A STUDY OF THE RECOMBINATION AND DNA DAMAGE REPAIR IN *ZYMOMONAS MOBILIS CP4* *AND ARTHROBACTER* ISOLATES

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1991

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfilment for
the Degree of
DOCTOR OF PHILOSOPHY
December, 1997

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AND DNA DAMAGE REPAIR IN

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ACKNOWLEDGMENTS

I wish to extend my gratitude and sincere thanks to all those who have helped put this thesis together. I wish to thank my major advisor Dr. Robert V. Miller for his guidance, assistance, and especially his patience as a mentor and teacher.

My sincere thanks and appreciation goes to my graduate committee members, Dr. Margaret Essenberg, Dr. Carol Bender, Dr. Robert Burnap, and Dr. Jeffery Hadwiger for their suggestions, input, and constructive comments throughout this endeaver.

My thanks are given to the special members of our lab, my friends and colleagues, Steven Ripp, Melissa Booth, Mohammed Elasri, Lori Van Waasbergen, and Shannon Walls.

I would like to extend my sincere thanks to many people in the Microbiology Department and on the Oklahoma State Campus that helped me adapt and succeed in my studies. Especial thanks go to, Dr. Kay Scheets who has never failed to answer a question and has been always helpful, and to the staff of the Microbiology Department for their support.

My love and appreciation to my soon to be wife Heidi Schmid for her constant support and her understanding. I would particularly like to thank my family, my brother Marwan, for being my long-distance advisor, my father without whom this would have been impossible, the rest of my Family who always gave me their love and understanding, Irmela, Haroun, Chaldoun, and of course Saladeen.

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CHAPTER I

Introduction

Zymomonas mobilis is a unique genus of bacteria that was first suggested as a potential organism for the production of fuel ethanol twenty years ago (85,15). Since then, the need for alternative fuel sources has sparked public interest in this extremely efficient ethanol producing microorganism. *Z. mobilis* has many attractive features that make it a perfect agent for the industrial production of ethanol: it has a high sugar-to-ethanol conversion rate; tolerance to ethanol concentrations of as much as 12%; tolerance to sugar concentrations in excess of 40%; growth naturally in low pH, and a fermentation process that produces mainly ethanol and CO_2 (98%). Also, very little biomass is produced (93). In addition, *Z. mobilis* is generally recognized as being superior to brewer's yeast with respect to both glucose conversion efficiency and ethanol productivity (73). The efficiency of sugar-to-ethanol conversion yield is strain specific, and it has been found that strain CP4 is the most competitive candidate (73).

Despite having several advantages over conventional yeast fermentation of sugars, *Z. mobilis* has a major disadvantage with respect to its narrow substrate range: it can only ferment glucose, fructose, and sucrose in the production of ethanol. Fermentation of more abundant substrate sources such as the pentose sugars found in lignocellulosic feedstocks cannot be accomplished without a modification of the metabolic pathway of *Z. mobilis*. Pentose sugars are abundant in nature as a major

component of lignocellulosic biomass. Furthermore, these sugars represent a potential resource for the production of ethanol. To overcome the narrow substrate range of *Z.mobilis*, genes encoding primary hydrolysis steps of more readily available substrates have been cloned into *Z. mobilis* to broaden its substrate range (12,13,14,77,86). A great many vectors carrying genes from a variety of microorganisms have been cloned and expressed in *Z. mobilis*. However, it has not yet been possible to stably maintain these genes in *Z. mobilis* . There are several reasons for this difficulty: low gene expression; instability and rearrangement of the cloned genes; interaction of the hetrologous DNA with native plasmids; and integration of the introduced DNA into both the host chromosome and native plasmids (17,66,86,91,92).

To avoid further work in this organism, several groups transferred the essential genes for fermentation from *Z. mobilis* into other bacteria. This was deemed necessary since little is known about the genetics of *Z. mobilis* other than the genes involved in the fermentation process. The two most important genes in the fermentation process, *pdc* and *adhII* of *Z. mobilis*, were cloned into *E. coli* initially on a plasmid vector and were later integrated into the *E. coli* chromosome (28,44,49,88). While it has been argued that integration of genetically engineered genes into the *Z. mobilis* chromosome would stabilize their expression, it also means a lower copy number of that gene and, therefore, lower expression of the protein. The initial success in genetically engineered *E. coli* for production of ethanol from a variety of sources was later overshadowed by the intolerance of *E. coli* to elevated ethanol concentrations (65). Additional reports confirmed the superiority of *Z. mobilis* in other aspects of the fermentation process as well (41).

The course of the genetic engineering of *Z. mobilis* for improved ethanol production has taken many years, and the question arises, do we know enough about this organism's genetics to manipulate its most fundamental process, fermentation of sugars? It is evident from the observations of the genetic instability of the cloned genes in *Z. mobilis*, that recombination and gene rearrangements of cloned genes and vectors occur.

The *recA* gene and its protein product, RecA, are central to the process of homologous recombination, DNA repair, and other processes involved in maintaining and diversifying the genetic material of a bacterial cell (71,72). In *E. coli recA* mutants such as *DH5α*, *DH5*, and *HMS174* have been created to obtain organisms that are genetically stable and to prevent rearrangement of genetic material introduced on plasmid vectors. Plasmid replication is also stabilized in *recA* mutants(80).

Is it possible that *Z. mobilis* has exceptional recombinational abilities, multiple copies of the *recA* gene or additional mechanisms that support DNA repair? In this study the recombinational efficiency of *Z. mobilis* CP4 was investigated and the presence of a *recA* analogue determined. In addition, the creation of a *Z. mobilis recA* mutant should enable genetic manipulations in a recombinational deficient strain.

Further research on *Z. mobilis* is essential and the results of this study present an opportunity to enhance genetic engineering of *Z. mobilis* by producing a recombinationaly stable background, an advantage that has been exclusive to few more thoroughly studied bacteria.

In the second part of this study the efficiency of DNA repair of deep subsurface *Arthrobacter* isolates is investigated. *Arthrobacter* is a Gram-positive microorganism commonly isolated from soil. DNA homology studies showed that *Arthrobacter* spp. don't exhibit a close relationship to

each other and are in fact diverse (45). In the presented work, samples from two DOE plant-sites were obtained for analysis. The repair potential of these samples was investigated by assaying for UV-mediated DNA damage. Despite being shielded from solar radiation for millennia we obtained surprising results similar to those obtained by Arage et. al (6).

The potential use of deep-subsurface microorganisms in environmental detoxification and bioremediation studies has raised interest in investigating the deep-subsurface micro flora (7). The study, presented here, like many others, will contribute to our overall knowledge of this new frontier in microbiology.

CHAPTER II

Literature Review

Zymomonas mobilis

Z. mobilis is a unique genus of bacteria that was first identified by Kluymer and Vaniel in 1936 (54, 55). It's natural niche is in or around plants (93). It causes "cider sickness" which is a secondary fermentation of sweet English Cider (9) and is considered a serious contaminant of beer but is not known to be pathogenic to man, animals, or plants (73). Unlike most Gram-negative bacteria, *Zymomonas* is a facultative anaerobe although some strains are obligatory anaerobes(93). The genome size is about 1.5 X 10^9 kilobase pair (kbp) and has a G+C content of 47.5-49.5% (93). It shows resistance to most antibiotics except tetracycline and rifampicin (73). *Z. mobilis* has a narrow catabolic substrate range; it ferments glucose, fructose, and sucrose to give ethanol and carbondioxide (85). Amino acids (aa) as the sole carbon source do not seem to be capable of supporting growth of *Z. mobilis* (86). The similarity of *Z. mobilis* to yeast in fermenting glucose, fructose and sucrose to ethanol led to the belief that *Z. mobilis* follows the glycolytic pathway of yeast. Gibbs and DeMoss *et al.* (35) however, discovered that *Z. mobilis* utilizes a modified Entner-Doudoroff Pathway.

Industrial Importance

The search for alternative sources of fuel combined with the recent demand for "cleaner" more environmentally sound fuel substitutes, has stimulated research into various aspects of the fermentation process. Traditionally, Yeasts have been the sole agent for the industrial production of ethanol until the monograph by Swings and De Ley in 1977 (93)on the biology of *Z. mobilis*. The authors suggested the employment of this unique microorganism for the production of ethanol. Indeed, *Z. mobilis* was found to have a higher sugar-to-ethanol conversion yield than yeast, however, the molar efficiency is strain specific and varies between 1.5-1.9 mole ethanol/ mole glucose (24).

The glycolysis of *Z. mobilis* occurs through a modified Entner-Doudoroff pathway to produce 1.9 mole of ethanol per mole of glucose fermented (73). This represents about 96% of the theoretical yield of ethanol production. The Entner-Doudoroff Pathway is extremely inefficient in energy production: while yeast produces 2 moles of adenosine triphosphate (ATP) per mole of glucose through the Embden-Meyerhoff-Parnas Pathway, *Z. mobilis* produces only 1 mole ATP per mole of glucose utilizing the Entner-Doudoroff pathway (73). The high yield of ethanol production contributes to the reduced biomass formation where only 2% of the fermented sugars is converted to biomass and for cell maintenance (78).

Z. mobilis has unique abilities to grow in high sugar and ethanol concentrations. Swings and De Ley found that half of their strains grew in a medium with 40% w/v glucose and 10% v/v ethanol (93). Other studies indicated growth of *Z. mobilis* in ethanol concentrations as high as 12%

(78). In a comparison between *Z. mobilis* ATCC12919 and Baker's Yeast*Sacharomyces cervisiae* in batch and continuos cultures the specific rate of glucose utilization and ethanol production were found to be several times higher in *Z. mobilis* (double the glucose rate and three times the ethanol rate). In addition, higher rates of ethanol yield (97% of the theoretical yield compared with 86% for Yeast) and higher ethanol tolerance were reported (86).

In conclusion, *Z. mobilis* demonstrates many of the traits sought in an ideal biocatalyst for ethanol production: it has high ethanol yield and tolerance, specific productivity, low biomass formation, high tolerance to elevated sugar concentrations and the ability to ferment sugars at extremely low pH, preventing foreign contamination.

Various strains of *Z. mobilis* were compared for their suitability for fermentation. The overall results clearly indicated that strain CP4 is the most competitive candidate (86). CP4 was found to produce ethanol at a considerably faster rate than the other strains. It was also found to be more ethanol tolerant and at temperatures higher than 30^0C appeared to produce the highest amount of ethanol among all the strains tested. In addition, and under the same conditions for all strains, CP4 showed the highest rate of glucose uptake and growth rate overall (86).

Despite all the favorable traits that would make *Z. mobilis* the perfect candidate for ethanol production, its application in industry, is limited by it's narrow substrate range (93). Therefore, abundant substrates such as the pentose sugars found in lignocellulosic feedstocks and lactose from dairy by-products cannot be fermented by this extremely efficient ethanologen unless a preliminary hydrolysis step is performed (15). Engineering of clones that are constructed to perform the primary

hydrolysis steps for utilization of more abundant substrates has been undertaken by many investigators in an effort to broaden the substrate range of *Z. mobilis.*

Genetic Engineering of bacteria for ethanol production

Many cloning strategies have been examined to introduce foreign genes into *Z. mobilis* in order to broaden its substrate repertoire. These efforts started over ten years ago in an attempt to create mutants showing improved ethanol tolerance and overall improvement in fermentation (86). These studies, however, did not address the substrate range of *Z. mobilis* and ways to broaden it.

In an attempt to enable *Z. mobilis* to convert cellulose to ethanol, *endoglucanase* (from *Xanthomonas albilineans*) and *b-glucosidase* (from *Pseudomonas* spp.) were linked on the same vector and transferred into *Z. mobilis*. Levels of expression of both genes was lower (one twelfth) in *Z. mobilis* than in *E. coli*, also Co-integration between the two plasmids (helper plasmid and vector) was observed (92). A similar situation occurred when Liu et al. (66) mobilized xylose catabolic genes carried on pRK404 into *Z. mobilis* by pRK2013.

Although plasmid vectors have been constructed for expression of heterologous DNA in *Z. mobilis* they are generally unstable and disappear (1,12,14) or lose their structural identity after several cell divisions (17). In order to construct vectors that can be stably maintained in *Z. mobilis*, the stability of native plasmid pZMO3 was investigated and subsequently used to construct plasmid vectors for the cloning of heterologous DNA in *Z. mobilis* (17). Alexandra and Drainas constructed pDS212, one of the

recombinant plasmids that combined a multiple cloning vector (pBR322) and a native *Z. mobilis* pZMO2 (1). The stability of this vector *in Z. mobilis* was proposed to be due to the origin of replication of pZMO2. It was also shown, however, that during transfer, helper plasmid and vector undergo genetic rearrangement within *Z. mobilis*, explaining the change in restriction pattern after transformation of the vector.

A set of expression vectors were constructed that combined *E. coli* plasmids, with a multiple cloning site of phage M13 amp18 under the control of the *Z. mobilis pdc* promoter (17) isolated in earlier studies (16) to create an expression vector. Low transfer rate and loss of the recombinant plasmid deemed this plasmid inappropriate despite having many desirable features.

In another study after failing to maintain IncW plasmid Sa in *Z. mobilis* (91), the same group (Rogers *et al.*,1990) created a new plasmid by Cointegrate formation with *Z. mobilis* native plasmid. This new plasmid pNSW301 was stably maintained in batch culture but was unstable in continuous culture. Also, deletions of the plasmid occurred, with decreasing frequency as the size of the cointegrate became smaller.

In general, some studies report plasmid stability in continuous culture but for less than 100 generations of growth (1,91). The obvious instability of cloned recombinant plasmids in *Z. mobilis* was explained by one group attempting to clone the α-amylase gene when they observed integration of the plasmid into the host genome (13). Initially, in that study a decrease in enzyme activity and antibiotic resistance was observed; however, both activities were higher than the basal level of the wild type. Similarly, there are other reports on the interaction of stably-replicating

plasmid vectors with indigenous plasmids or with host chromosome (4,16,91).

While the genes involved in ethanol fermentation and other metabolic genes have been cloned and studied, (99) little is known about the genetics of *Z. mobilis*. To circumvent this problem, Ingram et al. decided to move the genes involved in ethanol production from *Z. mobilis* into the genetically established *E. coli*. He felt the two most essential genes were the *pyruvate decarboxylase* (*pdc*) gene and the *alcohol dehydrogenase II* gene (adhII) (44,88). For added stability, and to avoid loss of the vector, these cloned genes were integrated into the *Z. mobilis* chromosome (49). Despite having success with the genetically engineered *E. coli* strains this approach did not result in sufficient ethanol production from *E. coli* since ethanol tolerance remains low with ethanologenic *E. coli* (62). In batch fermentation experiments, elevated levels of ethanol caused the early death of the genetically engineered *E. coli* strain (65). In addition, chemical induction of the integrated genes, with the use of antibiotics was an added expense in a process where low cost was the main concern (62).

To resolve confusion about which path to follow (either improvement of *Z. mobilis* or further engineering of *E. coli*), a study was carried out by Lawford and Rousseau (62) who compared glucose conversion to ethanol by *Z. mobilis* and recombinant *E. coli* under similar conditions. It was found that *Z. mobilis* fermentation was completed after thirteen hours as opposed to 33 hours for *E. coli* fermentation. With a doubling time of 84 minutes instead of 20 min for *E. coli*, fermentation efficiency of *Z. mobilis* was found to be superior.

Use of dried biomass as animal feed would be an important aspect in reducing the cost of fuel-ethanol production. While *Z. mobilis* is a

Generally Recognized As Safe (GRAS) for use as animal feed, *E. coli* would not sell as animal feed constituent especially after recent reports in the media of *E. coli* poisoning.

In conclusion, *Z. mobilis* has been recognized for its many unique features to be a perfect candidate for the industrial scale production of fuel-ethanol. It has high ethanol turnover, tolerance to high glucose and ethanol, and operates naturally at a low pH, preventing foreign contamination. Evolution has equipped this organism with features and abilities that cannot be replaced or simply cloned out because of the complexity of the network of ethanol resistance and the many factors involved in that process.

The observations reported in the literature suggest that the fermentation performance and characteristics of *Z. mobilis* are superior to recombinant *E. coli* and therefore further research and development is well justified in improving the productivity of *Z. mobilis*.

The *recA* gene product: characteristics and functions

The RecA protein is central to the process of homologous recombination, DNA repair, and other processes involved in maintaining and diversifying the genetic material of a bacterial cell (71,72).

In *E. coli*, the *recA* gene was first identified as an *E. coli* mutant unable to generate recombinants following bacterial conjugation (80). Further studies on this class of mutants mapped the dramatic reduction of recombination to a single loci termed *recA* (70). Later studies confirmed that *recA* is essential in pathways of bacterial homologous recombination

(60). In RecA$^-$ strains the level of normal recombinant formation involving homologous crossing over is reduced to practically zero (10).

The product of the *recA* gene RecA, is a multifunctional protein of an average molecular weight of 40,000 dalton (71). Its functions can be divided into these major activities: First, RecA acts as a co-protease by stimulating the repressor's autocatalytic cleavage and post-translational processess of mutagenesis protein activities (53,96); The other activity of *recA* promotes homologous pairing and strand exchange in recombination of DNA molecules in an ATP-dependent process (22,34,84).

Induction and mutagenesis role of *recA* In *E. coli* the number of RecA protein molecules increases following DNA damage (96). In fact, DNA damage induces the synthesis of 20 or more proteins in a repair system called the SOS repair regulon (72). Usually, mRNA synthesis from these genes is blocked by the LexA protein. The RecA protein acts as a coprotease cleaving LexA repressor at a specific Ala-Gly bond thereby destroying its ability to repress gene expression (64).

The *recA* gene is also involved in prophage induction. When activated by DNA damage, the *recA* gene product cleaves CI repressor of phage lambda, which maintains lysogeny (40). This cleavage eliminates maintenance of lysogeny and activates the lytic cycle. Phage lambda thus escapes the potential lethal consequences of damage to its host DNA.

RecA is also involved in SOS mutagenesis; induction of the SOS genes following LexA cleavage derepresses the proteins UmuD and UmuC which are essential for mutagenesis (53,96). Activated RecA acts as a coprotease again, this time targeting UmuD, cleaving it to produce UmuD' the functional protein. A UmuD'-UmuC complex then acts on polymerase III to produce an error-prone DNA repair mechanism (75).

Recombination and DNA repair: The RecA protein promotes homologous recombination in an ATP-dependent process (34). Activation of the RecA protein is believed to be by binding to single-stranded region of DNA (ssDNA) and formation of a complex which binds double-stranded DNA (dsDNA) (30). Assembly of the complex is unidirectional; 5' to 3' on ssDNA and is driven by the hydrolysis of ATP. This coherent movement in one direction enables the invading ssDNA to bypass damaged areas in the dsDNA target.

In many bacterial species, the *recA* gene has been well studied. In *E. coli*, the *recA* gene has been extensively studied since the discovery of recombination-deficient mutants. The *recA* gene has been isolated, characterized, and numerous mutants produced (48,63). The isolation of *recA* genes from other bacteria has been extensive (30). Perhaps the most studied *recA* gene of any Gram-negative bacteria other than *E. coli* is *Pseudomonas aeruginosa* (19,30,31,33). In a typical procedure for *recA* isolation, the *P. aeruginosa recA* gene was introduced into *E. coli recA* mutants and complementation of *recA*-associated phenotypes by the clone was observed (30,31,56). The cloned *recA* gene restored homologous recombination following Hfr-mediated conjugation, resistance to UV irradiation, and resistance to the alkylating agent methylmethane sulfonate, (MMS, causes ssDNA breaks). In addition, UV-induced mutagenesis was restored (30,56). It was also found that synthesis of the *P. aeruginosa* RecA protein was stimulated by the exposure of RecA$^+$ *P. aeruginosa* to various SOS-inducing compounds (42,56).

