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Induction of Gene Expression in Response to Stress in Pseudomonas Aeruginosa Pao

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INDUCTION OF GENE EXPRESSION IN RESPONSE TO

STRESS IN PSEUDOMONAS AERUGINOSA PAO

by

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For

Greg, Mom, Brenna

Michael

little John

Boma and Pa

Bart and Ange

John and Jim

and

Randy Adams

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CHAPTER I

GENERAL INTRODUCTION

While the pervasive soil and water microorganism Pseudomonas aeruginosa demonstrates sensitivity to ultraviolet (UV) radiation (94), this species possesses a recA gene that based upon structural and functional properties, appears capable of mediating damage inducible deoxyribonucleic acid (DNA) repair (89, 90, 124). This finding questions whether P. aeruginosa encodes a DNA damage response network analogous to the SOS network described in Escherichia coli (200). This question is significant due to recent interest in the use of P. aeruginosa for agricultural purposes and biodegradation of pollutants (43, 55), since the ability of microorganisms to repair and recombine DNA most likely will affect species survival and evolution in the environment (126). The objective of this dissertation research was to determine whether P. aeruginosa encodes SOS-like stress response genes. This question was addressed by studying the response of P. aeruginosa to DNA damaging agents including far (254 nm) ultraviolet (UVC) radiation, and the quinolone antibiotic norfloxacin. These inducing agents were chosen for investigation since they primarily induce an SOS response in E. coli.

The response of P. aeruginosa to DNA damaging agents was investigated by monitoring the expression of fusions linking P. aeruginosa promoters to a β-galactosidase reporter gene. These gene fusions were constructed by mutagenizing a

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P.aeruginosa genomic library (190) with the transposon Tn3-HoHoI, which encodes the promoterless lacZYA reporter gene (183). Examination of greater than 25,000 colonies allowed identification of about 50 gene fusions which increased their production of β-galactosidase following exposure to UVC radiation and quinolones. Restriction analysis revealed the presence of eight different gene fusions which did not contain the reporter gene linked to the P. aeruginosa recA gene. The designation sin for stress inducible was used to describe these novel fusions since it is not known whether DNA damage acts as the actual intracellular inducing signal.

Three of the gene fusions, sinA::lacZYA, sinB::lacZYA, and sinC::lacZYA, were selected for further investigation, including characterization of their stress responsive behavior. All three fusions were induced in response to UV and norfloxacin exposure, but not heat shock. Similar to E. coli SOS genes, the sin::lacZYA fusions were induced to different extents and with different kinetics following UV irradiation. However, for all three fusions, the maximal inductive response was obtained following UVC exposure of about 20 J/m^2.

As a start in defining P. aeruginosa stress responsive promoters, promoter--containing regions of the three gene fusions were roughly defined by subcloning experiments. Tn501 mutagenesis (13, 74) and exonuclease III/nuclease SI (63) digestion of sinA::lacZYA was carried out to more closely define 5' and 3' boundaries of the stress responsive sinA promoter.

Pulsed field gel electrophoresis mapping indicates that the sinA, sinB, and sinC genes are located between 34.6 and 37.4 minutes on the Holloway map of the P. aeruginosa chromosome (U. Romling and B. Tummler, personal communication). Based upon additional hybridization results, the sin genes do not appear to be located

within less than 2 kilobase pairs of each other. Results of genetic and hybridization experiments suggest that the three sin genes are not previously described UV inducible pyocin R2 structural genes, which also cluster within the same region of the chromosome (173). However, attempts to integrate sinA::lacZYA and sequences flanking sinB::lacZYA into the chromosome by a single crossover event resulted in pyocin expression in transconjugants. These results suggest sin::lacZYA constructs may encode or possess Tn3-HoHoI inserted into the pyocin R2 regulatory gene prtP, or previously uncharacterized UV inducible pyocin genes. Alternatively, an inability to replace chromosomal sin genes with the inactivated sin::lacZYA fusions may reflect a requirement for sin gene expression for cell viability. If sin genes function in P. aeruginosa recombination or DNA repair, then mutations in these genes might be expected to be lethal.

