1 was separated from the total DNA by isopycnic CsCl-EtBr ultracentrifugation.

A2. Buffers and Solutions

All buffers and solutions were sterilized by autoclaving at 121°C for 20 min unless otherwise indicated. Heat labile substances were sterilized by filtration through 0.22 mm membrane filters (Millipore).

A2.1 Alkaline agarose gels

Neutral buffer:

NaCl	2.92 g
0.5 M EDTA	2.0 ml
H_2O	up to 1.0 l

Alkaline electrophoresis buffer:

NaOH	1.2 g
0.5 M EDTA	4.0 ml
H_2O	up to 1.0 l

Alkaline tracking dye:

Bromocresol green	12.0 mg
glycerol	2.5 ml

Made up to 9.0 ml with H_2O; 1.0 ml of 5 M NaOH was added just before use.

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A2.2 SDS PAGE solutions

The method was adapted from Laemmli (1970).

1. Monomer Solution (30%T 2.7%Cbis)

Acrylamide	58.4 g
Bis	1.6 g
H_2O	to 200.0 ml

store at 4°C in the dark.

2. 4x Running gel buffer

Tris	36.3 g
adjust to pH 8.8 with HCl	
H_2O	to 200.0 ml

3. 4x Stacking gel buffer

Tris	3.0 g
adjust to pH 6.8 with HCl	
H_2O	to 50.0 ml

4. 10% SDS

SDS	50.0 g
H_2O	to 500.0 ml

5. Initiator

Ammonium persulfate	0.5 g
H_2O	to 5.0 ml

6. Running gel overlay

Tris	25.0 ml soln.2
SDS	1.0 ml soln.4
H_2O	to 100.0 ml

7. 2x Sample treatment buffer

Tris	2.5 ml soln.3
SDS	4.0 ml soln.4
glycerol	2.0 ml
2-mercaptoethanol	1.0 ml
H_2O	to 10.0 ml

divide into aliquots and freeze.

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8. Tank buffer

Tris	6.0 g
Glycine	28.8 g
SDS	20.0 ml soln.4
H_2O	to 2.0 l

This reagent can be made up non-sterile. The pH need not be checked.

9. Stain stock

Coomassie Blue R250	2.0 g
H_2O	to 200.0 ml

stir and filter.

10. Stain

Stain stock	62.5 ml
Methanol	250.0 ml
Acetic acid	50.0 ml
H_2O	to 500.0 ml

11. Water-saturated n-Butanol

n-Butanol	50.0 ml
H_2O	5.0 ml

shake, use top layer.

12. Destain

30 % Methanol
10 % Acetic acid

13. Glycerol

3 % gycerol in H_2O
soak for 2 h before drying at 80°C for 1 h on gel dryer.

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SDS-PAGE gels

Ingredients	10 % separating gel	15 % separating gel	4 % stacking gel
acryl. stock	6.67 ml	10.0 ml	1.33 ml
soln 2 (run)	5 ml	5 ml	
soln 3 (stack)			2.5 ml
SDS	0.2 ml	0.2 ml	0.1 ml
H_2O	8 ml	4.7 ml	6.1 ml
Amm.pers(5)	100 µl	100 µl	50 µl
TEMED	6.7 µl	6.7 µl	5 µl

-can pour separating gel the day before and keep at 4°C under running gel overlay and clingwrap
-don't pour stacking gel the day before, it shrinks
-wash separating gel well before pouring next gel over
-wash out wells before loading samples

A2.3 Exonuclease shortening solutions

1. 10 x S1 buffer

3 M KOAc	1.1 ml
5 M NaCl	5.0 ml
glycerol	5.0 ml
$ZnSO_4$	30.0 mg

2. Exo-buffer

1 M Tris HCl pH 8	660.0 µl
0.1 M $MgCl_2$	66.4 µl
H_2O	9.27 ml

3. S1 mixture

10 x S1 buffer	41.0 µl
H_2O	259.0 µl
S1 nuclease	60.0 units

4. S1 stop

0.3 M Tris-base
0.05 M EDTA

The pH of this solution was not adjusted as alkaline conditions are required to inactivate the S1 nuclease.

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5. Klenow mixture
20 mM Tris HCl pH 8
7 mM $MgCl_2$
Klenow enzyme 1 unit/1 µg DNA

6. Ligation mixture

10 x ligation buffer	144.0 µl
T4 ligase (1 unit/µl)	24.0 µl
H_2O	1.44 ml

A2.4 DNA Loading solution (6x)

Bromophenol blue	0.25 g
Sucrose	40.0 g
Distilled water	to 100.0 ml

The solution was stored at 4°C.