The *P. aeruginosa recA* gene nucleotide sequence is 54 % identical with *E. coli recA* gene while the amino acid sequence of the RecA protein

is 74 % identical to the *E. coli* protein (82). Generally the RecA protein is highly conserved in all bacteria from which *recA* has been isolated (30).

Clones of the *recA* gene of many bacterial strains (including Cyanobacteria) have been shown to complement UV-sensitivity and recombination deficiency following Hfr-mediated conjugal transfer in *E. coli recA* mutants. Prophage induction and MMS-resistance are also complemented (34,37).

In contrast to Gram-negative bacteria, few *recA* genes have been cloned form Gram-positive bacteria. This may be due to the difficulties in gene expression and complementation (100). Recent progress in PCR techniques, however, allowed researchers to overcome this problem to some degree. PCR-based cloning of *recA* genes has been reported for Gram-positive bacteria (28)

The deep subsurface program

Many recent studies have reported the existence of large bacterial populations in industrial and toxic waste sites. Several bacterial species isolated from these sites were able to degrade toxic waste products in addition to surviving harsh conditions in the lab. Interest in the microbiota of the subsurface has increased as aquifers have rapidly become contaminated with toxic byproducts and domestic waste (90). It is therefore believed that the presence of a large population of bacteria may play a role in the degradation of toxic ground water pollutants (7).

As part of it's Subsurface Science program the Department of Energy (DOE) initiated a program to investigate the microbial ecology of deeper subsurface environments, which has given scientists an insight into

environments deep below the surface. Some of the DOE's objectives were to identify the microbiota of the subsurface and to isolate organisms with exceptional abilities to degrade toxic byproducts and industrial waste.

Samples from the boreholes of two DOE plant sites: the Savannah River Site (SRS) Plant near Aiken, South Carolina, and the Hanford Reservation Plant in Washington, were made available for analysis to several laboratories across the country. The majority of the samples were aerobic chemoheterotrophic bacteria (90), but were otherwise very diverse. For instance, the G+C content of deep subsurface bacterial isolates chosen randomly from 3 DOE plant sites ranged from 20-76 G+C mol %(45), while isolates from the SRS plant showed a range of 36-60 G+C mol %. Variations in the physiological characteristics of surface and subsurface isolates have also been reported (7). Similar results were reported for shallow aquifer sediments at a site in Oklahoma (8).

In earlier studies of deep-subsurface microorganisms, 10^3-10^6 bacteria per gram of soil were found at depths up to 90 meters (5,7). Sediments at the SRS however, contained as much as 10^5-10^8 viable cells per gram soil.

Arthrobacter was found to be a frequent isolate from various depths of the SRS borehole and is very well characterized in addition to being frequently isolated from surface soils. Therefore, *Arthrobacter* was chosen as a representative organism to study the origin of deep-subsurface bacteria.

The ability of subsurface microorganisms to succeed *in situ* may depend on their ability to tolerate toxic levels of contaminants. Such contaminants may create adverse conditions in addition to the DNA-damage cells are exposed to in subsurface environments. Isolation of bacteria at

such high concentrations from the deep-subsurface may reflect the unusual survivability of these bacteria in their harsh environment. It is therefore expected that these deep subsurface isolates possess an efficient DNA repair mechanism. This study will attempt to shed some light on the DNA-repair capabilities of these bacteria.

Arthrobacter globiformis:

Arthrobacter globiformis 1080 is the type strain of *A globiformis.* and is commonly isolated from soil samples (38). It is a Gram-positive bacterium that goes through a rod-coccus growth cycle according to it's growth phase. At stationary phase, cultures are entirely coccoid; upon transfer to fresh medium the coccoid shape produces outgrowths, giving *Arthrobacter* an irregular rod-shape, characteristic of the exponential phase (38). *Arthrobacter* spp. are obligate aerobic chemoorganotrophic organisms and have an optimum growth temperature of 25-30^{0}C, however, most grow in the range of 10-35^0 C.

Arthrobacter spp. can use a wide and varied range of substrates as the main Carbon source (50). Studies on *Arthrobacter,* therefore, have been mainly on degradative enzymes since *Arthrobacter* showed an unusual wide range of substrates it can utilize as a sole Carbon source (94,103) This ability certainly contributes to their widespread presence in soils. *Arthrobacter* spp. have commonly been shown to form a numerically important fraction of the indigenous flora of soils from various parts of the world (38).

Identification of *Arthrobacter* spp. has been by morphological appearance, cell wall composition, and nutritional requirements. More

recently, DNA-DNA homology studies were performed on *Arthrobacter* spp. for taxonomic characterization (89). Sixteen strains of *Arthrobacter* and two strains of *Brevibacterium* were investigated by DNA-DNA hybridizations. Among all *Arthrobacter* spp., the homology data ranged between 10-70% DNA-homology (89). It was concluded that only a small number of species of *Arthrobacter* exhibit a close relationship with each other. In many studies *Arthrobacter* genes were cloned and expressed in *E. coli* (30,67) . The sequence of such genes was found in many instances to be closely related to their counterparts in Gram-negative bacteria. In other studies, however, doubt about recognition of *Arthrobacter* expression signals by *E. coli* forced investigators to adopt complementation studies in *Arthrobacter* spp. (94,67).

Genetic tools in *Arthrobacter* are relatively well established, yet transformation protocols of *Arthrobacter* rely on protoplast fusion and regeneration which is less efficient than transformation in *E. coli.*

To determine to what extent DNA-repair mechanisms have been conserved, uncharacterized subsurface isolates were examined for tolerance to DNA-damage mediated by UV radiation and hydrogen peroxide by Aarage et al.(6). In that study, it was assumed that these isolates would be very sensitive to UV radiation because of the length of time they had been separated from solar light. Since the resistance to UV light has been correlated to the amount of solar radiation in an organism's natural habitat (21). Surprisingly, the UV resistance of surface and subsurface isolates were similar (6).

Ultraviolet-Radiation-Mediated DNA-damage

Ultraviolet Radiation is part of the spectrum of electromagnetic waves covering the interval between x-rays and visible light (39). Ranging between 190 and 380 nm, UV is biologically divided into UVA, UVB, and UVC, the last being the most damaging to living cells. Solar radiation reaching the Earth's surface contains only UVA and UVB while UVC is shielded by the ozone layer (39).

The ionizing damage caused by UV radiation affects the whole cell, but the primary and most serious effect is on the photochemical alteration of the nucleic acids. Specifically, UV radiation causes cyclobutyl type dimers of two adjacent pyrimidines (39,98). The lethal effect of UV damage on bacterial cells becomes apparent after replication of the damaged cells. Postreplication mutagenesis is primarily due to the misrepair of radiation damage to the bacterial chromosome (98).

As a model for most of our understanding of microbiological genetics, *E. coli* has been most extensively studied in DNA-damage repair (98). In *E. coli* three types of enzymatic DNA repair effectively neutralize UV-damaged DNA. One is Photoreactivation by "photo-reactivating enzyme " which binds pyrimidine dimers in the dark but requires photoreactivating light in the range of 310-400 nm to monomerize them (98). Without the photreactivating flash of light, a second method of removal may be by a multienzymatic excision repair. This pathway

consists of five basic steps: damage recognition, incision, excision, repair synthesis, and ligation (43,46,98). The first three steps of excision repair are carried out by *UVrA*, *UVrB*, and *UVrC*, collectively known as UVrABC nuclease complex (95). This complex has the ability to recognize a variety of DNA-damage including: lesions, large chemical modifications thymine glycols and O-6-methylguanine (95). The third method by which *E. coli* overcomes DNA-damage is by the involvement of *recA* and *lexA* in a fascinating mechanism involving induction of genes and activational "autocleavage" of biomolecules, which is a process known as recombinational repair (56,57,58) (for a more detailed explanation see the section on *recA*).

CHAPTER III

MATERIALS AND METHODS

Bacterial Strains and Plasmids

In all manipulations in this study, involving *Z. mobilis*, strain CP4 "wild-type" kindly provided to us by Professor L. Ingram was used. *E. coli* strains used for UV-resistance studies and conjugation experiments were *E. coli* strain AB1157 (58) and various recombination deficient *E. coli* strains listed in Table I. Strain JC158 is a High Frequency of Recombination (Hfr) *E. coli* strain used as a donor cell in conjugation studies. All cloning and subcloning as well as recombinant plasmids construction, generation and purification were performed in *E. coli* strains and are listed in Table III. *Arthrobacter globiformis* ATCC 1080 (American Type and Culture Collection) type strain was used as a reference strain. In addition, soil isolates from the deep-subsurface are listed in Table IV. All isolates that have been studied for their UV-resistance were identified as *Arthrobacter* spp by rDNA analysis, and were kindly provided to us by David Balkwill. *Arthrobacter* isolates were obtained from two DOE plant sites: the Savannah River Site (SRS), and the Hannford Reservation Site.

Vector pUC18 is a multicopy plasmid lacking the *rop* gene and conferring resistance to ampicillin in *E. coli.* Plasmid pUC18 was the main plasmid used in this study, additional cloning vectors such as pBluescript and SK^+ were also used and are listed in table II. Several subclones and

TABLE I

E. coli STRAINS

Strain	Genotype	Source/Reference
AB1157	*argE3 his-4 leuB6 proA2 thr-1 Sm*	(58)
HB101	*leu hsd20 proA2 supE44 Sm recA13*	(57)
DH5α	*supE44 ΔlacY169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories
JC158	*HfrH thi-1 serA*	(58)
JC2926	As for AB1157but *recA*13	(58)
JC10289	*srlR300::*Tn10 (srl *recA*)Δ5, *thr-1,leuB6,ala-14,proA2,lacY1,tsx33, galC2, his-4, rpsl-31, xyl-5, mtl-1, argE3, thi-1*	(20)
JC11327	As for AB1157 but *rec*A56,*srl310*::Tn10	(61)

Sm: streptomycin

TABLE II

BACTERIAL STRAINS AND CLONING VECTORS

Strain	Genotype/Phenotype	Source/Reference
A. globiformis **ATCC1080**	Type strain	American Type and Culture Collection
B. subtilis **YB886**	Wild-type	R. E. Yasbin
Z. mobilis **CP4**	Wild-type	Ingram *et al.*
Z. mobilis **AM96**	Tc^R, Tc::*recA*	This study
pUC18	2.69 kb, Ap^R, *lacZ*	Stratagene
pBluescript SK+	2.9 kb, Cm^R, Em^R	Stratagene
pBR322	4.36 kb, Tc^R, Ap^R	des 101

Tc: tetracycline, Cm: chloramphenicol, Ap: ampicilln

TABLE III

RECOMBINANT VECTORS CREATED IN THIS STUDY

Recombinant vector	Parent Plasmid	Relevant Features
pADH11	pMMZ1	pMMZ1 nested deletion
pADH9	pMMZ1	pMMZ1 nested deletion
pADH6	pMMZI	pMMZ1 nested deletion
pZBX1	pBluescript SK+	XbaI fragment on SK+
pZD41	pMMZ1	pMMZ1 lacking NruI fragment
pZD21	pMMZ1	pMMZ1 lacking HincII fragment
pZD11	pMMZ1	pMMZ1 lacking EcorI fragment
pMMZ1	pUC18	a 7.0 kb *Z. mobilis* genomic insert
pZRD1	pMMZ1	tet cassette of pBR322 in pZDAH1
pZDAH1	pMMZ	pMMZ1 lacking the Afl II-HindIII fragment
pZD31	pMMZ1	pMMZ1 lacking AvaI fragment

TABLE IV

Arthrobacter deep subsurface isolates

Strain	Depth (m)	Site of Isolaion
422B	145	SRS
518B	204	SRS
537B	204	SRS
603B	244	SRS
620B	244	SRS
672B	259	SRS
703B	259	SRS
723B	265	SRS
755B	topsoil	SRS
776B	topsoil	SRS
ZAT002	567-570	Hanford Site
ZAT018	572-574	Hanford Site
G915	580-582	Hanford Site
G982	648	Hanford Site
G950	714-715	Hanford Site
G963	582-585	Hanford Site

nested deletion clones were constructed and are listed in Table II. To obtain a tetracycline resistance gene for the construction of pZDR1, plasmid pBR322 was utilized.

Media and Growth conditions

Z. mobilis CP4 was grown and stored in RM medium at 30^0 C without shaking. RM broth is a rich medium containing 10 g yeast extract, 2 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$ and 0.1 g $(NH_4)_2SO_4$ per liter of double distilled (dd) water. For solid medium, 1.5 % (w/v) agar was added to RM medium prior to autoclaving. After autoclaving, RM broth and RM agar were cooled to 60^0 C and 2% glucose was aseptically added. Tetracycline and Methyl methane sulfonate (MMS) in RM medium were used for the selection of *Z. mobilis* mutants at concentrations of 5mg/ml and 0.03% (v/v) respectively. *E. coli* strains were grown in rich Luria broth or Luria agar (Luria broth containing 1.5% agar) at 37^0 C. Antibiotics were used for selection at the following concentrations: ampicillin, 100 mg/ml for pUC18 based plasmids and 50 mg/ml for pBR322; tetracycline, 10 mg/ml. MMS at a concentration of 0.01% (v/v) was used for the initial screening of the recombinant *Z. mobilis* library in *E. coli.* Both the *Arthrobacter* type strain and deep subsurface isolates were grown on nutrient broth (NB) (Bacto Beef Extract and Bacto Peptone, Difco Laboratories) or Nutrient agar (Nutrient Broth supplied with 1.5% agar) at 30^0 C with vigorous shaking.

Enzymes, Chemicals, and Reagents

Restriction endonucleases, T4 DNA ligase, *E.coli* DNA polymerase I, DNA molecular weight standards, polyacrylamide, and Shrimp alkaline phosphatase (SAP) were purchased from Promega Biotec, Bethesda Research Laboratories (BRL), United States Biochemical Company (USBC), or New England Biolabs (NEB). Antibiotics, DNaseI, RNaseI, egg white lysozyme (gradel), Proteinase K, Vitamins, and bovine serum albumin (bovine serum albumin) were purchase from Sigma and Fisher Biotech. Bacteriological agar and media were purchased from Difco Laboratories. Agars, Low melting point agarose and Ultrapure agarose were purchased form FMC Bioproducts. [α-^{32}P] dCTP was purchased form New England Nuclear (NEN). Other chemicals, compounds and reagents were purchased from Sigma, Fisher, BRL and USBC.

UV sensitivity

In order to determine the sensitivity of bacterial cells to Ultraviolet Radiation (UV), cells were grown to a density of approximately 10^8 cfu/ml in the appropriate medium. A five ml sample was washed and resuspended in 0.85 % sterile saline solution (57,58). These cell suspensions were then irradiated with various doses of UV including a zero exposure and appropriate dilutions were plated on solid medium and wrapped in aluminum foil. Dilution, plating and irradiation were all done in the absence of visible light in a dark room, under a Kodak safe light. *Z. mobilis* CP4 and *Arthrobacter* spp. were incubated at 30^0 C for two days

and *E. coli* was incubated at 37^0 C overnight. Colony forming units (CFU) were counted and a UV-kill curve was established with the x-axis representing the UV dose and the y-axis representing the fraction of colony forming units surviving.

Conjugational proficiency

Donor and recipient cells were grown to mid-exponential phase, washed, resuspended in an equal amount of fresh media, and mixed at a ratio of 1:9 respectively. Conjugation was allowed to proceed for two hours at 37^0 C without shaking. Selection for transconjugants was by acquisition of amino acid prototrophies and streptomyocin (Sm^r) resistance. Streptomyosin was used as a Counterselection for donor cells.

Preparation of competence cells and Electroporation

Electro-competent *E. coli* cells were prepared as follows: Cells were grown in one liter of L-broth at 37^0 C with vigorous shaking to an OD_{600} of 0.5 to 1.0 (approximately 10^{10} cells/ml). The cells were briefly chilled on ice and spun at 4000x g for 15 minutes at 4^0 C. The cells were then washed in an equal volume of sterile, cold water. This wash was followed by three additional washes under the same conditions with decreasing volume of water. The second and third washes were one half of the original volume, while the fourth wash was in 2.0% of the original volume. Finally, the cells were resuspended in 0.002 volume of sterile, ice-cold 10% glycerol, aliquoted, and frozen at -70^0 C for immediate use.

An ECM 600 Electroporation System (BTX Inc.) was used at a setting of 2.5 kV for *E. coli* strains and 2.0 kV for *Z. mobilis*, in a 2.0 mm gap cuvette. Electrocompetent cells were thawed on ice for 5-10 min and 40 ml were mixed with 1 ml of plasmid DNA and chilled on ice for one minute. After discharge, 960 ml of SOC broth were added and quickly mixed. Electroporated cells were incubated for one hour at 37^0 C with shaking and 0.1 ml was plated on appropriate medium with antibiotic selection.

Chromosomal and Plasmid DNA Isolation

Chromosomal DNA isolation from *Z. mobilis*

Chromosomal extraction from *Z. mobilis* was performed as described by Ingram et al. (18). Cultures were grown in one liter of RM medium overnight at 30^0 C without shaking. The cells were pelleted and washed with 10 ml of TE buffer [10mM EDTA, 25mM Tris-HCl (pH 8.0)]. The pellet was resuspended in 10 ml of TE buffer containing 10% glycerol and 10 mg lysozyme and incubated at 37^0 C overnight. The cells were then moved to 50^0 C for 12 hours after the addition of protease K (200 mg/ml). N-laurylsarkosinate at a final concentration of 1 % was added and the cell were incubated for 12 more hours. At the end of the incubation two volumes of 95% ethanol were added and chromosomal DNA was then spooled out on a glass rod and rinsed with 70% ethanol. The DNA was briefly air-dried and redissolved in 4 ml of TE buffer (pH 8.0). This DNA was used in blot hybridizations and library construction.

Chromosomal DNA isolation from *Arthrobacter* spp

Chromosomal DNA was isolated by a modification of the method by Horn and Cutting (42). A bacterial culture of 250 ml was grown in NB medium overnight with shaking at 30^0 C. The cells were pelleted and resuspended in 0.02 M Tris-HCl (pH 8.0) buffer. Polyethylene glycol (PEG 4000) and lysozyme were added to a final concentration of 12% and 1 mg/ml respectively and incubated at 37^0 C for 2 hrs. The cell suspension was centrifuged and the pellet was resuspended in 0.02 M Tris-HCl (pH 8.0). Pronase E (1mg/ml) and SDS (1%) were added and the suspension was incubated for an additional hour. Chromosomal DNA was extracted twice with an equal volume of phenol/chloroform, precipitated with two volumes of 95% ethanol, spooled on a glass rod, washed with 70% ethanol, air dried, and eventually resuspended in 3 ml of TE (pH 8.0).