Regulation of the stress responsive gene fusions was investigated. Inducible expression of sinA::lacZYA and sinB::lacZYA is dependent upon P. aeruginosa RecA protein function. The UV-mediated induction of the three sin gene fusions was sensitive to rifampicin, which indicates that these sin genes are regulated at the level of transcription. The stress responsive behavior of sinA::lacZYA was also examined in E. coli. Even though the gene fusion appeared to be expressed in this host, UVC responsive gene fusion expression was not observed. Thus sinA::lacZYA is not regulated by the E. coli recA and lexA gene products. These results suggest that while RecA-dependent stress responsive genes are conserved in P. aeruginosa, regulation of these genes may be fundamentally different from regulation in E. coli.

CHAPTER II

REVIEW OF THE RELATED LITERATURE

The SOS Network of <u>Escherichia coli</u>

Of all the integral components which define the living cell, there is none so important as the deoxyribonucleic acid (DNA) molecule. DNA damage may result from normal cellular activities such as DNA replication and recombination, or as a consequence of exposure to DNA damaging agents such as the common environmental stressor, ultraviolet (UV) radiation. Since damage to DNA may lead to mutation or cell death, organisms have evolved processes for protecting their genomes.

Within the past decade, significant progress has been made elucidating the processes of recombination and repair used by cells to safeguard genomic DNA. These processes have been investigated with the greatest intensity in the bacterium Escherichia coli. The experiments of Weigle (210) gave the first indication that repair capacity of E. coli can be increased in response to exposure to stress agents. His experiments demonstrated that survival (Weigle reactivation) and mutagenesis (Weigle mutagenesis) of UV irradiated bacteriophages increases in a host irradiated with far (254 nm) UV radiation (UVC) as compared to an unirradiated E. coli host. Subsequent experiments have verified that E. coli possesses numerous genetic systems which are induced following exposure of the cell to various stresses (Table 1).

Table 1.--Stress responsive networks of <u>Escherichia coli</u>

Network	Description	Reference
SOS	At least 20 genes are induced upon exposure to DNA damaging treatments.	178
Heat Shock	More than 17 genes are induced upon increasing the temperature.	132
Adaptive Response	At least 4 genes are induced by alkylating agents.	100
Oxidative Response	About 30 genes increase their expression upon exposure to oxidative stress.	44, 205
Cold Shock	Approximately 13 genes are induced when the temperature is abruptly decreased.	78
Starvation	At least 30 genes are induced in response to glucose starvation.	163
Osmotic Pressure	<u>E. coli</u> modulates the expression of specific genes in response to changes in osmolarity.	65
Nitrogen Limitation	In response to nitrogen limitation, expression of nitrogen scavenging proteins proteins is increased.	156
Phosphate Limitation	In response to phosphate limitation, expression of phosphate scavenging proteins is increased.	209
Thiols	Several thiol responsive promoters have been identified in <u>E. coli</u>.	7

The SOS regulatory system was one of the first systems characterized in E. coli, and remains the largest, most complex, and best understood stress response network (202). This system specifically responds to agents which damage DNA such as UVC radiation, and agents which inhibit DNA replication such as the quinolone antimicrobial norfloxacin (200). Exposure of E. coli to these stressors results in the expression of a heterogeneous collection of physiological responses which include an enhanced capacity for DNA repair, induction of resident prophages, increased mutagenesis, and filamentous growth (103). These responses are now known to be due to the increased synthesis of more than twenty coordinately regulated and unlinked damage inducible (din) genes (200). Many of these genes encode products involved in the repair and recombination of DNA.