A2.5 Ligation buffer (10 x) (pH 7.6)

Tris-HCl (1 M, pH 7.6)	0.66 ml
$MgCl_2$ (1 M)	66.0 µl
Dithiothreitol	15.4 mg
Distilled water	0.274 ml

Stored in 50 ml aliquots at -20°C. Discarded remainder once defrosted. ATP was added to the ligation mixtures at a final concentration of 1 mM.

A2.6 Phenol (TE-saturated)

Phenol (200 g, Merck) was melted at 65°C and 0.3 g of 8-hydroxyquinoline was added. The phenol was extracted three times with TE (10 x) or until the pH of the aqueous phase was approximately pH 7.6. The phenol was stored under TE (1 x) at -20°C.

A2.7 SSC (20 x)

NaCl (3 M)	175.3 g
Sodium citrate (0.3 M)	88.2 g
Distilled water	to 1.0 l

Adjusted the pH to 7.0 with NaOH (10 N) and autoclaved.

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A2.8 T4 DNA polymerase buffer (10 x)

This buffer was prepared using restriction endonuclease buffer A (Boehringer Mannheim) and adding bovine serum albumin (BSA Pentax Fraction V) to a final concentration of 1 mg/ml. Buffer A has the following composition:

Tris-acetate (pH 7.9)	0.33 M
K-acetate	0.66 M
Mg-acetate	0.1 M
Dithiothreitol	5.0 mM

After addition of the BSA the buffer was divided into 100 ml aliquots and stored at -20°C.

A2.9 Tris-Acetate EDTA buffer (TAE) (pH 8.0) (50 x)

Tris base	242.0 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M, pH 8.0)	100.0 ml
Distilled water	to 1.0 l

For Tris-acetate buffered electrophoresis using SeaPlaque^R low melting point agarose, the addition of EDTA was omitted.

A2.10 TRIS-EDTA (TE) buffer (pH 8.0)

Tris base	1.21 g
EDTA (0.5 M, pH 8.0)	2.0 ml
Distilled water	to 1.0 l

Adjusted the pH to 8.0 with HCl (0.1 M) and autoclaved.

A2.11 TSB solution

LB	150.0 ml

pH to 6.1 with 2 drops conc. HCl.

PEG 4000	15.0 g
$MgSO_4$ (1 M)	1.5 ml
$MgCl_2$ (1 M)	1.5 ml

Dispensed in 20 ml aliquots and autoclaved. Added DMSO (1 ml) and glucose (0.5 M, 400 ml when necessary) immediately before use.

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A2.12 Ringer solution

NaCl	2.25 g
KCl	0.105 g
$CaCl_2$	0.12 g
$NaHCO_3$	0.05 g
Distilled water	to 1.0 l

A2.13 Lysis buffer

Tris HCl	50.0 mM
EDTA	10.0 mM
NaCl	400.0 mM

The pH of the solution was adjusted to 7.5.

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A3. Medium

The media were sterilized by autoclaving at 121°C for 20 min unless otherwise indicated. Heat labile substances were sterilized by filtration through 0.22 mm membrane filters (Millipore).

A3.1 Luria-Bertani medium (LB)

Ingredient	Amount/l
Bacto tryptone	10.0 g
Yeast extract	5.0 g
NaCl	5.0 g

Solid media contained 1.5% (w/v) agar.

A3.2 TYG medium

Ingredient	Amount/l
trypticase	10.0 g
yeast extract	5.0 g
glucose	2.0 g
1 M K-phosphate buffer (pH 7.2)	100.0 ml
TYG salts	40.0 ml
0.8 % $CaCl_2$	1.0 ml
resazurin	4.0 ml

The pH of the solution was adjusted to 7.2 before autoclaving. After cooling the following sterile solutions were added:

Ingredient	Amount/l
hemin-menadione	10.0 ml
1.0 % $FeSO_4.7H_2O$ *fs*	0.04 ml
5.0 % cysteine (free base) *fs*	10.0 ml

For agar plates 15.0 g/l of agar was added to the TYG broth.

TYG salts

Ingredient	Amount/l
$MgSO_4.7H_2O$	0.5 g
$NaHCO_3$	10.0 g
NaCL	2.0 g

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A3.3 M9 minimal medium for *E. coli*

Ingredient	Amount/l
Na_2HPO_4	6.0 g
KH_2PO_4	3.0 g
NaCl	0.5 g
NH_4Cl	1.0 g

The pH of the solution was adjusted to 7.4 before autoclaving. After cooling the following sterile solutions were added:

Ingredient	Amount/l
20% $MgSO_4.7H_2O$	1.0 ml
0.1 M $CaCl_2.2H_2O$	1.0 ml
20% glucose	10.0 ml

For Supplemented M9 medium used in the T7 promoter-directed expression system the following sterile solutions were added:

Ingredient	Amount/l
20 ug/ml thiamine (vitamin B_1)	1.0 ml
all 20 aa (20 mg/ml) except cys and met	1.0 ml each

For the anaerobic T7 experiment
-add 2 ml/100 ml of 20% Na_2CO_3 to buffer against acidity.
-add Na-thioglycolate at 0.1 g/100 ml to help reduce media.