Plasmid DNA Isolation form *E. coli*

Alkaline-SDS lysis based plasmid isolation was done as described by Sambrook *et al.* (80) One and a half ml of an overnight culture of bacteria was pelleted in a microfuge tube at 16,000x g at room temperature for 1 min in a microcentrifuge (Eppendorf 5415 C). Cells were resuspended in 100 ml of cell resuspension solution [50mM glucose, 25 mM Tris-HCl (pH 8.0), and 10 mM EDTA] and kept at room temperature for 5 min. Cells were then lysed with 200 ml of cell lysis solution [0.2 N NaOH and 1% SDS] on ice for 5 min. The lysate was then mixed with 150 ml of ice cold neutralization solution [3 M potassium acetate (pH 4.9) and 11.2% (v/v) glacial acetic acid] and kept on ice for an additional 5 min. The lysate was

centrifuged at room temperature for 5 min and the supernatant fluid was transferred to a new tube. Plasmid DNA was precipitated with two volumes of 95% ethanol at -20^0 C for 10 min. The precipitated DNA was then pelleted at 16,000 X g for 5 min, vacuum dried, and resuspended in 50 ml of TE buffer containing Dnase-free pancreatic RNase (10 mg/ml). For rapid visualization of the recombinant plasmid, 5 ml of DNA was electrophoresed on an agarose gel. For further work or restriction endonuclease analysis, prior to ethanol precipitation, the plasmid DNA was extracted with equal volumes of phenol-chloroform, and chloroform-isoamyl alcohol. A plasmid DNA isolation kit was also used for analysis of a large number of samples as described by the manufacturer (Wizard minipreps, Promega). Large scale plasmid DNA purification was done essentially the same as the mini-plasmid preparation except for the additional CsCl-gradient purification step. A one liter overnight culture was pelleted at 4000 rpm in a RC 5B Sorvall centrifuge for 15 min and washed in 10 ml of 50 mM Tris-HCl (pH 8.0) buffer. The pellet was resuspended in 10 ml of 20% sucrose (w/v) in 50 mM Tris-HCl (pH 8.0), 10 mg of lysozyme, and 3 ml of 250 mM EDTA and placed on ice for 30 min. The cell suspension was lysed with 8 ml of 0.4% Triton X-100, 50 mM Tris-HCl (pH 8.0), and 25 mM EDTA and allowed to stand at room temperature for 10 min. The cleared solution was centrifuged at 10,000 rpm for 20 min and the clear supernatant fluid was transferred to a fresh tube. One gram of CsCl/ml of supernatant fluid and 0.8 ml of EtBr (10 mg/ml stock) were added to the supernatant fluid and mixed.

The resulting mixture was wrapped in aluminum foil and centrifuged at 8,000 rpm for 5 min at RT. The clear red solution was transferred with a Pasteur pipette into quick seal tubes and centrifuged at 65,000 rpm for 16 h

at 20^0 C in a Beckman Ultracentrifuge. The plasmid DNA band was recovered under UV light with a 16-G needle and extracted with 20X SSC-saturated isopropanol until all the EtBr was removed. The sample was then diluted 5 times with 50 mM Tris-HCl (pH 8.0) and one-tenth volume of 3M potassium acetate was added. The plasmid DNA was precipitated with an equal volume of ethanol at -20^0 C, air-dried, and resuspended in TE. Occasionally, plasmid DNA was dialyzed against 2 liter of TE buffer (pH 8.0) in the coldroom for twelve hours.

DNA Manipulations and Analysis

Conditions employed for DNA digestion with restriction endonucleases, ligation, alkaline phosphatase treatment of vector DNA, random-primed labeling of DNA, and, gel purification of DNA fragments were all essentially done as recommended by the manufacturers or Sambrook *et al.*(80).

Agarose Gel Electrophoresis of Recombinant DNA

Agarose gel electrophoresis of plasmid and chromosomal DNAs was performed as described by Sambrook *et al.* (80). For measurement of chromosomal DNA fragments larger than 2.0 kb, 0.4 to 0.8% agarose gels were used. For the measurements of fragments smaller than 2.0 kb, 1.0% to 2.5% agarose gels were used. DNA samples were mixed with a tracking solution containing 5% glycerol, 3 mM EDTA (pH 8.0), bromophenol blue, and xylene cyanol. Electrophoresis was done in TAE buffer [0.04 M Tris-acetate and 0.001 M EDTA (pH 8.0)] at room temperature. Gels were

stained for 15 min in 1 mg ethidium bromide per ml of double-destilled water and destained in water for 30 min. DNA bands were visualized by transillumination with 312 nm ultraviolet light and photographed with a Kodak Polaroid MP-4 Land camera. Fragment sizes were estimated by comparing to molecular weight standards [*Hin*dIII-digested l phage DNA, a one kb DNA ladder (BRL), or a 100 bp ladder (BRL)]. Purification of DNA restriction fragments for either labeling or cloning was performed using Ultrapure agarose (FMC). The gel pieces were destained and the DNA was gel purified using a QIA quick-gel extraction kit according to manufacturers recommendations (Qigen Inc.).

Southern Hybridization

Preparation of Membrane-bound Denatured DNA

Southern Blot Hybridization was essentially carried out as described by Southern (87, and Sambrook *et al.* (80) and in the case of non-radioactive labeling, according to manufacturers recommendations (Genius labeling kit, Boehringer Mannheim). After electrophoresis, the gel was soaked in 250 mM HCl for 10 min to depurinate large DNA fragments. The gel was immediately washed briefly with several volumes of double distilled water, soaked in denaturing solution (0.5 N NaOH and 1.5 M NaCl) for 45 min, and then neutralized in neutralization solution [1.0 M Tris-HCl (pH 8.0) and 1.5 M NaCl] for additional 45 min. The gel was placed on a precut nylon membrane (Nytran maximum strength, Schleicher & Schuell) that has been wetted in dd water and 2X SSC for 10 min each. The DNA was transferred to the membrane by a vacuum blotter using 10X

SSA according to the manufacturer's specifications (Model 785 Vacuum blotter, Bio-Rad). Transfers was continued for 90 min. at 5Hg/inch. After transfer the membrane was briefly rinsed in 2X SSC and the DNA on the damp membrane was corss-linked using a UV corss-linker (Stratalinker, Stratagene) at optimum cross-linking setting. The membrane was then air-dried between two filter papers and kept under vaccum until further use.

Prehybridization and Hybridization

The blotted membrane was placed in a heat-sealable plastic bag containing 20 ml of prehybridization solution (6X SSC, 5X Denhardt's, 0.5% SDS, and 100 mg/ml denatured, fragmented salmon sperm DNA) per 100 cm^2 of membrane. The bag was incubated at 68^0 C for over 3 h with gentle agitation. After prehybridization, the membrane was placed in a new bag and the prehybridization solution was replaced with the hybridization solution (essentially the same as the prehybridization solution less the Denhardt's solution) containing a heat denatured probe. Hybridization commenced for over 12 h at the same temperature. At the end of the hybridization, the membrane was removed from the bag and washed under high stringency conditions. The membrane was washed as follows: once at RT for 5 min in 2X SSC and 0.5% SDS, once at RT for 15 min in 2X SSC and 0.1% SDS, once at 37^0 C for 45 min in 0.1X SSC and 0.5% SDS, once at 68^0 C for 45 min in 0.1X SSC and 0.5% SDS, and finally at RT for 2 min in 0.1X SSC. The damp membrane was wrapped in plastic wrap and exposed to X-ray film (Kodak X-OMAT AR) with an intensifying screen at -70^0 C for varying times.

Random Primed Labeling

Random primed labeling of denatured probe utilizing radio-labeled phosphorous [α-^{32}P] was performed using the Megaprime labeling systems according to manufacturers recommendations (Life Sciences, Amersham).

Analysis of Nucleotide sequence

Nucleotide sequencing and primer synthesis was performed by the Oklahoma State University Core facility. Automated sequencing was done using a 373 DNA Sequencer, (Applied Biosystems). Sequence analysis, design of sequence primers, restriction analysis, open reading frame alignments, and homology searches were performed by Sequence analysis software packages including MacVector, MacDnasis, Plasmid artist, and Oligos 5.0. In addition internet search engines and database analyses were performed using databases such as BLAST, Genebank, and SWISS PROT.

CHAPTER IV

ISOLATION AND CHARACTERIZATION OF THE *Z.mobilis recA* GENE

UV resistance of *Z. mobilis* CP4

To obtain a measure of the repair abilities of wild-type *Z. mobilis* strain CP4, a UV survival curve was performed. After growing the culture to mid-exponential phase, the cells were irradiated at the following flunkies: 0.0, 10, 20, 25, 30, and 50 J/cm^2 (Figure).

It was observed that 90% killing of *Z. mobilis* occurred between 25 and 30 J/cm^2, and while a dramatic drop in cell viability occurred in that range, further exposure did not exert a similar degree of damage on the cells. Since light repair was not allowed to occur by performing the experiment in the dark, a relatively high *recA*-mediated resistance to UV radiation by strain CP4 was suggested.

Creation of a *Z. mobilis* genomic library in pUC18

The *Z. mobilis* CP4 chromosome was partially digested with *Sau3*AI and the resulting fragments were cloned into pUC18. *Z. mobilis* genomic DNA was prepared as described in the methods section and was partially digested with *Sau3*AI as evidenced by a sample that was electrophoresed on a small gel. Approximately, 500 μg of partially digested genomic DNA were loaded on an Ultrapure agarose gel and run at 5V/cm for over 20 h in

1X TAE buffer. Fragments ranging in size from 7.0 to 10.0 kb were excised from the gel and purified using a GeneClean II gel extraction kit (Bio 101). Purified DNA concentration was determined and a fraction was ligated to plasmid pUC18. The plasmid vector was linearized with *Bam*HI and dephosphorylated prior to ligation to genomic fragments. Ligation was carried out at RT for 1.5 h as recommended by the manufacturer (BRL), and terminated by the addition of EDTA and heating the sample to 70^0C for 10 min. The ligation reaction was diluted 5 fold and approximately 25 ng of ligated DNA was transformed into 200 µl competent *E. coli* HB101 (*recA*13) cells using the $CaCl_2$ transformation method as described below. *E. coli* cells were made competent using 100 mM $CaCl_2$ and transformation was facilitated by heat shocking competent cells for 1 min at 42^0 C. One milliliter of L broth was added to the transformation mixture, and the mixture was incubated at 37^0 C for 60 min to allow for the expression of the antibiotic marker gene before plating 0.1 ml on selective media.

Isolation of a clone complementing the *recA*$^-$ phenotype of mutant *E. coli*

Approximately 2500 transformants were screened on LB medium containing 100µg/ml ampicillin and 0.01% MMS. A plasmid profile was obtained for all 78 MMS^R clones by digestion with *Pvu*II and visualization on an 0.8% agarose gel. The restriction endonuclease *Pvu*II was chosen because two restriction sites exist for *Pvu*II on pUC18 just outside the Multiple Cloning Site on both sides, so that the cloned genomic fragment(s) could be liberated from the vector. In addition, the remaining pUC18

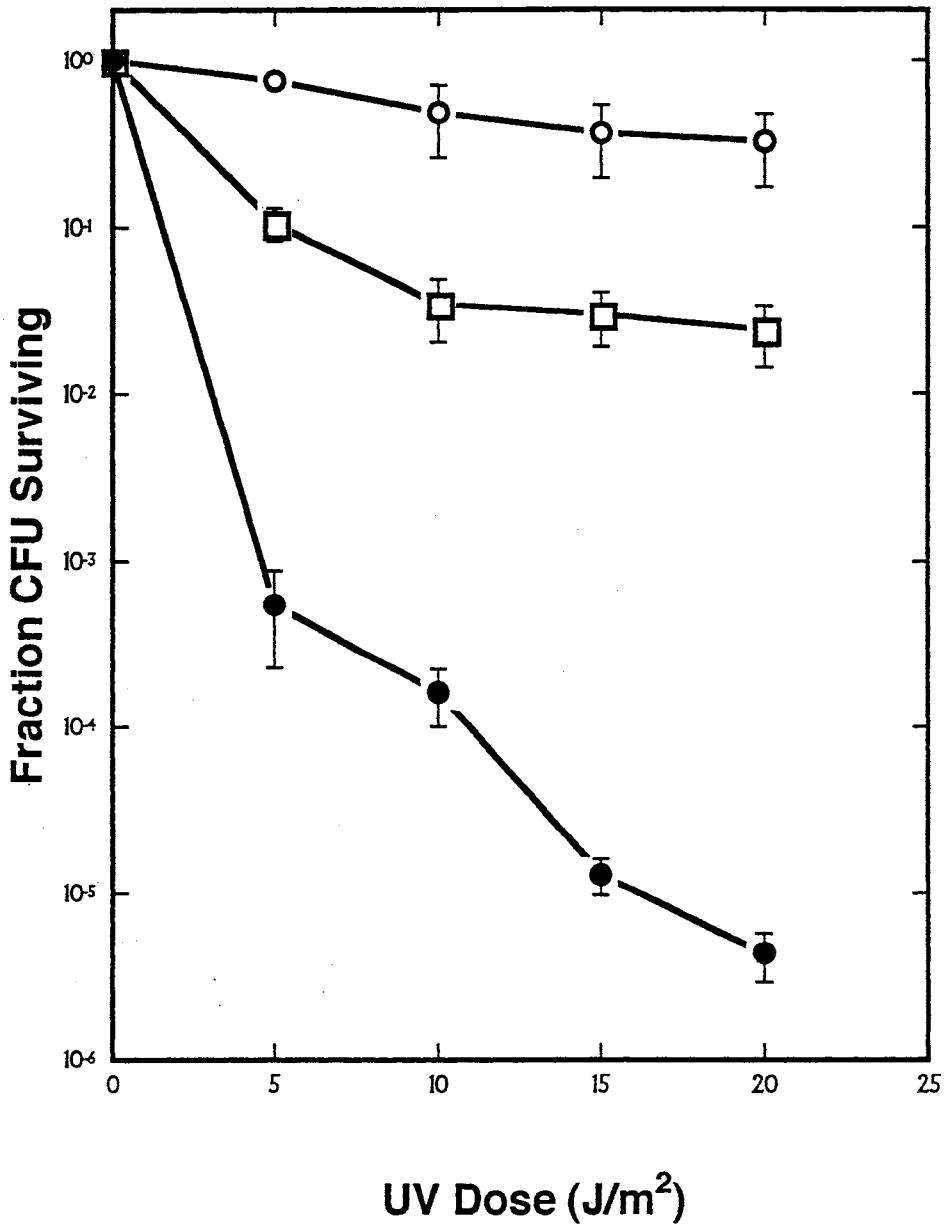
fragment could be used as a reference for proper digestion. An approximately, 7.0 kb insert was the dominant fragment in the plasmid profile analysis of the 78 MMS^R clones that complemented the $RecA^-$ phenotype of HB101. Three clones each with an approximate. 7.0 kb fragment, were randomly selected for further analysis. Quantification of the UV resistance of HB101 containing these plasmids was conducted to determine the clone that best complements the $RecA^-$ phenotype of the mutant *E. coli* cells. It was observed that all three clones complemented the $RecA^-$ phenotype equally well, and, therefore, one clone was selected to characterize the putative *recA* analogue of *Z. mobilis*. This plasmid was named pMMZ1. The genomic insert in pMMZ1 was confirmed to originate from the *Z. mobilis* chromosome by southern hybridization using the pMMZ1-*Pvu*II fragment as probe.

Complementation of three *E.coli recA* mutants by pMMZ1 utilizing UV survival curves

Complementation of *recA*-associated phenotypes such as UV sensitivity by the putative *Z. mobilis recA* construct has been widely used in the isolation of *recA* analogues (30,57,58). Similarly, in this study repair of UV-induced DNA damage was investigated. To obtain a relative measure of complementation of the RecA phenotype by pMMZ1 in *E.coli*, *recA* mutant strains, are needed that originate from a known wild-type strain. Complementation of the UV^s of these *recA* mutants by pMMZ1 can then be compared to the level of UV sensitivity of the parent wild-type strain. The putative *Z. mobilis recA* analogue was, therefore, moved from

Figure 1. Restoration of resistance by the *Z. mobilis recA* analogue to killing by UV irradiation of *E. coli recA13* strain HB101.

Cells were grown to a density of approximately 10^8 CFU/ml in Luria broth, pelleted, suspended, in saline, and exposed to various fluences of UV irradiation. After appropriate dilution in saline, the radiated cells were plated in duplicate on L agar and incubated at 37^0 C overnight in the dark Mean value ∓ standard error are plotted. Symbols: (○), *E coli* AB1157 *recA*$^+$; (❑), *E coli* HB101 *recA13* (pMMZ1); (●), *E coli* HB101 *recA13*.


Fraction CFU Surviving
10⁰
10⁻¹
10⁻²
10⁻³
10⁻⁴
10⁻⁵
10⁻⁶
0
5
10
15
20
25
UV Dose (J/m²)

HB101 to three *E. coli recA* mutants each containing a different *recA* allele (see Table I). Two of these mutants (JC2926 and JC11372) share a common parent, AB1157, which is an *E. coli* wild-type strain. It was used in this study as a reference of RecA+ phenotype. In the third mutant (JC10289), the *recA* gene has been almost completely deleted. All three mutants display a null phenotype. Except for the ability to bind single-stranded DNA, these mutants have lost all the *in vitro* enzymatic activities associated with the RecA protein (20,61).

UV resistance was measured in each of these mutant strains after introduction of pMMZ1 (see Figures 1, 2, and 3) This was compared to the levels of resistance exhibited by the wild-type strain AB1157. All experiments were repeated at least three times and each dilution was plated in duplicate.

Cells were exposed to 5, 10, 15, 20, and 0 J/cm^2. Introduction of pMMZ1 into the *recA* mutants increased the survivor rate JC2926 (*recA13*) 10-to-100 fold, (Figure 2), similarly, it increased the survivor rate of JC11372 (*recA56*) 10-to-100 fold (Figure 3). The survivor rate of JC10289 (*recA* deletion) was increased by 50-to-over-100 fold (Figure 4). Repair of UV-induced DNA damage is accomplished in bacteria, however, by more than one mechanism (see literature review and 95). While photoreactivation repair of UV-induced DNA damage can be eliminated by performing the experiments in the dark, excision repair may still account for a substantial portion of the repair observed in surviving bacteria (39). Complementation of other *recA*-associated phenotypes in these mutants by pMMZ1 was therefore investigated.

Figure 2. Restoration of resistance by the *Z. mobilis recA* analogue to killing by UV irradiation of *E. coli recA13* mutant in an AB1157 background.
Cells were grown to a density of approximately 10^8 CFU/ml in Luria broth, pelleted, suspended, in saline, and exposed to various fluences of UV irradiation. After appropriate dilution in saline, the radiated cells were plated in duplicate on L agar and incubated at 37^0 C overnight in the dark Mean value ∓ standard error are plotted. Symbols: (■), *E coli* AB1157 *recA*$^+$; (●), *E coli* JC2926 *recA13* (pMMZ1); (○), *E coli* JC2926 *recA13*.