Expression of din genes is controlled by the recA and lexA gene products (103, 200, 201). In the uninduced cell, SOS gene expression is repressed by the binding of the LexA protein to a consensus operator sequence (the SOS box) present in one or more copies in the promoters of each SOS gene or operon. Since din operator/promoter regions differ in affinity for LexA protein, in the repressed state many SOS genes including recA and lexA are expressed at significant levels. Exposure of E. coli to inducing treatments generates an intracellular signal which interacts with and alters RecA protein to an activated form, RecA*. RecA* functions as a coprotease to facilitate a latent capacity of the LexA protein for autodigestion at a unique -Ala-Gly- bond (102). This inactivation of the LexA monomer results in a decrease in intracellular pools of functional LexA, derepression of various SOS network genes, and observation of the SOS responses mediated by the products of the induced din genes (103, 200, 201). Repressors of the lytic growth cycle of certain bacteriophages which

have homology to LexA are similarly cleaved by RecA* at an -Ala-Gly- bond, resulting in prophage induction. Following repair of the damaged DNA or removal of the DNA replication block, intracellular levels of the inducing signal decrease, and RecA protein is no longer activated. As levels of LexA rise within the cell, repression of SOS genes is reestablished since the affinity of the lexA operator for LexA protein is such that LexA is present in adequate amounts to repress all other din genes before its own synthesis is repressed.

The most compelling evidence for a role of RecA protein in sensing the DNA damage-generated signal(s) is the existence of recA protease constituitive (Prt^c) mutants in which RecA protein is continually activated for LexA coproteolytic cleavage, even in the absence of inducing treatments (186). Despite intensive study in this area, the identity of the intracellular SOS inductive signal(s) detected by RecA protein remains elusive. Mutations in several other E. coli genes have been identified which result in either subinduction or constituitive expression of SOS responses. These include mutations in dam, dnaB, dnaE, dnaG, lig, polA, recF, ruv, ssb, and uvrD genes (145). All of the mutations are alike in that they primarily affect DNA metabolism, suggesting that interruptions of DNA synthesis generate a signal or signals capable of activating RecA. Candidates for the activator(s) include single stranded regions of DNA resulting from inhibition of DNA synthesis following DNA damage, single stranded DNA (ssDNA) gaps opposite lesions, oligonucleotides resulting from DNAse degradation of damaged DNA, alterations in nucleotide pools following DNA damage, and changes in DNA superhelicity (140). Biochemical evidence suggests that RecA activation requires the formation of a ternary complex comprised of RecA protein, a nucleoside triphosphate such as ATP or dATP, and a polynucleotide such as

poly(dT) (128). It is generally believed that this _in vitro_ requirement of a polynucleotide for RecA proteolytic activity mirrors an _in vivo_ generation of ssDNA regions by SOS inducing treatments.

DNA Repair Activities of the SOS Network

A primary factor responsible for recognition of genes involved in the SOS response network was the development of the Mud(lac) bacteriophage (30). Using this phage, Kenyon and Walker (85) generated random bacteriophage insertions into the E. coli chromosome. These insertions were screened to identify fusions linking the promoterless Mud lac operon to promoters of genes demonstrating increased expression following DNA damaging treatments. The investigators were able to identify both previously characterized genes and novel genes of unknown function, and establish that expression of these genes was controlled by the RecA and LexA proteins (84, 203). This approach has subsequently been used to determine whether genes whose products appear to function in DNA repair or mutagenesis (e.g. uvrA (86), umuDC (6)) are part of the SOS regulatory network.

Results of the gene fusion studies have helped to clarify strategies of SOS--mediated DNA repair in E. coli. The study of these strategies is complicated by the fact that repair processes are capable of interacting in lesion repair, and are influenced by DNA replication (163). Furthermore, "crosstalk" may occur between SOS network gene products and protein components of other stress responsive networks. For example, recA gene expression is inhibited by the adaptive response regulatory protein Ada (196), and the UV inducible inhibitor of cell septation SulA protein is degraded by the heat shock regulated Lon protease (161). Despite these

barriers to experimental progress, three main repair activities have been demonstrated to be associated with the SOS network: excision repair, postreplication recombination repair, and mutagenic repair.