A3.4 BHI medium

BHI broth

Ingredient	Amount/l
BHI	37.0 g
Yeast extract	5.0 g
Sodium thioglycolate	1.1 g
Na_2CO_3	4.0 g

Steam for 30 min, add 10 ml haemin menadione stock, gas with CO_2 and autoclave.

BHI agar

Ingredient	Amount/l
BHI	37.0 g
Yeast extract	5.0 g
Difco agar	15.0 g

Autoclave medium, cool and add sterile cysteine stock (10.0 ml), Na_2CO_3 (20.0 ml), haemin menadione stock (10.0 ml).

BHI stocks

Menadione stock:

Menadione	100.0 mg
Ethanol	20.0 ml

Filtersterilize and store at 4°C.

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Haemin stock:

Haemin	50.0 mg
NaOH 1N	1.0 ml
H_2O	up to 100.0 ml

Autoclave and store at 4°C.

Haemin menadione stock:

Menadione stock	1.0 ml
haemin stock	100.0 ml

Cysteine stock:

Cysteine hydrochloride	1.0 g
H_2O	up to 20.0 ml

Sodium carbonate stock:

Na_2CO_3	20.0 g
H_2O	up to 100.0 ml

A3.5 Antibiotics

Antibiotic	Conc. used	Stock
Ampicillin	100 µg/ml	100 mg/ml in water
Chloramphenicol	20 µg/ml	20 mg/ml in ethanol
Erythromycin	10 µg/ml	10 mg/ml in ethanol
Gentamycin	200 µg/ml	20 mg/ml in water
Kanamycin	50 µg/ml	25 mg/ml in water
Metronidazole	0-2000 µg/ml	10 mg/ml in water
Rifampicin	200 µg/ml	50 mg/ml in DMSO
Tetracycline	10 µg/ml	10 mg/ml in water

The stock solutions of the antibiotics disolved in water were filtersterilized. Except for ampicillin, which could be stored stored at 4°C, all solutions were made fresh.

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A4. *Escherichia coli* strains

strain	genotype	reference/origin
C600	*thi*1, *thr*1, *leuB*6, *lacY*1, *tonA*21, *supE*44	Appleyard (1954)
AB1157	*rac*, *ara*, *argE*, del(*gpt-proA*)62, *galK*, *hisG*, *kdgK*, *lacY*1, *leuB*6, *mgl*51, *mtl*1, *qsr*, *rfbD*1, *rpsL*, *supE*44, *thi*1, *thr*1, *tsx*33, *xyl*5	ATCC 29055
AB1886	*uvrA*6, otherwise as AB1157	Howard-Flanders *et al*, 1966
AB1885	*uvrB*5, otherwise as AB1157	Howard-Flanders *et al*, 1966
AB1884	*uvrC*34, otherwise as AB1157	Howard-Flanders *et al*, 1966
JC5519	*recB*21, *recC*22, otherwise as AB1157	A.J. Clark
JC8679	*recB*21, *recC*22, *sbcA*23, *rac*$^+$, *phi*1, otherwise as AB1157	ATCC 47001
TK603	*uvrA*6, *thr*1, *leu*6, *proA*2, *his*, *ilv*325, *thi*1, *lacY*1, *galK*2, *ara*14, *xyl*15, *mtl*1, *tsx*33, *rpsL*31, *supE*44	Kato and Shinoura, 1977
CC118	*recA*1, *araD*139, del(*ara-leu*)7697, del *lacX*74, *galE*, *galK*, *thi*, *rpsE*, *rpoB*, *argEam*	Manoil and Beckwith 1985
F19	*ntr*, *phoA* del20, otherwise as CC118	Santangelo *et al.*, 1991
DK1	del(*srl-recA*)306, del(*ara*, *leu*)7697, *lacX*74, *galU*, *galK*, *hsr*, *araD*139, *strA*	ATCC 35691
HB101	*recA*13, *hsdS*20, *ara*14, *proA*2, *lacY*1, *galK*2, *rpsL*20, *xyl*5, *mtl*1, *supE*44	Boyer and Roulland-Dussoix, 1969

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