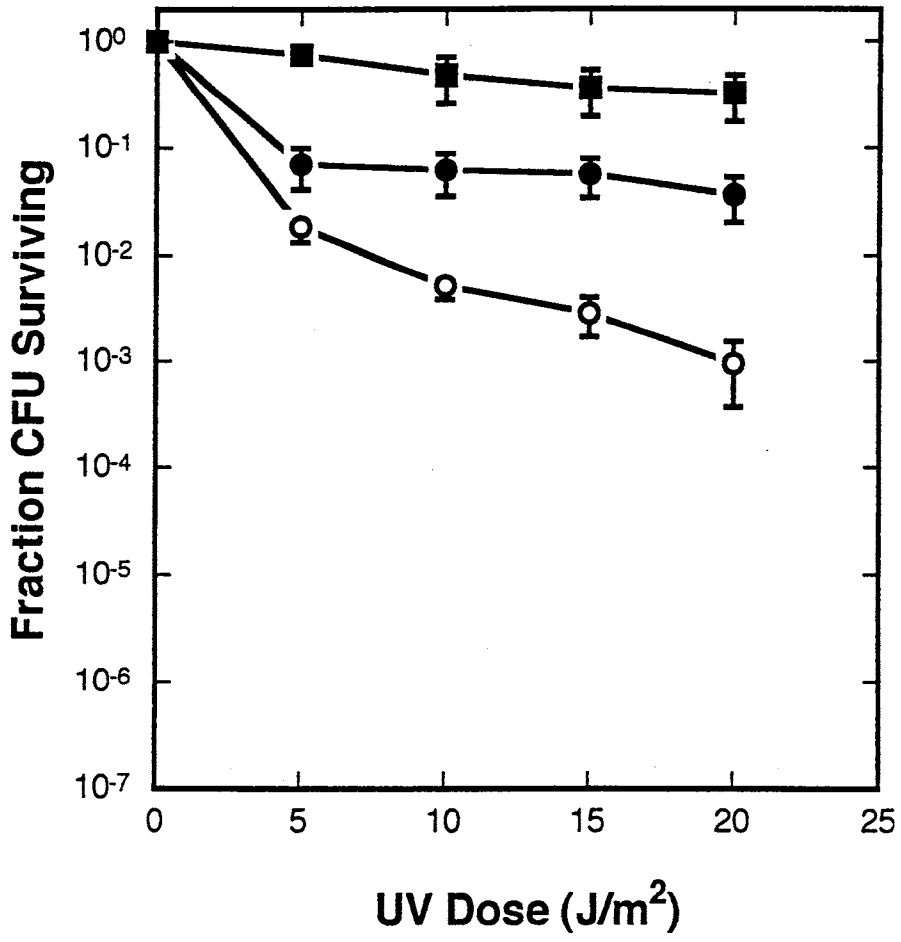

Fraction CFU Surviving
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
0
5
10
15
20
25
UV Dose (J/m^2)

Figure 3. Restoration of resistance by the *Z. mobilis recA* analogue to killing by UV irradiation of *E. coli recA56* mutant in an AB1157 background.
Cells were grown to a density of approximately 10^8 CFU/ml in Luria broth, pelleted, suspended, in saline, and exposed to various fluences of UV irradiation. After appropriate dilution in saline, the radiated cells were plated in duplicate on L agar and incubated at 37^0 C overnight in the dark. Mean value ∓ standard error are plotted. Symbols: (■), *E coli* AB1157 *recA*+; (●), *E coli* JC11372 *recA56* (pMMZ1); (○), *E coli* JC11372 *recA56*.

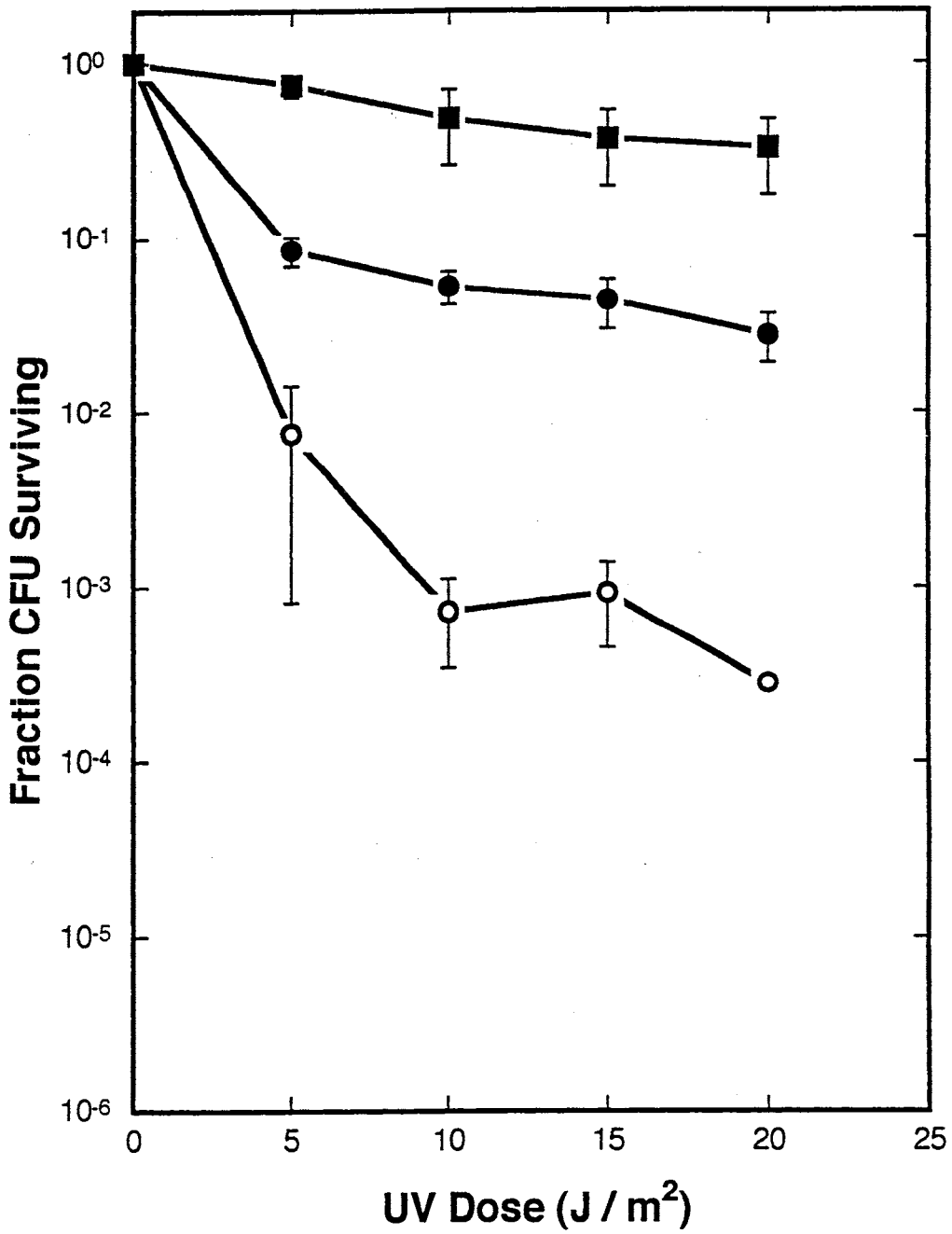
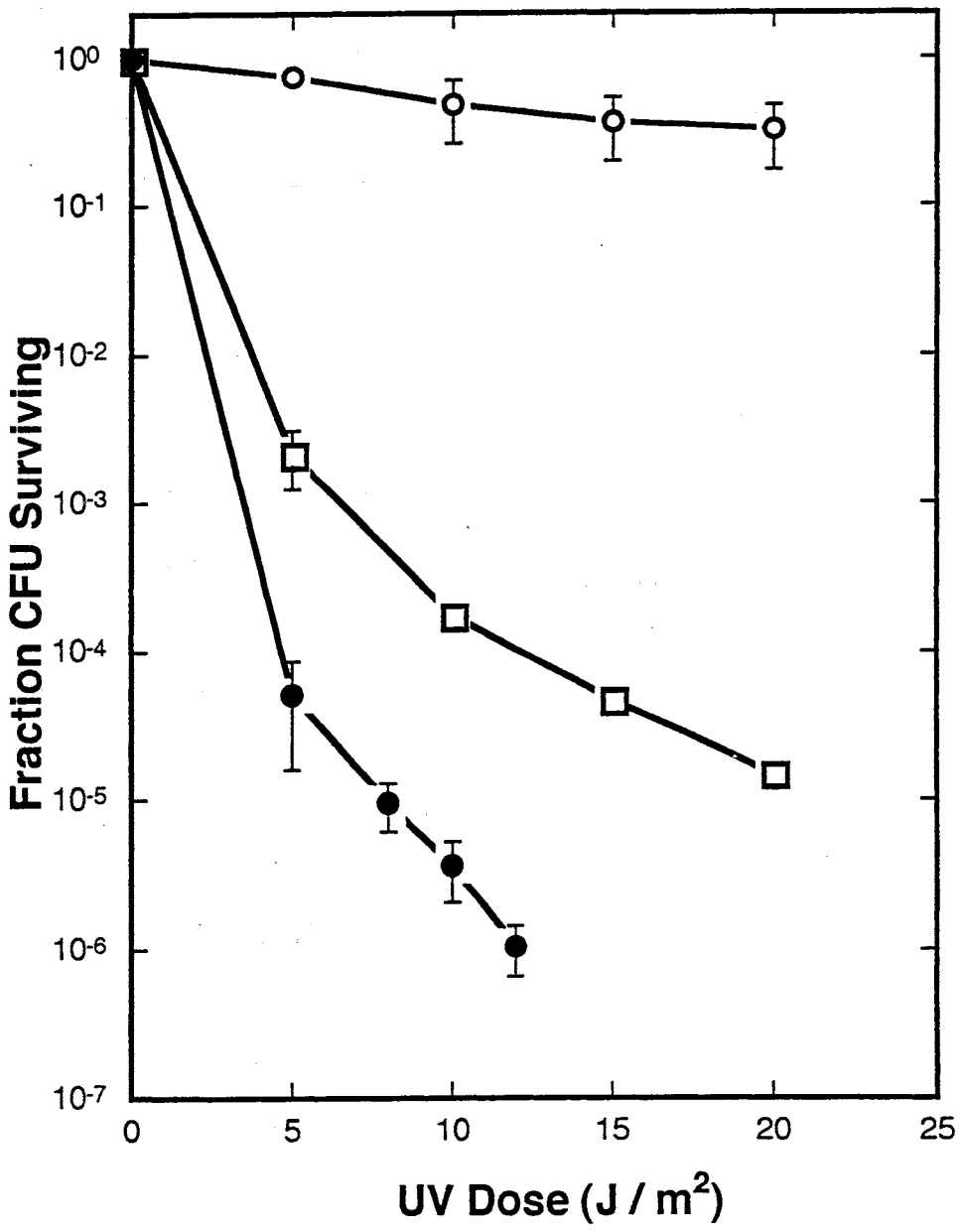

Fraction CFU Surviving
10⁰
10⁻¹
10⁻²
10⁻³
10⁻⁴
10⁻⁵
10⁻⁶
0
5
10
15
20
25
UV Dose (J / m²)

Figure 4. Restoration of resistance by the *Z. mobilis recA* analogue to killing by UV irradiation in an *E. coli recA* deletion mutant.

Cells were grown to a density of approximately 10^8 CFU/ml in Luria broth, pelleted, suspended, in saline, and exposed to various fluences of UV irradiation. After appropriate dilution in saline, the radiated cells were plated in duplicate on L agar and incubated at 37^0 C overnight in the dark. Mean value ∓ standard error are plotted. Symbols: (○), *E coli* AB1157 *recA*$^+$; (❑), *E coli* JC10289 Δ*recA* (pMMZ1); (●), *E coli* JC10289 Δ*recA*.


Fraction CFU Surviving
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
0
5
10
15
20
25
UV Dose (J / m^2)

Complementation of three *E.coli recA* mutant alleles by pMMZ1 utilizing conjugational-mediated recombination with the Hfr- strain JC158

The RecA protein is central to the process of homologous recombination. We therefore investigated the ability of pMMZ1 to complement the recombinational deficiency of the above-mentioned *E. coli recA* mutants following conjugation. Mating of each mutant with the Hfr strain JC158 was carried out with and without pMMZ1 as explained in the methods section. In addition, the wild-type strain AB1157 was also mated with JC158 as a positive control. A proficiency index for each strain was obtained by dividing the percent recombination in the mating by the percent recombination observed in a wild-type mating. The proficiency index of AB1157 with JC158 mating was set at one. Again the obtained results confirmed the ability of pMMZ1 to complement *recA*-associated phenotypes in *recA* mutants of *E. coli* strains (see Table V). An examination of these data revealed that pMMZ1 increased the proficiency index of JC2926 by forty fold, the proficiency index of the deletion mutant (JC10289) increased by as much as 2000 fold, however, the proficiency index of JC11327 was increased by only sixteen fold.

The observed inability of pMMZ1 to completely complement *E. coli recA* mutants to wild-type levels in UV-resistance studies and conjugation experiments, can be explained as follows: in general a decrease in the ability of foreign genes to express their proteins in a new host system is due to a decrease in the strength of recognition of the promoter sequence by the host's replication and translation machinery. In addition, many factors and requirements,

TABLE V. Recombinational Proficiency of *recA* mutants containing pMMZ1 [a]

Strain	pMMZ1	%Recombinants [b]	Recombinational Proficiency [c]
AB1157 (WT)	NO	3.06	1.0
JC2926 (*recA13*)	NO	0.0016	0.0007
JC2926	YES	0.033	0.03
JC11372 (*recA56*)	NO	0.006	0.0003
JC11372	YES	0.014	0.005
JC10289 (*ΔrecA*)	NO	0.0004	0.0001
JC10289	YES	0.047	0.2

a *E.coli* Hfr-strain JC158 was the donor strain. Cells were mixed in a donor-to-recipient ratio of 1:9. Matings were performed at 37o C for 2 hours without shaking. An average of at least three experiments were performed for each mating and the average of the three was reported.

b ProA+ recombinants / 100 donors

c % recombinants / % recombinants of AB11157

perhaps not present in the host system, may contribute, to a decrease in the expression of such foreign genes.

Another explanation would be the negative effect exerted by defective *recA* allele protein on wild-type RecA protein. There are two ways a defective allele can interfere negatively with the wild-type protein. First, by competition for available binding sites on the DNA. Second, by the formation of mixed multimers of, wild-type and defective protein, resulting in the formation of "poisoned" heteromultimers that have been shown to be unable to catalyze all or some of the *recA* activities (61). Particularly, it was reported that the *recA56* allele, present in JC11327, differentially affects various wild-type RecA protein activities *in vivo* (61). This allele has no effect on the UV resistance of wild-type *recA/recA*56 heterodiploids, but is codominant for homologous recombination.

In this study, the results obtained were similar to the above mentioned reports on the behavior of *E. coli recA56/recA* mixed multimers. In the UV survival curves, complementation of the *recA56* and *recA13* allele by pMMZ1 were similar. However, complementation of the *recA56* allele by pMMZ1 in the conjugational recombination experiment, was noticeably lower than the complementation of the *recA13* mutant by pMMZ1. The most noticeable affect of heterodimer interference was seen when pMMZ1 was used to complement the *recA*-deletion mutant proficiency in recombination. Since the *recA*-deletion strain, JC10289, lacks the RecA protein almost entirely, almost no interference with the putative *Z. mobilis recA* gene was expected. Despite being unable to elevate the recombinational proficiency of the *E. coli recA*

mutants to that of the wild-type *E. coli*, pMMZ1 did increase the recombinational index of JC10289 by a significant 2000 fold.

Establishment of a physical map of pMMZ1

After identifying a *recA* analogue on pMMZ1, a physical map was established to allow for the cloning of the smallest DNA fragment that would complement *recA*-associated phenotypes.

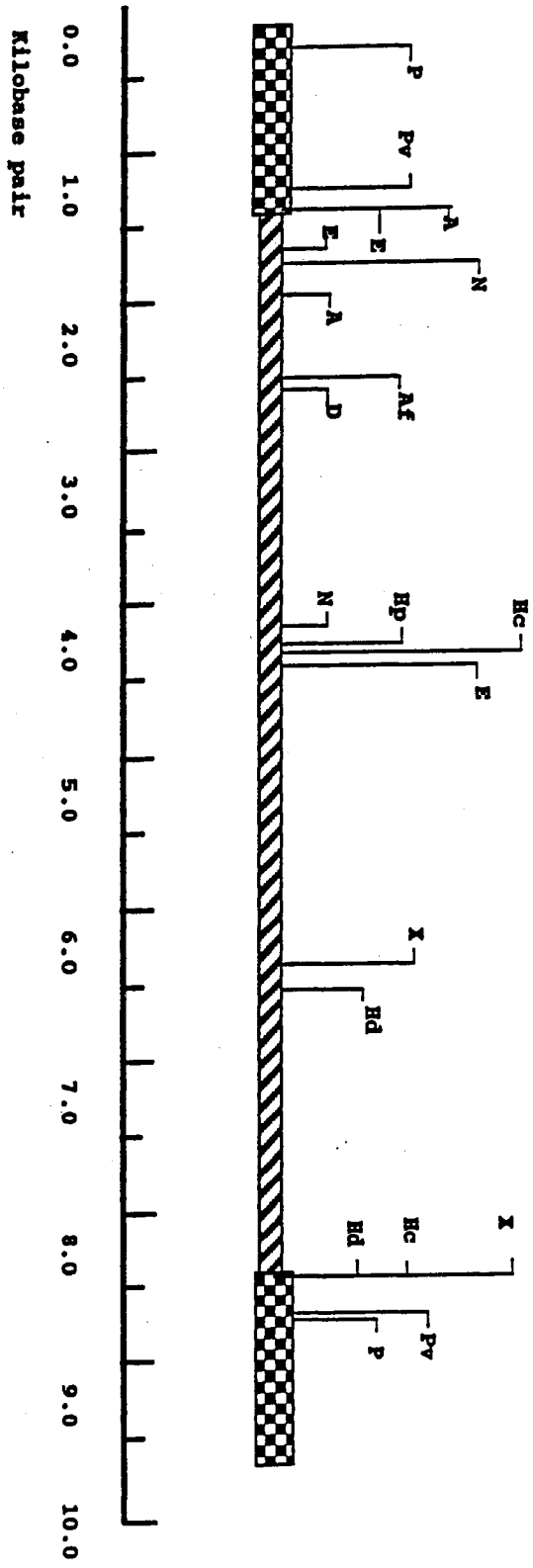
pMMZ1 was subjected to several rounds of digestion with restriction endonucleases: in each round pMMZ1 was digested first with enzyme "A" then with enzyme "B" and subsequently with both enzymes either in the same reaction or in two separate reactions depending on the compatibility of the two reaction conditions. Another approach was to digest with enzyme "A" gel purify the DNA fragment(s) and digest each fragment with enzyme "B". Eventually a sufficient restriction endonuclease map was created (Figure 4). This map was then used to create several subclones.

Subcloning of pMMZ1 and the creation of pMMZ1 nested-deletions to narrow the *recA*-complementing region

Four subclones of pMMZ1 were created by digesting pMMZ1 with restriction enzymes that have two, or more, sites on pMMZ1 one site was in the multiple cloning region of pUC18. The resulting fragments were separated on an agarose gel, purified, recircularized by hexamine cobalt chloride facilitated ligation (79), and transformed into an *E. coli recA* mutant for complementation studies. The four subclones were designated: pZD11, pZD21, pZD31, and pZD41. Each subclone essentially

Figure 5. Physical map of pMMZ1.

Plasmid vector pUC18 is shown in the checkered box, while *Z mobilis* DNA is shown in the stripped box. Restriction sites are designated as follows: Af, *Afl*II; A, *Ava*I; D, *Dra*I; E, *EcoR*I; Hc, *Hinc*III; Hd, *Hind*III; Hp, *Hpa*I; N, *Nru*I; P, *Pvu*I; Pv, *Pvu*II; X, *Xba*I.


P
Pv
A
E
E
N
A
Af
D
N
Hp
Hc
E
X
Hp
X
Hp
Hc
Pv
P
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
Kilobase pair

is pMMZ1 lacking one of the following fragments respectively: *EcoR*I fragment, *Hinc*II fragment, *Ava*I fragment, and the *NruI* fragment. In addition, the *XbaI* fragment of pMMZ1 was subcloned in the plasmid vector pBluescript SK+. All the subclones were tested for complementation of UV^S and MMS^S (Table VI). The smallest subclone which complemented the *recA*-associated phenotypes was pZD21. These complementation studies reduced the region containing the *recA* analogue of *Z. mobilis* to the left half on the pMMZ1 map (Figure 4) on the first 3.0 kb. From this result sequencing of the *recA* analogue was performed by initiating sequencing from the left side of pMMZ1.