The SOS repair strategy utilized by the cell appears to be dependent upon the amount of genetic information lost as a consequence of DNA damage (164). When damage occurs to only a single strand of DNA, sufficient information is available in the complementary strand to allow repair of the duplex by excision repair. This is the only repair pathway which literally repairs or removes the DNA damage. When damage is present in both DNA strands, repair can occur by either one of two pathways. If the damaged duplex is capable of interacting with an intact homologous duplex, DNA structure and integrity will be restored by postreplication recombination repair. In the case where the damage cannot be repaired through recombination, the third strategy of repair will operate to prevent complete loss of genetic information. This third strategy of mutagenic repair simply allows the cell to tolerate the DNA damage, and processes this damage in such a fashion that mutations result.

Excision repair in E. coli is an error-free process which recognizes and removes damage induced distortions of the DNA helix (53, 141, 145, 211). This repair mechanism requires the uvrA, uvrB, uvrC and uvrD gene products, as well as DNA polymerase I and ligase. The uvrA, uvrB, uvrD, but not uvrC gene products have been shown to be regulated by the SOS network. The SOS-associated excision repair activity differs from excision repair processes which depend upon specific glycosylases and AP (apurinic or apyrimidinic) endonucleases in that it can repair a wide variety of structurally dissimilar DNA lesions. A 'repairosome' complex consisting of the six proteins has been suggested to effect the entire SOS associated

excision repair process (211). In the incision step of this process, UvrA, UvrB, and UvrC proteins act in concert to generate two endonucleolytic breaks on either side of the lesion in an ATP dependent reaction. The 5'- endonucleolytic break is positioned seven nucleotides 5' to the damaged site whether the lesion is a pyrimidine dimer, psoralen monoadduct, or cisplatin diadduct (195). In contrast, the 3' endonucleolytic break occurs either three or four nucleotides 3' to the damaged site depending upon the type of damage generated. The distance separating the two incisions is approximately one helical turn of B-DNA (53), suggesting that the complex interacts with one face of the damaged helix.

The incision step is followed by removal and resynthesis of DNA which requires DNA polymerase I, UvrD, and DNA ligase. The release of the damage--containing fragment and repair of the resulting duplex gap is effected by the polymerase and 5'-->3' exonuclease activities of DNA polymerase I, and helicase properties of UvrD acting in conjunction. Whereas the majority of the repair replication patches are 10 to 20 nucleotides long, some repair patches may be up to 1500 nucleotides in length (38). While both short and long patch repair appear to be inducible functions in that Uvr proteins increase following DNA damage (145, 201), long patch repair is most definitely an inducible function since such repair is impeded in the absence of protein synthesis and does not occur in RecA⁻ mutants (38).

Excision repair can generate substrates for postreplication recombination repair (180). Two substrates which can be produced by excision repair are excision repair gaps formed when a lesion in the replicated portion of the chromosome is removed, and double strand breaks (DSB) resulting from overlapping excision gaps. These lesions can also be induced by other cellular mechanisms. For example, daughter

strand gaps (DSG) can be produced in nascent DNA when the replication complex skips past damage induced lesions and reinitiates downstream of the damaged site (206). Similarly, DSB can be induced by the action of hydroxyl radicals (159).

Regardless of the manner in which the damage is generated, E. coli appears to possess two postreplication recombination repair strategies responsible for correcting DSG and DSB. These SOS network recombination repair strategies are analogous to the cellular pathways of chromosomal recombination, and are primarily defined on a genetic basis since the activities of some of the enzymes involved, and the structures of DNA substrates and intermediates are largely undefined (113). The recombination pathways or repair strategies are named to reflect a gene product or enzyme acting uniquely within that process (178). While many gene products have been shown to act in more than one recombination pathway or repair strategy, the RecBCD enzyme and the RecF gene products characterize the two distinct processes for the conversion of substrates (parental DNA or damaged DNA) to products (recombined DNA or repaired DNA).

In both RecBCD and RecF recombinational repair strategies, repair is effected through the physical exchange of DNA sequences from an intact sister duplex to join discontinuous DNA ends of a homologous damaged duplex into high molecular weight DNA (180, 206). The resulting gap in the sister duplex is then filled by repair replication. While the RecBCD dependent strategy allows repair of DSB, the RecF dependent strategy is primarily responsible for the repair of DSG. These and other results suggest that substrates for recombination and repair presenting double stranded blunt ends follow a RecBCD dependent process, whereas substrates presenting relatively long single stranded tails follow a RecF dependent process.

The RecBCD pathway is the major recombinational pathway in E. coli. Mutations in recB and recC reduce recombinational efficiency to no more than 1% of wild-type (33), and result in defects in DNA repair (3). Wild-type levels of DNA recombination and repair can be restored by the extragenic suppressor mutations sbcA, sbcB and sbcC. Single mutations in sbcA activate a RecE pathway by promoting expression of Exonuclease VIII, an ATP- independent nuclease specified by the recE gene of the cryptic Rac prophage present in some strains of E. coli (52). The recE gene product digests each strand of linear double stranded (dsDNA) in the 5'-->3' direction to produce dsDNA with 3'-ended ssDNA tails (178). Mutations in sbcA are cis-acting and dominant to the wild-type allele. Such mutations promote recE expression by creating a new promoter for mRNA synthesis, or allow translation of RecE by altering the reading frame, creating a new translation initiation codon, or creating a new ribosome binding site (81). Combined mutations in sbcB (95) and sbcC (104) activate a RecF pathway by inactivating Exonuclease I. Since Exonuclease I digests ssDNA in the 3'-->5' direction (178), it has been suggested that this exonuclease converts substrates for the RecF pathway possessing single stranded 3' tails into substrates for RecBCD-mediated processes (95). While the sbcC single mutation has no apparent effect on the phenotype of Rec$^+$, recB or recC strains, this mutation is required for full suppression by sbcB of the mutant recB or recC phenotype (104).

RecBCD dependent recombination has been shown to require the recB, recC, and recD gene products (17, 109, 207) along with RecA protein, ssDNA binding protein (SSB), DNA gyrase, DNA polymerase I, and DNA ligase (178). The recB, recC and recD gene products comprise the three subunits of Exonuclease V (17, 207).

This multifunctional enzyme possesses helicase, dsDNA exonuclease, ssDNA endonuclease, ssDNA exonuclease, and ATPase activities (36). The RecBCD dependent capacity to repair DSB appears to be an inducible process since pretreatment of cells with inducing agents including UVC radiation and nalidixic acid results in an increased resistance to further irradiation which is dependent upon the RecB⁺ phenotype (149). This induced radioresistance is controlled by the RecA and LexA proteins, and requires ongoing transcription and translation since it can be blocked by rifampicin or chloramphenicol (92). Additionally, a RecBCD dependent process has been implicated in at least three inducible phenomena: (i) the alleviation of lambda restriction observed following UV irradiation or nalidixic acid treatment (187); (ii) prophage induction in permeabilized or thymine-starved cells (73, 138); and (iii) prophage inactivation in irradiated lysogens incapable of successful DNA repair (193).

Mutations in recD reduce exonucleolytic degradation of duplex DNA (3, 17), whereas mutations in recB or recC decrease nuclease activity and recombinational proficiency, and increase sensitivity to DNA damage (39). These results suggest that the RecB and/or RecC subunits of Exonuclease V possess the DNA helicase activity, whereas recD encodes the exonuclease activity (39). This interpretation is supported by the identification of recD mutants which were found to lack nuclease activity, and yet demonstrate the same viability, recombinational proficiency, and resistance to UVC irradiation as their Rec⁺ parent (33). These results further suggest it is the helicase activity, or some other unidentified enzymatic activity of Exonuclease V which is critical in repair and recombination. The demonstration of RecA dependent induction of SOS genes in mutants lacking nuclease activity and yet retaining helicase activity supports this hypothesis (7, 35).