Sequencing of the *Z. mobilis recA* gene

Sequencing of pMMZ1 was performed by automated sequencer as described in the methods section. Various primers ranging from 17 bp to 20 bp were designed with the aid of a DNA analysis software (Oligos 5.0, Macintosh, Inc.) and were sent for synthesis at the local core facility, Biochemistry Department, Oklahoma State University. The putative *recA* gene was sequenced in both directions with a significant overlap between each round of sequence. Both strands of the DNA fragment were sequenced. The sequence revealed three open-reading frames (ORFs). ORF2 was further analyzed and compared for similarity with other genes in different data banks. This analysis revealed that ORF2 had strong similarity to *recA* genes from a large and varied number of bacterial species. Figure (6) shows the *recA* gene sequence of *Z. mobilis* and its

deduced amino acid (aa) sequence in addition to putative recognition sites. It is evident from the sequence data obtained that the stop codon and

TABLE VI

recA PHENOTYPE COMPLEMENTATION

plasmid	**fragment missing**	**UV^R**	**MMS^R**
pMMZ1	none	+	+
pZD11	*EcoRI* fragment	-	-
pZD21	*HincII* fragment	+	+
pZD31	*AvaI* fragment	-	-
pZD41	*NruI* fragment	-	-
pZBX1	*XbaI* fragment on pBluescript	-	-

Figure 6. Nucleotide Sequences of the *Z. mobilis recA* gene
The codons and deduced amino acid sequence are indicated. The reading frame begins with a **Met** start codon in bold face at bp 271-274. Probable Shine-Delgarno sequence is underlined Numbering of the amino acid sequence begins at the start codon.

```
AAG ATC AGC TCT TTC CCG CCA CCC GTC CTG ATG TTT TGA CCT ATG AAA   48
 K   I   S   S   F   P   P   P   V   L   M   F   *   P   M   K

CAC CGG TTC TTG ACC ATC CCG TAA AAT TAA GAG GTG CGC CTT TCG CCA   96
 H   R   F   L   T   I   P   *   N   *   E   V   R   L   S   P

ATC TCT TAG CCG CAA CGA CTG GAA GTG ATG TTG ATT GGG TGG TGA AGT  144
 I   S   *   P   Q   R   L   E   V   M   L   I   G   W   *   S

TGA TTG ACG TTT ATC CCG ATG AGA TAC CCT CTG ATC CCA AAA TGG GAG  192
 *   L   T   F   I   P   M   R   Y   P   L   I   P   K   W   E

GCT ATC AAC TAG CGA TCA GCA TGG ATA TTT TCA GAG GCC GTT ATC GCA  240
 A   I   N   *   R   S   A   W   I   F   S   E   A   V   I   A

ACA GTT TTG AGA AGC CGT CAC CCG TGC CAG CCG GAA AAG TCC AAC AAT  288
 T   V   L   R   S   R   H   P   C   Q   P   E   K   S   N   N

ATC GCT TCC GTT TAC CGG TTG TTG ATC ATG TTT TTC TGC CGG GGC ATC  336
 I   A   S   V   Y   R   L   L   I   M   F   F   C   R   G   I

GGA TTA TGG TTC AAA TTC AGT CAA GTC TTT TCC CGC TTT ATT GAT CGT  384
 G   L   W   F   K   F   S   Q   V   F   S   R   F   I   D   R

AAT CCG CAG CGT TAT GTC GAA AAT ATT ATG TTC GCC AAG CCT TCT GAT  432
 N   P   Q   R   Y   V   E   N   I   M   F   A   K   P   S   D

TAT GCG NCA NCC GTA GAA NCG GTC ATG CAT TCA CCG GAT CAG GCA AGC  480
 Y   A   X   X   V   E   X   V   M   H   S   P   D   Q   A   S

TCG GTT GAA TTG CCG ATT ATC CCA TAA ATC TTT GAG AAT AAA TTC ACT  528
 S   V   E   L   P   I   I   P   *   I   F   E   N   K   F   T

GCT GGG GAA GCT GTT TTT CAG CTT TCT CGG TAG TAG AAA AAC AAG TTT  576
 A   G   E   A   V   F   Q   L   S   R   *   *   K   N   K   F

TAA ATT CTG TCA GTT AAG ACT TGC TAT TCT GTT TCT GCT CTC TAT GTT  624
 *   I   L   S   V   K   T   C   Y   S   V   S   A   L   Y   V

CAC CTT ATG TTC TTC ATG CTG TTC CGG TAT ATT CGA CGC AAT CCT GAC  672
 H   L   M   F   F   M   L   F   R   Y   I   R   R   N   P   D

ATT ATG CCG GTT GAT GCC TTA AGA GAG ACG TTA TAG GAG AAG GGG GTA  720
 I   M   P   V   D   A   L   R   E   T   L   *   E   K   G   V

GCG ATG GCT CCG CCA CAT AAG GTT ACG ACT TCC GGT AAG GAT AAT AAT  768
 A   M   A   P   P   H   K   V   T   T   S   G   X   D   N   N   15

ATG GAC AAC CAA CAA GCC CTT GAA GCG GCT TTA GCT CAG AAT GAC CGC  816
 M   D   N   Q   Q   A   L   E   A   A   L   A   Q   N   D   R   31

GCT TTT GGT GAA GGT TCG GTT ATG CGC TTG GGG TCT CGT GAG GAA ATC  864
 A   F   G   E   G   S   V   M   R   L   G   S   R   E   E   I   47

GAG ATT GAT ACA ATT TCC AGC GGA TCA CTT GGC CTT GAT ATT GCG CTG  912
 E   I   D   T   I   S   S   G   S   L   G   L   D   I   A   L   63
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GGC ATT GGC GGC TTG CTC CGT GGC CGT ATC GTC GAA ATC TAT GGT CCT   960
 G   I   G   G   L   L   R   G   R   I   V   E   I   Y   G   P    79

GAA AGC TCG GGT AAA ACA ACT TTA GCC CTC CAT GCT ATC GCT GAA GCA   1008
 E   S   S   G   K   T   T   L   A   L   H   A   I   A   E   A    95

CAC ACA GCA GGT GGA ACA GCG ACC TTT GTC GAT GCT GAA CAT GCG CTT   1056
 H   T   A   G   G   T   A   T   F   V   D   A   E   H   A   L   111

GAT CCG GTC TAT GCC AAA AAA CTG GGC GTT AAT ACG GAT GAT CTA ATC   1104
 D   P   V   Y   A   K   K   L   G   V   N   T   D   D   L   I   127

GTC TCT CAG CCC GAT ACG GGT GAG CAG GCT CTG GAA ATT ACA GAT ACC   1152
 V   S   Q   P   D   T   G   E   Q   A   L   E   I   T   D   T   143

CTG ATC CGT TCT AAC GCT GTT GAT ATA TTG GTT GTC GAT TCT GTT GCG   1200
 L   I   R   S   N   A   V   D   I   L   V   V   D   S   V   A   159

GCC TTA ACA CCC CGC GCT GAA ATC GAA GGC GAA ATG GGC GAT AGT CAC   1248
 A   L   T   P   R   A   E   I   E   G   E   M   G   D   S   H   175

GTT GGT TTG CAA GCC CGT TTG ATG AGT CAG GCG CTT CGC AAA ATT ACC   1296
 V   G   L   Q   A   R   L   M   S   Q   A   L   R   K   I   T   191

GGA TCA ATC AAC CGC TCT CAA ACT TTG GTT ATT TTT ATC AAC CAA GTC   1344
 G   S   I   N   R   S   Q   T   L   V   I   F   I   N   Q   V   207

CGG ATG AAA ATC GGG GTA ATG TAT GGC AAC CCT GAA ACG ACA ACG GGC   1392
 R   M   K   I   G   V   M   Y   G   N   P   E   T   T   T   G   223

GGT AAT GCT TTG AAA TTC TAT GCC AGC GTT CGG CTT GAT ATC CGT CGT   1440
 G   N   A   L   K   F   Y   A   S   V   R   L   D   I   R   R   239

GTA GGA CAA ATT AAA GAT CGC GAT GAA ATC GTT GGT AAT CCC ACC CGT   1488
 V   G   Q   I   K   D   R   D   E   I   V   G   N   P   T   R   255

GTC AAA GTG GTA AAA AAC AAG CTG GCT CCG CCT TTC AAG CAG GTT GAA   1536
 V   K   V   V   K   N   K   L   A   P   P   F   K   Q   V   E

TTC GAT ATC ATG TAT GGA GAA GGT GTC TCC AAG ATG GGC GAA ATT CTC   1584
 F   D   I   M   Y   G   E   G   V   S   K   M   G   E   I   L   271

GAT CTG GGC GTA AAA GCC GGT ATT ATC GAC AAG TCA GGC TCA TGG TTC   1632
 D   L   G   V   K   A   G   I   I   D   K   S   G   S   W   F   287

AGT CAT GAT TCC GTT CGC ATC GGT CAG GGA CGT GAA AAT NCC AAA ACG   1680
 S   H   D   S   V   R   I   G   Q   G   R   E   N   X   K   T   303


TTC TTG CGT GAA CAT CCT GAA ATG ACT GAA AAA ATT GAA AAA ATG ATT   1728
 F   L   R   E   H   P   E   M   T   E   K   I   E   K   M   I   319

CGT CAT AAT ACG GCT GAA ATT GCC GAC GAA ATG CT                   1763
 R   H   N   T   A   E   I   A   D   E   M   L                  331
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therefore the complete gene is not present. Attempts to obtain the remaining *recA* gene fragment were unsuccessful. Some of the methods attempted include: southern hybridization utilizing internal *recA* gene probes; colony hybridization of the partial library; polymerase chain reaction (PCR) amplification of the library; and inverse-PCR of the whole genome. We hope to obtain the missing fragment from personal collaboration with Professor Milton Typas at the University of Athens, Greece, who has obtained partial sequence of the *Z. mobilis recA* gene (personal communication).

The *recA* gene is very conserved among all bacterial strains sequenced to this date (30,31). Conservation of the amino acid sequence is much higher in the amino terminus of the proteins than the carboxyl terminus. This observation reflects the fact that the functional domains on the *recA* gene are condensed near the amino -terminus of the gene (30). By aligning the deduced amino-acid sequence of the *Z. mobilis recA* gene with the sequence of other closely related RecA protein sequences (Figures 6 and 7), it is observed that only a few amino acids were missing from the carboxyl end of the *Z. mobilis* RecA deduced amino acid sequence. In addition, it was clearly demonstrated that the cloned *recA* gene was functional and exhibited all *recA*-associated phenotypes. From the results obtained during this search I have to conclude that the carboxyl end of the *Z. mobilis recA* gene may be lethal in *E. coli*, or an attached sequence downstream of the missing fragment is lethal.

Figure 7. Alignment of the *Z. mobilis* RecA protein.
The deduced amino acid sequence of the *Z. mobilis* RecA protein was aligned with various randomly chosen RecA proteins using the MACCAW protein analysis software . Capital letters represent aligned similar amino acids, while lower case letters represent unsimilar amino acids. The absence of any amino acid at a particular site is represented with a dash. Bacterial names are designated as follows: Pm, *Proteus mirabilis*, Va, *Vibrio anguillarum*; Pa, *Pseudomonas aeruginosa*; Ec, *Escherichia coli*; Hv, *Helicobacter variabilis*; Ss,Synechococcus sp; Ng, *Nisseria gonorrhoeae*; Am, *Aquaspirillum magnetotacticum*; Ba, *Brucella abortus*; Sm, *Serratia marcescens*; Sp, *Streptococcus pyogens*; Al, *Acholeplasma laidlawii*; Tt,*Thermus thetaiotaomicron*; Tf, *Thiobacillus ferooxidans*

```
Zm      Mxpphkvttsgxdnnmdnqqa-LeAALaQnDRAFGeGsVMRLGsreeieidtisS-GSLG
Pm      Mai-------DEN-----KQKALAAALGQIEKQFGKGSIMRLGEDRSMn-VETISTGSls
Va      M---------DEN-----KQKALAAALGQIEKQFGKGSIMRLGDRTMD--VETISTGSls
Pa      M---------DEN-----KKRALAAALGQIERQFGKGAVMRMGDherqa-IPAISTGSlg
Ec      Mai-------DEN-----KQKALAAALGQIEKQFGKGSIMRLGEDRSMd-VETISTGSls
Hv      Maintdtsg---------KQKALTMVLNQIERSFGKGAIMRLGDatrmr-VETISTGAlt
Ss      Msaisnnp--D-------KEKALNLVLNQIERNFGKGAIMRLGDaaqmk-VATIPSGAlt
Nq      Msd-------D-------KSKALAAALAQIEKSFGKGAIMKMDGsqqeenLEVISTGSlg
Am      M---------D-------RQKALEAAVSQIERAFGKGSIMKLGGkdqvveTEVVSTRIlg
Ba      Msqnslrlve DNSvd---KTKALDAALSQIERAFGKGSIMRLGQndqvveIETVSTGSls
Sm      Mai-------DEN-----KQKALAAALGQIEKQFGKGSIMRLGEDRSMd-VETISTGSls
Sp      Makkpkkleeiskkfgae REKALNDALKLIEKDFGKGSIMRLGEraeqk-VQVMSSGSla
Al      Ms--------DNK-----KQQALELALKQIEKQFGKGSIMKLGDgadhs-IEAIPSGSia
Tt      M---------DES-----KRKALENALKAIEKEFGKGAVMRLGEmppkq-VDVIPTGSla
Tf      M---------DEQ-----RSKGLSAALSQIDKQFGKGAVMRLGDhd----IEVYSTGSLG

Zm      LDIALGIGGLlRGRIVEIYGPESSGKTTLaLHAIAEAhtAGGTAtFVDAEHALDPvYAkk
Pm      LDvAlGaGGLpRGRIVEIYGPESSGKTTLtLqvIAsAQREGKICAFIDAEHALDPiYAqk
Va      LDiAlGaGGLpmGRIVEVYGPESSGKTTLtLelIAaAQRVGKTCAFIDAEHALDPiYAkk
Pa      LDiAlGiGGLpkGRIVEIYGPESSGKTTLtLsvIAEAQKQGATCAFVDAEHALDPdYAgk
Ec      LDiAlGaGGLpmGRIVEIYGPESSGKTTLtLqvIAaAQREGKTCAFIDAEHALDPiYArk
Hv      LDlAlG-GGLpRGRVIEIYGPESSGKTTVaLHAIAEVQKEGGIAAFVDAEQALDPtYAsa
Ss      LDqAmG-GGFpRGRIVEIYGPESSGKTTVaLHAIAEVQKAGGVAAFIDAEHALDPtYSaa
Nq      LDlALGvGGLrRGRIVEIFGPESSGKTTLcLeAvAQCQKNGGVCAFVDAEHAFDPvYArk
Am      LDvALGiGGvpRGRIIEVYGPESSGKTTLaLHiIAEAQKkGGtcAFVDAEHALDPsYArk
Ba      LDiALGvGGLpkGRIVEIYGPESSGKTTLaLHtIAEAQKKGGIcAFVDAEHALDPvYArk
Sm      LDIALGaGGLpmGRIVEIYGPESSGKTTLtLqvIAaAQREGKTCAFIDAEHALDPiYAkk
Sp      LDIALGsGGypkGRIIEIYGPESSGKTTVaLHAvAQAQKEGGIAAFIDAEHALDPaYAaa
Al      LDIALGiGGypRGRIIEVYGPESSGKTTLtLHAmAsAQKQGGTVAFIDAEHALDPnYAka
Tt      LDlALGIGGipRGRIVEIYGPESGGKTTLaLTiIAqAQRRGGVAAFVDAEHALDPlYAqr
Tf      LDlALGvGGLpRGRVVEIYGPESSGKTTLtLHAIAsCQAAGGTAAFIDAEHALDPgYAhk

Zm      LGVntDdLivSQPDTGEQALEItDtLiRSnAVDilVVDSVAALTPRAEIEGEMGDSHVGL
Pm      LGvdiDnLlcSQPDTGEQALEIcDaLsRSGAVDviVVDSVAALTPKAEIEGEiGDSHVGL
Va      LGvniDeLlvSQPDTGEQALEIcDaLaRSGAIDviViDSVAALTPKAEIEGEMGDSHmGL
Pa      LGvnvDdLlvSQPDTGEQALEItDmLvRSNAVDviiVDSVAALvPKAEIEGEMGDaHVGL
Ec      LGvdiDnLlcSQPDTGEQALEIcDaLaRSGAVDviVVDSVAALTPKAEIEGEiGDSHmGL
Hv      LGvdiqnLlvSQPDTGESALEIvDqLvpSAAVDivViDSVAALvPRAEIEGDMGDaHVGL
Ss      LGvdienLlvAQPDNGESALEIaDqLvRSAAVDliViDSVAALvPRAEIEGEMGDvQVGL
Nq      LGvkveeLylSQPDTGEQALEIcDtLvRSGGIDmvVVDSVAALvPKAEIEGDMGDSHVGL
Am      LGaldelLi-SePDaGEQALEIaDtLvRpgavDvlVVDSVAALvPRGELEGEMGDnHmGL
Ba      LGVhlenLliSQPITGEQALEItDtLvRSGAIDvlVVDSVAALTPRAEIEGEMGDSH-GL
Sm      LGVdidnLlcSQPDTGEQALEIcDaLtRSGAVDviiVDSVAALTPKAEIEGEiGDSHmGL
Sp      LGVniDeLllSQPDSGEQGLEIaGkLidSgAVDlvVVDSVAALvPRAEIdGDiGDSHVGL
Al      LGVdlDnLvlSQPDTGEQALDIaEaLiKSGSIDmiViDSVAALvPeAEIaGDMSAnHVGL
Tt      LGVqvedLlvSQPDTGEQALEIvElLaRSgAVDviVVDSVAALvPRAEIEGEMGDqHVGL
Tf      LGVdlenLliSQPDTGEQALEIaDmLvRSGAVDliViDSVAALTPKAEIEGEMGDSHVGL
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Zm      QARLMSQALRkiTGsInrSqTLvIFINQvRMKIGVMYGnPETTTGGNALKFYASVRLDIR
Pm      aARmMSQAmRKLaGnlknSnTLlIFINQIRMKIGVMFGNPETTTGGNALKFYASVRLDIR
Va      QARmlSQAmRKLTGnlkqSncmcIFINQIRMKIGVMFGNPETTTGGNALKFYASVRLDIR
Pa      QARlMSQALRKITGnIknancLvIFINQIRMKIGVMFGNPETTTGGNALKFYASVRLDIR
Ec      aARmMSQAmRKLaGnlkqSnTLlIFINQIRMKIGVMFGNPETTTGGNALKFYASVRLDIR
Hv      QARlMSQALRKITGnIgkSgctvIFINQLRqKIGVTYGSPETTTGGNALKFYASVRLDIR
Ss      QARlMSKALRKIaGnmgrSgctvIFLNQLRqKIGISYGNPEVTTGGtALKFYASVRLDIR
Nq      QARlMSQALRKLTGhIkktnTLvVFINQIRMKIGVMFGSPETTTGGNALKFYSSVRLDIR
Am      hARlMSQALRKLTGsvskSkTivIFINQIRMKIGVMfgnpETTTGGNALKFYASVRMEIR
Ba      QARlMSQAvRKLTGsIsrSncmvIFINQIRMKIGVMFGSPETTTGGNALKFYASVRLDIR
Sm      aARmMSQAmRKLaGnlknanTLlIFINQIRMKIGVMFGNPETTTGGNALKFYASVRLDIR
Sp      QARmMSQAmRKLgasInktkTiaIFINQLReKVGVMFGNPETTpGGrALKFYASVRLDVR
Al      QARmMSQAmRKMsGvIskSnvvaIFINQIReKVGVMFGNPETTpGGrALKFfsSVRLEIR
Tt      QARlMSQALRKLTavlakSnTaaIFINQVReKVGVtygnPETTpGGrALKFYASVRLDVR
Tf      QARLMSQALRnLTanIsrSnTLvIFINQIRMKIGVMYGSPETTTGGNALKFYASVRLDIR