In contrast to RecBCD, RecF appears to function primarily in DNA repair, since recF single mutants show increased sensitivity to DNA damaging agents, and only a modest deficiency in genetic recombination (68). This increased sensitivity of recF mutants has been correlated with a decreased ability to perform DSG repair (208). Furthermore, it has been demonstrated that RecF dependent processes are inducible and are under SOS control (4, 107). RecF dependent activities have been shown to require the recF, recA, recJ, recN, recO, recQ, ruv, and uvrD gene products (141). While recA, recN, recQ, ruv, and uvrD are regulated by the RecA and LexA proteins, recF is not an SOS gene, and regulation of the remaining genes has not yet been determined (112, 141). Aside from RecA, an activity has been reported for only the recJ gene product, a ssDNA exonuclease (178). Mutations in genes of the RecF pathway result in differing phenotypes when exposed to the various stress agents. For example, recA, recF, ruv and uvrD single mutations result in sensitivity to both UVC radiation and mitomycin C, whereas recN mutants are sensitive to mitomycin C but not UV exposure (145). Single mutations in recJ (108) and recQ (132) result in UVC sensitivity only in a recBC sbcB genetic background. These results may suggest that separate proteins of the RecF pathway are needed to repair different types of chromosomal damage.

Mutations in recF, recB and recC genes have also been examined for their effect upon recA mRNA and protein induction in response to various stress agents. Mutations in recF inhibit RecA protein and mRNA induction following UVC but not nalidixic acid exposure, whereas mutations in recB or recC genes impede RecA protein and mRNA induction by nalidixic acid but not UV radiation (82, 120). These results imply that the products of these other rec genes are capable of recognizing

DNA damage and promoting activation of RecA protein (73, 82). It has been suggested that RecA activation and SOS induction by UVC radiation may be mediated by the RecF protein, while quinolone induction may require a functional RecBCD enzyme. However, a new recF mutation has been identified which results in impaired induction but not complete inhibition of SOS genes in response to both UV irradiation and nalidixic acid in the presence of a recA operator constituitive mutation (188). This suggests that RecF may play a more general role in the induction process, modulating interaction of the SOS inducing signal with RecA protein. One way in which this could take place would be through an interaction of the RecF protein with RecA protein in reactions leading to LexA coproteolytic cleavage. This hypothesis is supported by the ability of recA441, a mutation which results in constituitive RecA coproteolytic activity at 42°C, to partially suppress the UV sensitivity of a recF mutant (199). Other mutations in recA (i.e. srf or suppressor of recF) have been identified which suppress RecF recombination deficiencies without affecting SOS protein induction (197, 198, 208). In both types of recA mutations, suppression of recF has been shown to require the recJ gene product and increased amounts of RecA protein (189). These results might suggest that RecF is required for two different changes in RecA activity following DNA damage. One change would be RecF modulation of RecA protein activation, allowing induction of repair processes. The second change would be RecF modulation of the ability of RecA protein to participate directly in RecF dependent recombination and repair.

Mutagenic repair, or SOS processing is the third type of repair strategy associated with the SOS regulatory network. Mutagenesis has been shown to require

the products of at least three genes: recA, umuC, and umuD (6, 144), with the umuC and umuD genes constituting an operon (144). RecA appears to have three functions in UVC- and chemical-mediated mutagenesis. First, activated RecA coproteolytically cleaves LexA, promoting derepression of both recA and umuDC encoded proteins. Second, RecA post- translationally activates UmuD for mutagenesis by mediating its cleavage at a Cys-Gly bond (26, 134, 169). Third, RecA protein appears to participate directly in SOS processing. While the mechanism of RecA participation in SOS processing remains to be discovered, this additional role of RecA is indicated by experiments in which the carboxyl terminal polypeptide of UmuD which is active in mutagenesis is able to suppress the UVC nonmutability of a lexA (Def) strain (or a strain containing defective LexA repressor) possessing reduced RecA protease activity, but not of a lexA (Def) strain suffering a deletion of recA (134).

Bacteria possessing umuC mutations are normally not mutable by UVC radiation and other SOS inducing treatments (6). However, Bridges et al. (22, 24) clearly demonstrated that mutagenesis can occur in the absence of UmuC and UmuD, provided that irradiated cells are photoreactivated after prolonged incubation in rich medium. Based on these results, the investigators propose a two step model of SOS processing (23, 24, 25). In the first step, misincorporation, bases are misincorporated close to or opposite a segment of damaged DNA. The presence of this damaged DNA constitutes a block to DNA replication. This block can be overcome in the second bypass step, which requires increased levels of UmuDC proteins. In the umuDC strain, the mutagenic process is somehow interrupted, and potential mutants fail to survive due to an inability to remove the block to DNA replication. However,

delayed photoreversal allows the direct removal of pyrimidine dimers. Following this event, DNA polymerization can continue, allowing the observation of mutagenesis in a umuDC host.

The misincorporation step was initially thought to be mediated by activated RecA. In support of this hypothesis, investigators were able to demonstrate that the yield of mutants obtained varied with the recA allele present in photoreactivated umuC bacterium (24). Specifically, umuC mutants containing recA430, which is defective in proteolytic activity gave a lower frequency of mutations in the delayed photoreversal assay than did umuC recA441 mutants. However, the significance of RecA cleavage activity in the misincorporation step was questioned by the finding that frequency of mutations in the presence of RecA441 protein showed no difference with temperature, even though the cleavage activity of the protein is much greater at 42°C than at 30°C. Furthermore, UVC induced mutagenesis of photoreactivated phages can be obtained in the absence of both UmuC and RecA function (185). These results suggest that the DNA replication complex may normally be able to replicate unassisted past UV induced lesions with a relatively high frequency, and in this fashion contribute to cell mutagenesis without the need of intervention by an induced cellular repair activity. Subsequent studies of UV mutagenesis following delayed photoreversal in recA mutants indicate that RecA is unnecessary for misincorporation and may even act to inhibit this step of mutagenesis (20).

Induced mutagenesis may also rely upon properties of the DNA polymerases involved in repairing the DNA damage. An altered form of DNA polymerase I--DNA PolI*--has been identified in cells induced for SOS functions (96). DNA PolI* possesses lower replication fidelity than does DNA polymerase I present in uninduced

cells, and could conceivably be involved in the misincorporation or bypass steps of SOS mutagenesis. However, it is unlikely that DNA polymerase I plays any major role in SOS processing since mutagenesis is unimpaired in strains carrying a deletion of the polA gene (11).

In contrast to DNA polymerase I, genetic and physiological studies tend to support a role for DNA polymerase III in SOS mutagenesis (21). It has been demonstrated that RecA binds UVC irradiated DNA more efficiently than unirradiated DNA, and is capable of inhibiting the 3'-->5' exonuclease proofreading activity of the epsilon subunit of DNA PolIII encoded by dnaQ (111). These results suggest that RecA may act in the lesion bypass step of mutagenesis by preferentially binding to damaged regions of DNA and allowing DNA PolIII to replicate across the damaged site with reduced fidelity, leading to the misincorporation of bases. In support of this hypothesis, the mutagenic potential of recA alleles was found to correlate with the capacity of RecA to bind irradiated as compared to unirradiated DNA (110). Whereas wild-type RecA protein binds more efficiently to irradiated DNA, RecA441 protein binds equally well to both unirradiated and irradiated DNA, and RecA430 protein binds poorly to both types of DNA. Correspondingly, when compared to wild-type E. coli, recA441 strains demonstrate constituitive mutagenesis whereas recA430 strains are defective for mutagenesis.

The hypothesis of an in vivo interaction between SOS regulated proteins and the epsilon subunit was further supported by the finding that UmuDC and RecA* proteins enhance the mutator phenotype associated with the defective epsilon subunit encoded by the dnaQ49 allele (50). Additionally, overproduction of epsilon markedly decreases the frequency of mutations induced by UVC radiation and methyl methanesulfonate

through an antimutagenic process which is partially suppressed by excess UmuDC proteins (51, 77). In this context, it is interesting that dnaQ has been demonstrated to be induced upon exposure to the mutagenic stress agent 2-aminopurine (150). However, since a dominant allele of dnaQ (mutD5) does not restore mutability to a umuC mutant strain, it appears as if loss of DNA PolIII proofreading activity may be necessary, but not sufficient for SOS associated mutagenesis (216).