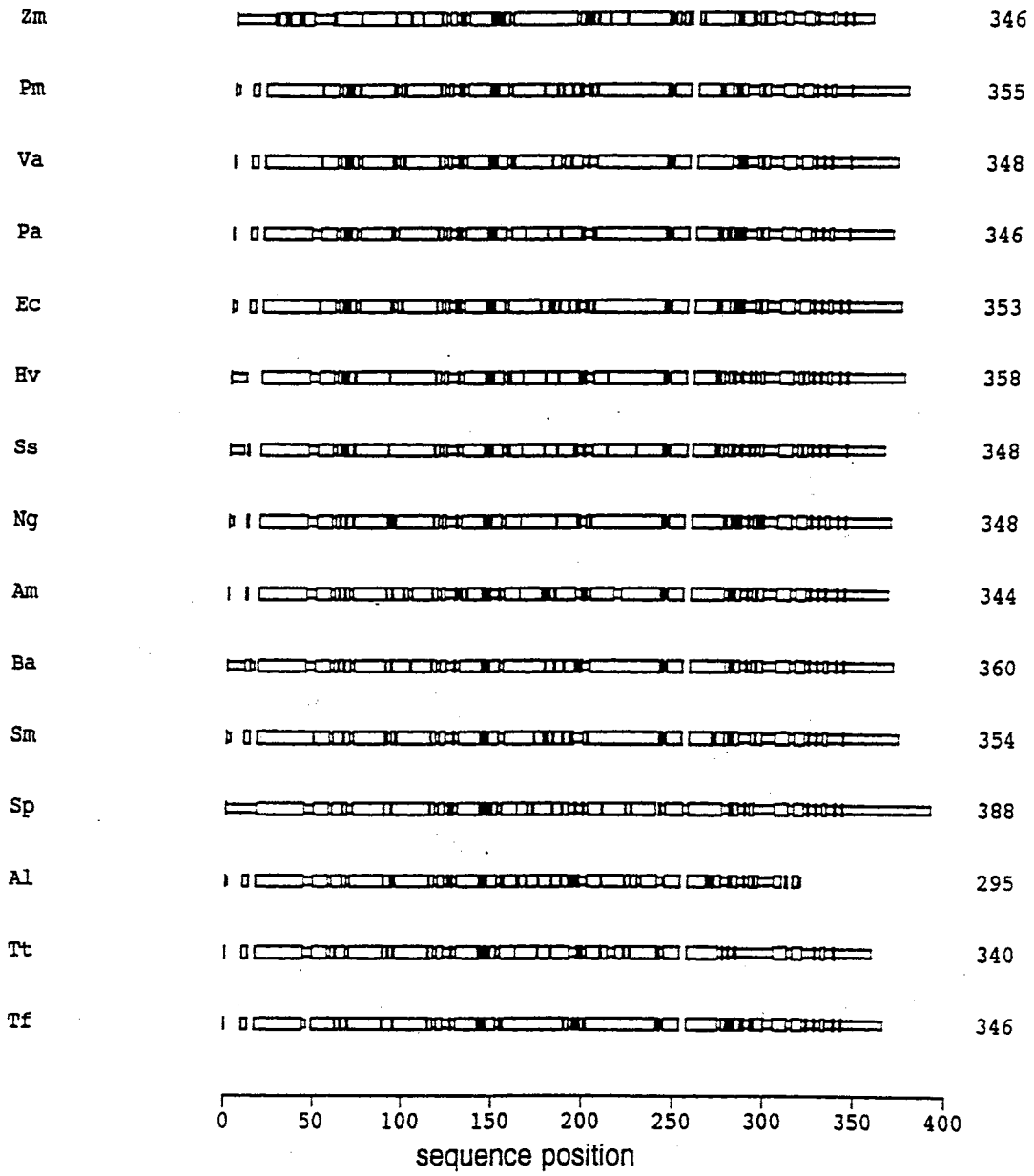
Zm      RvGq--IKdrDEiV----GNpTRVKVVKNKLAPPFKQvEFdI-MYGEGvSkmGEilDLGV
Pm      RiGs--VKNGDEVI----GSETRVKVVKNKvAaPFKQaEFqI-MYGegintyGEliDLGv
Va      RtGs--IKEGDEAV----GNETRIKVVKNKIAAPFKQadtqIlY-Gqgfnre GElvDLGv
Pa      RtGa--VKEGDEVV----GSETRVKVVKNKvSpPFrQaEFqIlY-GkgiyrtGEiiDLGv
Ec      RiGa--VKEGENVV----GSETRVKVVKNKiAaPFKQaEFqIlY-GeginfyGElvDLGv
Hv      RiQt--LKKGTDEF----GNRVKVKVAKNKvApPFriaEFdIif-GkgvStlGclvDLae
Ss      RiQt--LKKGSEGEF---GIRAKVKVAKNKvApPFriaEFdIif-GkgiSrvGcmlDLae
Nq      RtGs--IKKGEEVL----GNETRVKVIKNKVAPPFrQaEFdIlY-GegiSweGEliDiGv
Am      RvGa--IKDRDEVV----GNQTRVKVVKNKLAPPFKvvdFdI-MYGegiSkmGEliDLGv
Ba      RiGs--IKERDEVV----GNQTRVKVVKNKLAPPFKQvEFdI-MYGagvSkvGElvDLGv
Sm      RiGa--IKEGDEVV----GSETRVKVVKNKiAaPFKQaEFqI-MYGeginsrGElvDLGv
Sp      --GntqIKGTGDQKEtnvGKETKIKVVKNKVAPPFKeavveI-MYGegiSktGEllkias
Al      Raea--IKQGSEMI----GIKSNVKVVKsKvApPLKtasidI-MYGtgiSrsGEvlDLsv
Tt      KsGqp-IKVGNEAV----GVKVRVKVVKNKLAPPFreaEleIyf-Grgldpvadlvnvav
Tf      RiGa--IKKSDEVV----GNDTRVKVVKNKVAPPFreaEFaIyY-GEGiSrlsElvDLGV

Zm      KagiidKSGSWfshdsvRIGQGreNxktflrehPEmtekIekmIRhntaeiadem-----
Pm      khklveKAGAWYSYngeKIGQGkaNatnyLkehPEmynelntklReml lnhageftsaad
Va      khklveKAGAWYSYngdKIGQGkaNackfLrenPaaamaldtklReml lnpaeliveepi
Pa      qlglveKSGAWYSYqgsKIGQGkaNaakyLednPEigsvlektIRdqllaksgpvkadae
Ec      keklieKAGAWYSYkgeKIGQGkaNatawLkdnPEtakeIekkvRelllsnpnstpdfsv
Hv      etgillRKGAWYSYngdNIsQGrdNaikyLeekPEfaeqIkqqvRekldkgavvsansva
Ss      qtgvitRKGAWYSYegdNIaQGrdNavkyLeenPdvaaivtqkvRenldmssmgfgdehh
Nq      kndiinKSGAWYSYngaKIGQGkdNvrvwLkenPEisdeIdakIRalngvemhitegtqd
Am      kanvvkKSGAWFSYnstRIGQGreNakqfLrdnPamaaeIegaIRqnagliseala avpd
Ba      kagvveKSGAWFSYnsqRLGQGreNakqyLkdnPEvareIettlRqnagliaeqflddgg
Sm      khkmieKAGAWYSYngeKIGQGkaNacnfLkenPAiaaeldkklRdlllhsggelvaasg
Sp      dldiikKAGAWYSYkdeKIGQGseNakkyLaehPEifdeIdkqvRskfglidgeevseqd
Al      elnlvnKSGAW--Y---NIGe-------------------------------------
Tt      aagvieKAGSWFSYgelRLGQGkekaaeaLrerPElleeIrakvlersdqvvlaagedeg
Tf      KfdiveKSGAWYSYqghRIGQGkdNarqyLkvhPElaanIeqrIRaaaaghplafaeeve
```

```
Zm  ----------
Pm  fageesdsdaddtke----------
Va  lsempqeeel----------
Pa  evadaead----------
Ec  ddsegvaetnedf----------
Hv  kaneedeedvdldeee----------
Ss  tteee----------
Nq  etdgerpee----------
Am  ldgtpvae----------
Ba  peedaagaaem----------
Sm  ddfeddeaetseqf----------
Sp  tenkkdepkkeeavneevpldlgdeleieiee
Al  ----------
Tt  e----------
Tf  spqrsas----------
```

Figure 8. Schematic representation of the RecA protein alignment

The *Z. mobilis* RecA protein was aligned with several RecA proteins from various bacteria using the MACCAW protein analysis software and schematically represented as a function of similarity. Bacterial names are designated as follows: Pm, *Proteus mirabilis*, Va, *Vibrio anguillarum*; Pa, *Pseudomonas aeruginosa*; Ec, *Escherichia coli*; Hv, *Helicobacter variabilis*; Ss,Synechococcus sp; Ng, *Nisseria gonorrhoeae*; Am, *Aquaspirillum magnetotacticum*; Ba, *Brucella abortus*; Sm, *Serratia marcescens*; Sp, *Streptococcus pyogens*; Al, *Acholeplasma laidlawii*; Tt,*Thermus thetaiotaomicron*; Tf, *Thiobacillus ferooxidans*


Zm
346
Pm
355
Va
348
Pa
346
Ec
353
Hv
358
Ss
348
Ng
348
Am
344
Ba
360
Sm
354
Sp
388
Al
295
Tt
340
Tf
346
0
50
100
150
200
250
300
350
400
sequence position

CHAPTER V

INSERTIONAL INACTIVATION OF THE *Zymomonas mobilis recA* GENE BY GENE REPLACEMENT

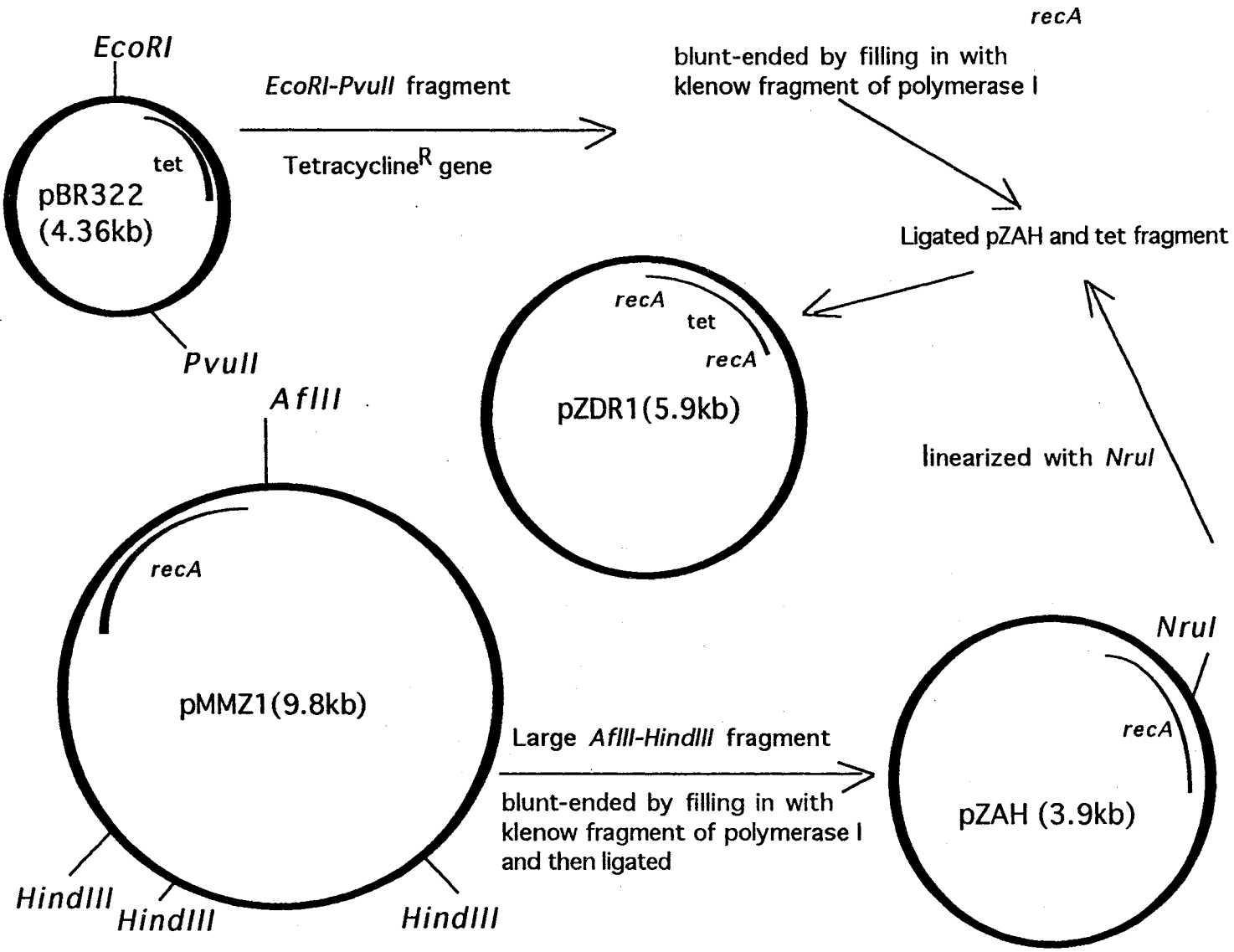
Construction of a Plasmid vector for the insertional inactivation of wild-type *Z. mobilis recA* gene

To construct a recombination-deficient *Z. mobilis* strain, insertional inactivation mutagenesis was carried out using strain CP4. *Z. mobilis* CP4 is resistant to almost all antibiotics except tetracycline, rifampicin, and chloramphenicol (54). The strategy for creating a *recA* mutant was to insert a tetracycline-resistance gene from pBR322 into the cloned *recA* gene on pMMZ1 and to transform *Z. mobilis* CP4 with a replication-deficient version of this construct, and eventually to select for tetracycline-resistant/MMS-resistant clones.

Plasmid vector pBR322 was digested with *Eco*RI and *Pvu*II in a double digest and the resulting bands were run on a small gel to confirm complete digestion. This digest resulted in two fragments: a 2067 bp fragment and a 2296 bp fragment that were separated on a 1.0% agarose gel. The smaller fragment was gel purified and resuspended in deionized, sterile Water. This fragment was expected to contain the tetracycline-resistance gene (86-1273bp fragment on pBR322) in addition to some pBR322 DNA. To confirm that the smaller gel-purified band on the gel was the correct fragment, it was digested with various restriction enzymes.

The presence of known restriction sites was confirmed by comparison with bands resulting from digestion of pBR322. The fragment containing the tetracycline-resistance gene, was blunt-ended (Filling in) using the klenow fragment of DNA polymerase I, and deoxyribonucleotide triphosphate (dNTP) as described by Sambrook et al.(80).

The next step after preparation of the tetracycline-resistance cassette, was to remove all unnecessary DNA from pMMZ1 leaving the intact *recA* gene, replicon, and the ampicillin-resistance gene of pUC18. This was done by digesting pMMZ1 with *Afl*II and *Hin*dIII in a double digest. After completion the reaction was stopped, and the total reaction mixture was first blunt-ended and then ligated overnight at 14° C according to manufacturers recommendation (Gibco, BRL). The fragments in this reaction mixture were: a 180 bp *Hin*dIII fragment, a 2.0 kbp *Hin*d III fragment, a 3.8 kbp *Hin*dIII-*Afl*II fragment, and a 3.7 kbp *Afl*II-*Hin*dIII fragment which contains the functional *recA* gene of *Z. mobilis* in addition to the replicon and ampicillin-resistance gene of pUC18 (see Figure 9). It was expected that all these fragments in this reaction would ligate, however, by screening for ampicillin resistance on ampicillin-suplemented LB agar plates, only the clone consisting of the 3700 bp *AflII-HindIII* fragment should grow, since it contains the origin of replication. Several ampicillin-resistant clones were obtained, and the one chosen for the construction of the insertional inactivation vector was named pZAH. The blunt-ended tetracycline-resistance cassette of pBR322 was then inserted into the linearized plasmid pZAH by ligation. The tetracycline-resistance cassette was placed inside the *recA* gene of *Z. mobilis* on pZAH such that the tetracycline-resistance gene was flanked on both sides with *recA* gene DNA (for an overview of the cloning strategy see Figure 9). This


EcoRI
EcoRI-PvuII fragment
Tetracycline^R gene
tet
pBR322
(4.36kb)
PvuII
recA
blunt-ended by filling in with
klenow fragment of polymerase I
Ligated pZAH and tet fragment
recA
tet
recA
pZDR1(5.9kb)
linearized with NruI
AflII
recA
pMMZ1(9.8kb)
HindIII
HindIII
HindIII
Large AflII-HindIII fragment
blunt-ended by filling in with
klenow fragment of polymerase I
and then ligated
NruI
recA
pZAH (3.9kb)

construct was termed pZDR1 and was selected for by resistance to tetracycline and ampicillin.

Introduction into *Z. mobilis*

Z. mobilis was essentially transformed with pZDR1 by electroporation as recommended by the manufacturer using an Electro Cell Manipulator 600, (BTX). Some modifications of the original methods of electroporation were adapted to account for the small size of *Z. mobilis* cells as described by Nakamura *et al.* (76). Prior to transformation of pZDR1, the origin of replication of pZDR1 (pUC18 ori) was removed by digestion of pZDR1 with *Ava*I, separation of the fragments on an agarose gel, and gel purification of the linearized plasmid DNA. Linear pZDR1 thus lacked an origin of replication, and was then used for the transformation of *Z. mobilis* CP4 with selection on agar plates containing 5.0mg/ml tetracycline. Since pZDR1 is unable to replicate in *Z. mobilis*, and *Z. mobilis* is tetracycline sensitive, it was expected that transformation would give rise to derivatives that had undergone homologous recombination between the parental chromosome and the cloned *recA* gene. Twenty nine tetracycline-resistant colonies were obtained and plated again on tetracycline-containing agar plates for confirmation of tetracycline-resistance. All twenty nine clones were found to be tetracycline resistant. Furthermore, all twenty nine clones were found to be MMS sensitive on MMS-suplemented agar plates.

Characterization of *Z. mobilis* AM96

Further characterization of one of the *Z. mobilis* derivatives was performed to ensure the loss of *recA*-associated phenotypes. This strain was named *Z. mobilis* AM96 The sensitivity of AM96 to MMS was tested by growing the cells to mid-exponential phase and resuspending in sterile saline solution, then 25 ml of cell suspension was dropped on an agar plate containing a known concentration of MMS. Cells were allowed to grow for twelve hours at 30^0 C. The concentration of MMS in the agar plates ranged from 0.01-to-0.04 % (see Table VII). The *Z. mobilis* wild-type strain (CP4) and *E. coli* wild-type strain (AB1157) were also tested for MMS sensitivity. *Z. mobilis* AM96 was very sensitive to all MMS concentrations tested, while both *Z. mobilis* and *E.coli* wild-type strains were resistant to elevated concentrations of MMS (see Table VII). To ensure the presence of the tetracycline-resistance gene in the mutant strain, AM96 was grown in liquid media in the presence of tetracycline at various concentrations as shown in table VIII. Growth of AM96 in tetracycline was observed after less than six hours of incubation. It was shown that strain AM96 grew in as much as 100 ug tetracycline/ml of broth, while the wild-type strain CP4 did not survive 50 ug tetracycline/ml of broth.

Additional characterization was done by performing a UV survival curve (see Chapter 3) on the mutated strain. *Z. mobilis* strains AM96 and strain CP4, were exposed to UV radiation ranging from ten to fifty joules/m^2 and surviving colonies were counted. Figure 10 shows that strain AM96 was very sensitive to UV radiation and had very low DNA repair abilities when compared to strain CP4 (Figure 10).

TABLE VII

MMS SENSITIVITY OF BACTERIAL STRAINS [a]

Strain	0.01% MMS	0.02% MMS	0.03% MMS	0.04% MMS
Z. mobilis CP4	+	+	+	+/-
Z. mobilis AM96	-	-	-	-
E. coli AB1157	+	+	+/-	-

[a] Bacteria were grown to mid-exponential phase, washed twice in saline, diluted in saline to appropriate concentrations, and plated on either LB nutrient agar plates (*E. coli*) or on RM nutrient agar plates (*Z. mobilis*) containing MMS at the indicated concentrations.

TABLE VIII

TETRACYCLINE RESISTANCE OF *Z. mobilis* AM96 [a]

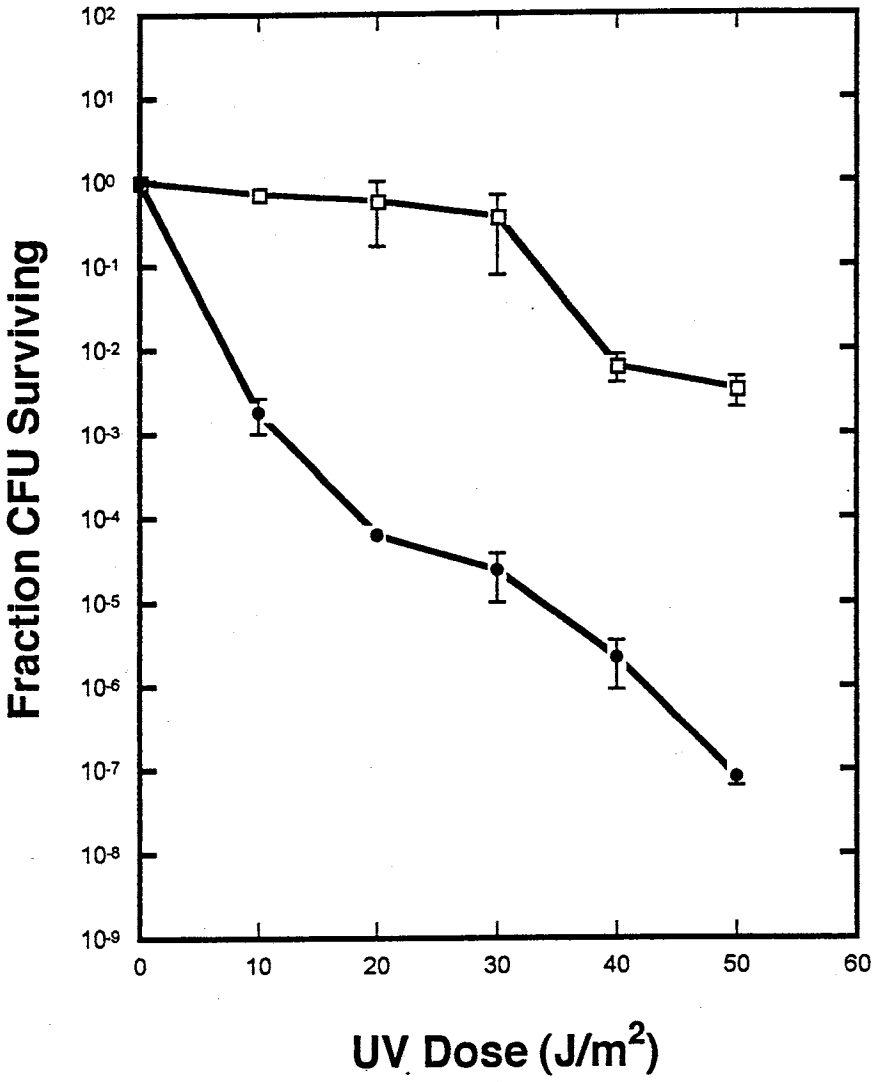
Strain	25 μg/ml	50 μg/ml	75μg/ml	100μg/ml
Z. mobilis CP4	+	-	-	-
Z. mobilis AM96	+	+	+	+/-

[a] Bacteria were picked from a fresh plate, grown to mid-log phase in RM nutrient broth, washed twice in saline, resuspended in saline, and a 0.1 ml was taken and added to 9.9 ml of RM nutrient broth containing the appropriate concentration of tetracycline.

Figure 10. Comparison of resistance by the *Z. mobilis* wild-type strain CP4 and the *recA* mutant *Z. mobilis* strain AM96 to killing by UV irradiation.

Cells were grown to a density of approximately 10^8 cfu/ml in RM broth, pelleted, suspended, in saline, and exposed to various fluences of UV irradiation. After appropriate dilution in saline, the radiated cells were plated in duplicate on RM agar and incubated at 30^0 C overnight in the dark Mean value $\mp$ standard error are plotted.

Symbols: (❑), *Z. mobilis* CP4 wild-type; (●), *Z. mobilis* AM96 *recA* insertional inactivation mutant.


Fraction CFU Surviving
10²
10¹
10⁰
10⁻¹
10⁻²
10⁻³
10⁻⁴
10⁻⁵
10⁻⁶
10⁻⁷
10⁻⁸
10⁻⁹
0
10
20
30
40
50
60
UV Dose (J/m²)

A one-to-ten thousand fold decrease in UV resistance is seen in strain AM96. This suggests that the *recA* gene has been insertionally inactivated in strain AM96.

It was shown in this study that the created *recA*-deficient *Z. mobilis* strain AM96, is tetracycline- resistant, MMS-sensitive, and has very low DNA-damage mediated repair as seen from the UV survival curves. In order to examine directly the integration of the tetracycline/*recA* gene fragment of pZDR1 into the *Z. mobilis* chromosome, a southern hybridization study was performed.

Genomic DNA from *Z. mobilis* strain AM96 and CP4 were digested, to completion, with *Hin*dIII and used in a southern blot analysis. When Probed with an internal *Ava*I fragment of the *Z. mobilis recA* gene, and using stringent conditions, the wild-type strain CP4 showed an approximately 7.0 kbp band presumed to contain the *recA* gene (Figure 11 lane C). Genomic DNA form strain AM96, however, showed an approximately 9.0 kb band (Figure 11 lane B), indicating that the chromosomal fragment containing the *recA* gene had increased in size by 2.0 kb, the same size as the tetracycline-resistance gene cloned into pZAH. This clearly indicates that the tetracycline-resistance gene had integrated directly into the *recA* gene of strain AM96.

On the basis of the experimental results presented in this study a *recA*-deficient strain of *Z. mobilis* has been obtained by insertional inactivation of the *recA* gene. The mutant strain created in this study should provide a valuable tool in future studies of *Z. mobilis*. It would be of particular interest to study plasmid expression and stability in this interesting recombination-deficient strain. Furthermore, introduction of the tetracycline resistance gene into *Z. mobilis* to inactivate the *recA* gene,

should provide additional selection marker for genetic studies on this strain.

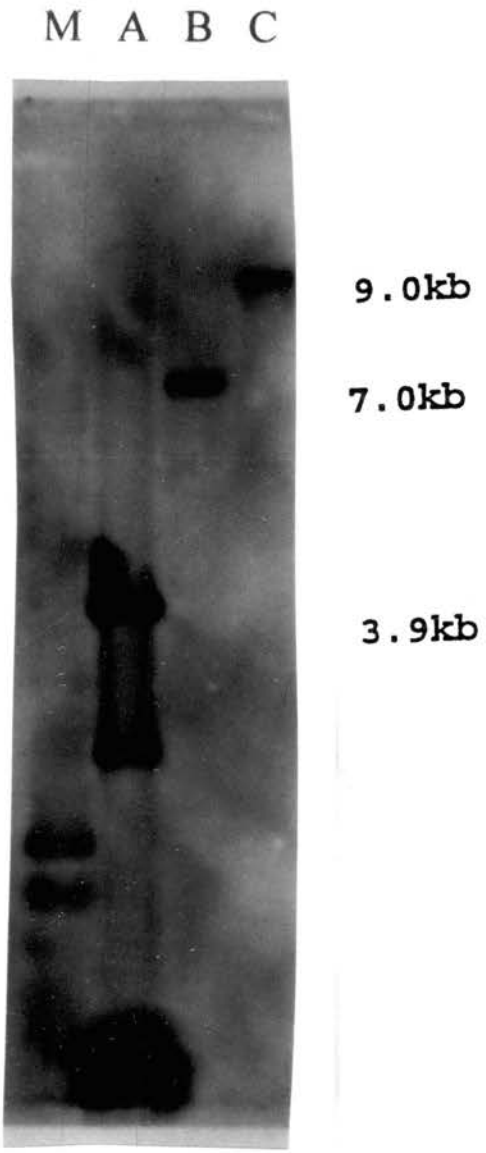

M A B C
9.0kb
7.0kb
3.9kb

Figure 11. Southern blot of *Z. mobilis* AM96

Southern blot analysis of *Z. mobilis* AM96 utilizing an internal fragment of the *Z. mobilis recA* gene. Lane designation: lane A, pZAH linearized with*Nru*I, the lower band is undigested plasmid DNA; lane B, *Z. mobilis* CP4 digested with *Hin*dIII to completion, a single band of an approximate size of 7.0 kbp is observed; lane C, *Z. mobilis* AM96 digested with *Hin*dIII to completion, a single band of 9.0 kbp is observed; lane M, molecular size marker of 1-kbp fragments, the lower two bands observed are non-specific binding.

CHAPTER VI

UV-mediated DNA-damage repair by deep-subsurface *Arthrobacter* isolates

UV resistance of bacteria has been related to the amount of solar radiation that the bacteria has been exposed to in it's natural habitat (6,21). It has also been observed that environmental isolates are generally more resistant to UV radiation than bacteria isolated from human or animal internal environments (2,6).

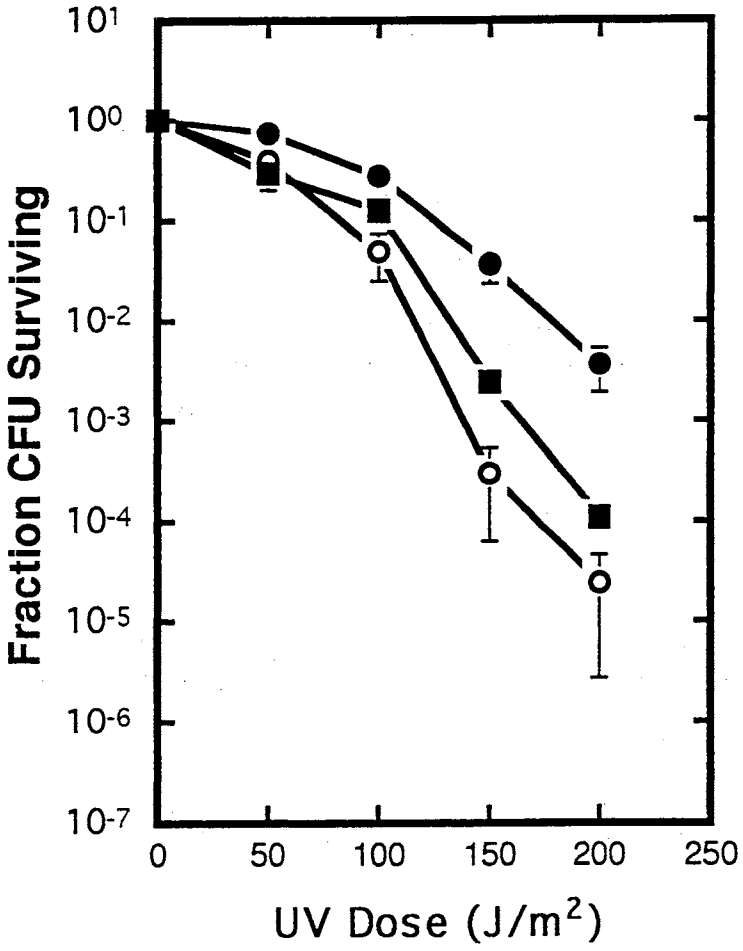
In this study, UV resistance of deep-subsurface *Arthrobacter* isolates from the bore holes of two Department of Energy (DOE) plant-sites as well as two surface isolates were examined. Deep-subsurface isolates from the DOE's Savannah River Plant Site are divided according to depth and geological formation. Four isolates (422B, 518B, 537B, 543B) originated form the Black Creek and Pee Dee geological formation (see Table IV), which ranges in depth from 145 to 204 meters. The remaining five Svannah River Site (SRS) isolates, 603B, 620B, 672B, 703B, and 723B are from the Middendorf formation. Topsoil isolates 755B and 776B (Figure 12) are soil isolates collected at the surface of the bore hole. Deep-subsurface isolates from the Hanford DOE plant site are grouped together (Table IV).

All bacterial cultures were tested for UV resistance during the mid exponential growth phase since cellular activity, and therefore DNA-repair, is generally more active during that stage (57,58). As a standard

Figure 12. Sensitivity to UV irradiation of *Arthrobacter* topsoil isolates.

Cells were grown to a density of approximately 10^8 cfu/ml in Nutrient broth, pelleted, suspended, in saline, and exposed to various fluences of UV irradiation. After appropriate dilution in saline, the radiated cells were plated in duplicate on Nutrient agar and incubated at 30^0 C for 24 hours in the dark Mean value $\mp$ standard error are plotted.

Symbols: (●), Arthrobacter 776B; (○), Arthrobacter 755B; (■), *Arthrobacter globiformis* 1080.


Fraction CFU Surviving
10¹
10⁰
10⁻¹
10⁻²
10⁻³
10⁻⁴
10⁻⁵
10⁻⁶
10⁻⁷
0
50
100
150
200
250
UV Dose (J/m²)

Arthrobacter globiformis type strain 1080 was utilized for the UV studies and was included on each curve for comparison.

As can be determined form the UV survival curve of topsoil *Arthrobacter* isolates (Figure 12), the UV resistance of *A. globiformis* type strain was found to be similar to the UV resistance of the topsoil *Arthrobacter* isolates. A ten-to-one hundred fold difference in resistance between topsoil isolates 776B and 755B was observed over the entire UV dosage range. The UV resistance of all deep-subsurface isolates from the Savannah River Site (SRS) plant (Black Creek, Pee Dee and Middendorf formation isolates) were higher (10-100 fold) than that of *A. globiformis* type strain (Figures 13 & 14). It was also observed that the UV resistance of the deep-subsurface isolates was similar. From table IX it can be seen that the difference in the D_{90} and D_{99} values for all SRS isolates fell within a range of 100 to 240 with the D_{99} value of isolate B518 being the highest. The differences in the resistance to UV radiation, however minor, could not be linked to the depth or geological formation of the isolate.

Our laboratory has also received a number of deep subsurface *Arthrobacter* isolates form the Hanford DOE plant-sites, collected from various depths of the drill hole. From each depth point, a representative isolate was chosen for the UV studies. The following isolates in addition to the *A. globiformis* type strain and *Bacillus subtilis* were used to establish a UV survival curve: G915, G950, G963, G982, ZAT002, and ZAT018 (Figure 15).

It was shown that *B. subtilis* had the lowest UV tolerance (Table IX and Figure 15). Isolates G915 and G963 showed relatively high UV resistance with isolate G915 having the highest resistance and D_{99} value of

TABLE IX

Bacteria	D_{90} (J/m^2)	D_{99} (J/m^2)	Bacteria	D_{90} (J/m^2)	D_{99} (J/m^2)
A. globiforms	100	135	*A.* isolate 703B	100	150
Bacillus subtilis	60	64	*A.* isolate 723B	130	175
A. isolate 422B	145	225[a]	*A.*isolate 755B	80	118
A. isolate 518B	170	240[a]	*A.* isolate 776B	128	180
A. isolate 537B	120	165	*A.* isolate G915	152	235[a]
A. isolate 543B	130	180	*A.* isolate G950	150	180
A. isolate 603B	165	190	*A.* isolate G982	62	105
A. isolate 620B	112	160	*A.* isolate ZAT002	75	108
A. isolate 672B	145	190	*A.* isolate ZAT 018	87	120

Table IX is showing the D_{90} and D_{99} values of deep-subsurface isolates from the DOE plant at the SRS, and the DOE plant at the Hanford plant site. The D_{90} and D_{99} values represent the doses required (expressed in J/m^2)to obtain 90% and 99% killing respectively.

a Expected values of D_{99}.

Figure 13. Sensitivity to UV irradiation of *Arthrobacter* isolates from the Black Creek and Pee Dee geological formations.

Cells were grown to a density of approximately 10^8 cfu/ml in Nutrient broth, pelleted, suspended, in saline, and exposed to various fluences of UV irradiation. After appropriate dilution in saline, the radiated cells were plated in duplicate on Nutrient agar and incubated at 30^0 C for 24 hours in the dark Mean value ∓ standard error are plotted.

Symbols: (▲), *Arthrobacter globiformis* 1080; (●), Arthrobacter 422B; (○), Arthrobacter 518B; (■), Arthrobacter 537B; (❑), Arthrobacter 543B.

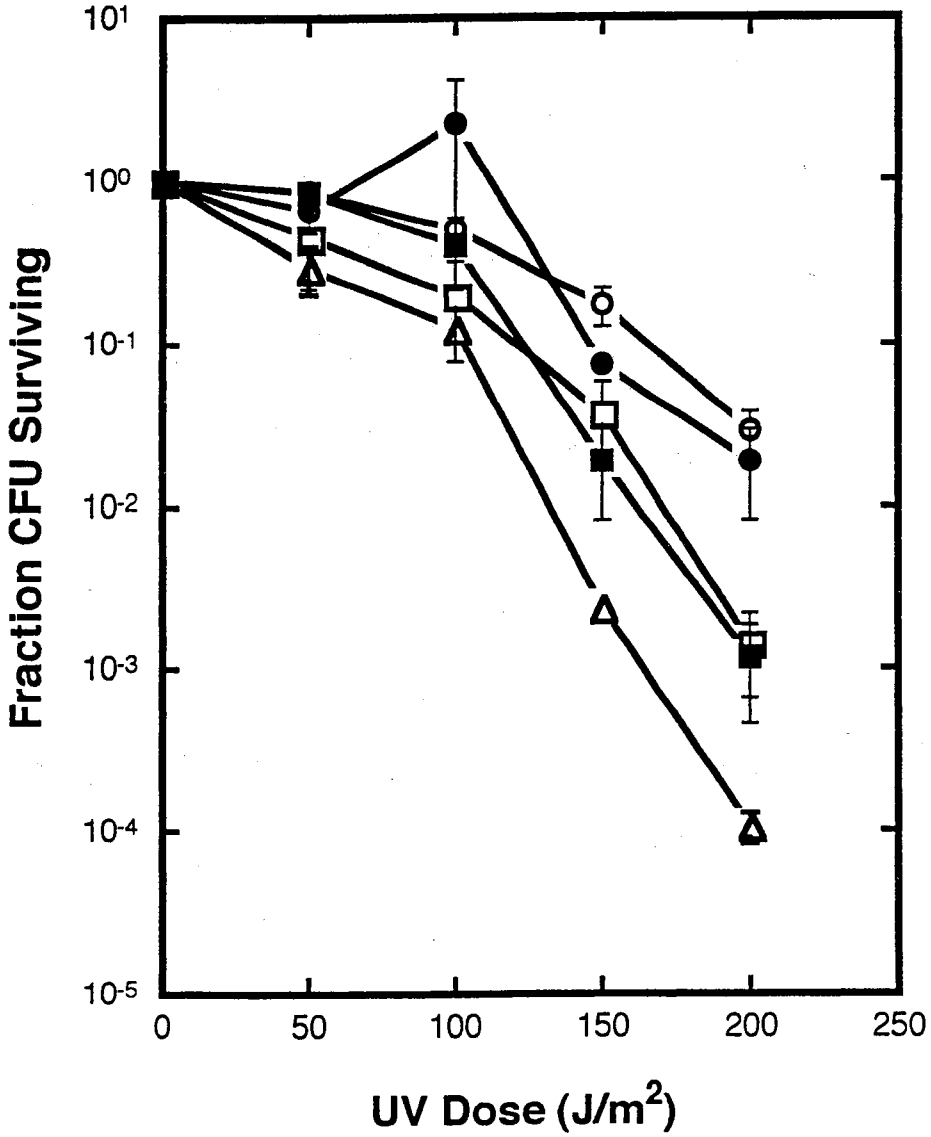

Fraction CFU Surviving
10¹
10⁰
10-1
10-2
10-3
10-4
10-5
0
50
100
150
200
250
UV Dose (J/m²)

Figure 14. Sensitivity to UV irradiation of *Arthrobacter* isolates from the Middendorf geological formation.

Cells were grown to a density of approximately 10^8 cfu/ml in Nutrient broth, pelleted, suspended, in saline, and exposed to various fluences of UV irradiation. After appropriate dilution in saline, the radiated cells were plated in duplicate on Nutrient agar and incubated at 30^0 C for 24 hours in the dark Mean value ∓ standard error are plotted.

Symbols: (▲), *Arthrobacter globiformis* 1080; (●), Arthrobacter 620B; (○), Arthrobacter 703B; (■), Arthrobacter 723B; (❑), Arthrobacter 603B; (▼), Arthrobacter 672B.

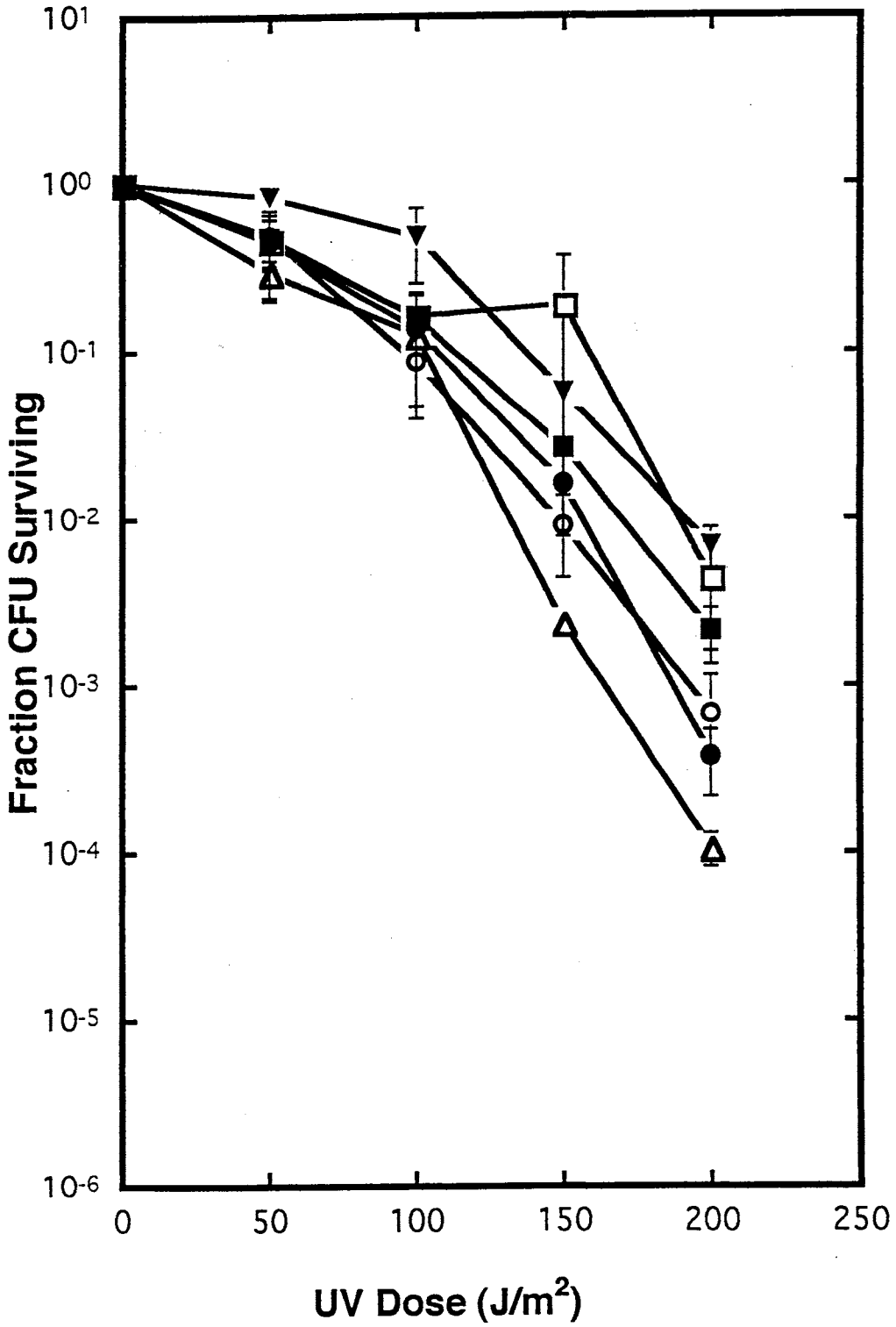
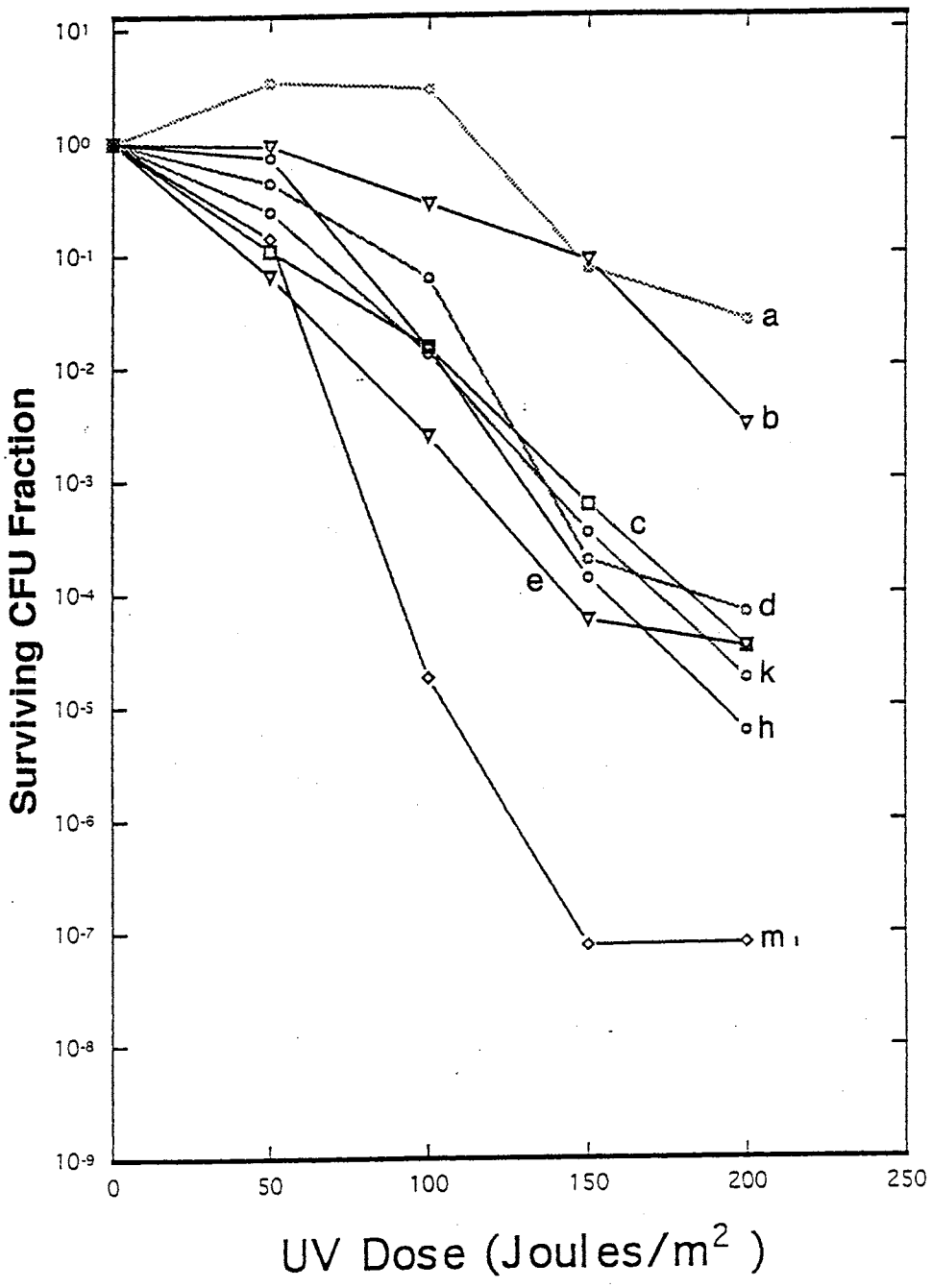

Fraction CFU Surviving
10¹
10⁰
10⁻¹
10⁻²
10⁻³
10⁻⁴
10⁻⁵
10⁻⁶
0
50
100
150
200
250
UV Dose (J/m²)

Figure 15. UV survival curves for *Arthrobacter* isolates from the Hanford site.

Cells were grown to a density of approximately 10^8. cfu/ml in Nutrient broth, pelleted, suspended, in saline, and exposed to various fluences of UV irradiation. After appropriate dilution in saline, the radiated cells were plated in duplicate on Nutrient agar and incubated at 30^0 C for 24 hours in the dark A representative experiment is shown. Lines: (m), *Bacillus subtilis*; (c), *Arthrobacter globiformis* 1080; (b), Arthrobacter G950; (e), Arthrobacter G963; (h), Arthrobacter ZAT002; (a), Arthrobacter G915; (d), Arthrobacter ZAT018; (k), Arthrobacter G982.


Surviving CFU Fraction
10¹
10⁰
10⁻¹
10⁻²
10⁻³
10⁻⁴
10⁻⁵
10⁻⁶
10⁻⁷
10⁻⁸
10⁻⁹
0
50
100
150
200
250
UV Dose (Joules/m²)
a
b
c
d
e
k
h
m

all the isolates tested. Again in this UV survival curve, it was observed that the UV resistance of deep-subsurface isolates was similar to each other.

It was interesting to notice that both G915 and G963 were isolated from a depth of 580-585 meters, which represents the most shallow point of depth at this drill hole. Since these isolates were shielded from solar radiation the lease amount of time when compared to the rest of the isolates, they (G915 and G963) may be more tolerant to UV radiation. This assumption would also imply that deposition of the isolates at each depth point occurred at the time that layer was formed millions of years ago. If however, the bacterial isolates migrated with the downwards flow of water, the age difference between the isolates would not explain the difference in UV resistance. It has not yet been demonstrated how these bacteria have reached their destination. Molecular and geological studies, however, do not support the idea that surface microorganisms have been transported to the deep-subsurface (6,81). It was also suggested that the geological formation of the area studied does not support the percolation of surface water to the subsurface (6,81). Furthermore, had such transport occurred it would have taken thousands of years to reach the depths at which the studied isolates were collected. Therefore, it seems that there are multiple answers to the question of how long these isolates really have been separated from solar radiation. It is therefore safe to conclude that regardless of the actual process the deep-subsurface bacteria were isolated from solar radiation for very long time.

The presented results suggest that these deep-subsurface isolates have good, or even better tolerance to UV radiation and DNA damage repair as surface bacteria. Furthermore, it was shown that the DNA repair

mechanisms have been well conserved in these isolates and this fact may be useful for future in situ bioremediation studies.

CHAPTER VII

CONCLUSION

Attempts to broaden the narrow substrate range of the ethanol-producing microorganism *Z. mobilis* by molecular cloning of enzymes encoding primary hydrolysis steps have been unsuccessful. The lack of success, despite the availability of the proper tools, has been shown to be mainly due to the instability and low expression of the cloned genes. Integration of the cloned genes into the chromosome has been later observed. This suggested the presence of strong recombinational activity in *Z. mobilis*.

The *recA* gene and it's product, RecA, are central to the process of homologous recombination and DNA repair in prokaryotic species. RecA acts as a regulator of viral expression and a regulator of the SOS-repair system. The *recA* gene has been well characterized and has been shown to have a highly conserved amino-acid sequence throughout the prokaryotic world. This gene has been found in every microorganism in which the presence of a *recA*-like gene has been sought.

The presence of a *recA* gene was investigated in *Z. mobilis* by intergeneric complementation of *recA*-deficient *E. coli* mutants. Indeed, a *recA* gene was isolated that complements several *recA*-associated phenotypes in various. *coli recA* mutants. Amino-acid-sequence analysis of the *Z. mobilis recA* gene revealed sequence homology to various microorganisms ranging from Gram-negative bacteria to Gram-positive soil bacteria. The cloned *recA* gene was found to be similar to other cloned

recA genes in size, conserved amino acid domains, and phenotypic expression. In this study, we were able to demonstrate that the cloned *recA* gene of *Z. mobilis* was able to rescue *recA*-associated function in *E. coli recA* mutants in several experiments. However, full expression of the *Z. mobilis recA* gene in *E. coli recA* deletion mutant did not seem to be achieved, probably due to the difference in promoter recognition sequence between *E. coli* and *Z. mobilis*, specifically, and due to the difference in the bacterial system, in general. Comparison of the nucleotide sequence of the *Z. mobilis recA* gene obtained in this study and its deduced amino acid sequence, revealed that only a small part (a few amino acids) is missing from the complete gene. The missing region from the cloned *Z. mobilis recA* gene was from the carboxyl end of the protein. Previous studies showed that the more important domains of this protein, such as the ATP-binding site and the RecA-binding site, are clustered on the amino-terminus of the protein. Therefore, despite being unable to obtain the complete *recA* gene, we were successful in investigating and studying a *recA*-like gene in *Z. mobilis*. This finding was then utilized in the subsequent insertional inactivation of the *Z. mobilis recA* gene. The necessity of obtaining a complete gene was not neglected, however, in this study a more practical goal was sought in creating a *recA* mutant of *Z. mobilis*.

A *Z. mobilis recA* mutant was created by insertional mutagenesis of a tetracycline resistance cassette. The insertional inactivation of the *Z. mobilis recA* gene is thought to have created a suitable strain for genetic manipulation. Studies utilizing the *recA* "minus" background of this mutant, are expected to show a reduction if not an elimination of integration of cloned genes into the *Z. mobilis* chromosome. It will be of great value for a future study to examine plasmid stability in the new *recA*

mutant strain of *Z. mobilis*. It is anticipated that cloned plasmids will stably replicate in the new *Z. mobilis* strain similar to the observed stability of replication of introduced plasmids in *E. coli recA* mutants that are widely used as hosts for genetic manipulations. It was reported in earlier studies, that the stability of engineered plasmids introduced in *Z. mobilis* to genetically manipulate it, was a major obstacle in the ongoing efforts to broaden its substrate range. This instability was the primary reason genetic manipulations in *Z. mobilis* were halted. In fact, many studies reported the utilization of low cost substrates by engineered *Z. mobilis* strains and the production of ethanol. However, the earlier discussed problems in maintaining stable plasmid expression in *Z. mobilis*, remains a major obstacle in their use in industrial production. The *recA* mutant strain of *Z. mobilis* produced in this study will allow scientists to proceed in genetically manipulating *Z. mobilis* in an effort to construct an improved strain with a broad substrate range for the production of ethanol on an industrial scale. In addition, this study examined *Z. mobilis* from a genetic point of view, rather than the metabolic studies traditionally performed.

In the second part of this study, the UV-repair capacities of deep-subsurface various *Arthrobacter* spp. isolates was investigated. Surprisingly high levels of resistance of these bacteria to UV radiation was observed. Previously it has been assumed that tolerance to UV radiation by bacteria meant previous exposure to UV radiation by the bacteria in its natural habitat. The findings in presented in this study reveal surprisingly high resistance to UV radiation by these deep-subsurface isolates despite their being shielded from any solar radiation for thousands or possibly millions of years. The actual age of these isolates is believed to be equal to the geological age of the layers in which the *Arthrobacter* spp. isolates

were found. This has been estimated to be in the millions of years. There are, however, hypothesis that attempt to explain the age of these isolates and their origin but are beyond the scope of this study. These hypothesis are each backed up by reasonable evidence, but it is undecided which one is correct. However, it can be safely assumed that these isolates have been shielded from solar radiation for at least thousands if not millions of years. It was, therefore, expected that these bacteria would have lost their ability to repair UV-mediated DNA damage. There are many genetic mechanisms involved in DNA repair in bacteria, there is extensive overlap of DNA repair processes and other stress-induced responses, such as oxidative protection (106), there are wide variations in phenotypic expression of UV radiation survival among different bacterial strains and isolates (6). The maintenance of an efficient DNA-repair mechanism by deep-subsurface *Arthrobacter* spp. isolates suggests that the deep-subsurface environment may pose serious hazards to the bacterial nucleic acid. One explanation for the conservation of DNA repair in subsurface bacteria is that this mechanism is necessary for protection against chemical toxicity. In similar studies, it has been shown by Aarage *et al* (6) that highly UV resistant deep-subsurface isolates were also more resistant to hydrogen peroxide (H_2O_2). Peroxide toxicity in bacteria is attributed in part to DNA damage (107). In addition, it is believed that subsurface bacteria have been subjected to extreme conditions of starvation and desiccation (81).

The results of this study demonstrate that subsurface bacteria are as competent as surface bacteria in tolerating DNA damage induced by UV light. This suggests that these bacteria have maintained an efficient DNA repair mechanism despite the lack of exposure to solar light. Knowing that these bacteria are millions of years old, it is clearly demonstrated that

DNA-repair systems in subsurface bacteria have been highly conserved through the course of evolution. Also, the findings presented here will allow for an insight into the bacterial environments of the deep-subsurface and will add to our general knowledge about subsurface bacterial communities. They also benefit remediation studies through the possible utilization of these newly isolated bacteria in the cleanup of contaminated soils. Such studies may involve enrichment of bacterial species isolated from the deep subsurface that demonstrate unusual abilities in degrading industrial toxic waste in addition to survival in harsh soil conditions. Subsequently, these enriched bacteria can be utilized in injection wells to clean up contaminated subsurface sites.

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VITA

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