E. coli RIN Functions are recA-Dependent and lexA-Independent

Several RecA promoted activities have been identified in E. coli which are not directly repressed by the LexA protein. For reason of clarity, these activities will be referred to as RIN functions to signify their independent regulation by RecA protein. These RIN activities all share in common the characteristic that they can be observed in the presence of mutations which render the LexA protein noncleavable, provided that RecA protein is produced in these hosts as a result of a repressor-insensitive operator constituitive recA mutation. RIN functions identified in this fashion include the partial alleviation of K-specific DNA restriction observed as a consequence of UVC exposure (187), the recovery of DNA synthesis following UVC irradiation or induced replisome reactivation (214), and the constituitive stable DNA replication not dependent upon concomitant protein synthesis observed for rnh mutants deficient in ribonuclease H (192). Induced replisome reactivation has been shown to require the postirradiation synthesis of one or more protein(s) not regulated by LexA (87). An additional RIN function(s) required for the reactivation and mutagenesis of UV irradiated lambda phage was identified by examining the chloramphenicol sensitivity of these processes in an E. coli host defective for LexA repressor activity (27).

Specifically, in a lexA (Def) E. coli host, the protein synthesis inhibitor chloramphenicol was found to inhibit in a dose-dependent fashion UV inducible repair and mutagenesis despite a high level of expression of umuDC and other din genes under the same conditions.

While all of the identified RIN functions appear to require stable or increased levels of RecA protein, the activities differ in their dependence upon RecA activation. The RecA dependent stable DNA replication observed in E. coli rnh mutants apparently depends upon some constituitive function of the RecA protein since mutations which block RecA activation fail to nullify the RIN activity of these mutants (192). In contrast, restriction alleviation, and expression of a RIN gene product(s) which mediates bacteriophage lambda repair and mutagenesis are similar to SOS regulated damage inducible phenomena in their dependence upon RecA protein activation for induction (27, 187). It is currently unclear whether induced replisome reactivation is mediated through RecA or RecA* (87, 214). These experiments suggest that RecA and/or RecA* can promote activities independently of an interaction with LexA. In this capacity, RecA or RecA* may promote nascent protein synthesis by functioning as a transcriptional activator, or by cleaving an unidentified repressor in a reaction analogous to that observed for LexA and bacteriophage repressors. Alternatively, the recA gene product may participate directly in RIN processes, or allow their occurrence through posttranslationally modifying enzymes involved, similar to the posttranslational activation of umuD.

SOS-Like Genes and Responses of Other Bacterial Species

The recA gene is highly conserved among bacteria, and has been identified in and cloned from a variety of strains other than E. coli including Pseudomonas aeruginosa (89, 137), Thiobacillus ferrooxidans (151), Proteus mirabilis (1), Agrobacterium tumefaciens (48), Synechococcus sp. (131), Vibrio anguillarum (176), Serratia marcescens (8), Bacteroides fragilis (58), Haemophilus influenzae (10, 166), Proteus vulgaris (83), Erwinia carotovora (83), Bacillus subtilis (116), Anabaena variabilis (142), and Shigella flexneri (83). Genes which have been sequenced and compared to E. coli recA demonstrate identity values ranging from 56 to 73% (8, 131, 151, 158). While an SOS consensus sequence has been identified in the recA promoter regions of P. aeruginosa (158), P. mirabilis (1), S. marcescens (8), P. vulgaris (83), E. carotovora (83), and S. Flexneri (83), examination of the upstream region of T. ferrooxidans (151), and Synechococcus sp. recA genes fails to reveal the presence of an SOS box. Of the strains exhibiting the conserved SOS promoter element, all appear to be induced in an E. coli recA host following exposure to DNA damaging agents (8, 83, 90) with the exception of the P. mirabilis recA gene, for which this experiment was not yet performed. In cases where the ability of the recA analogue to complement various repair and recombination deficiencies of E. coli recA mutants was examined, P. aeruginosa (89, 137), V. anguillarum (175), P. vulgaris (83), E. carotovora (83), S. flexneri (83), A. variabilis (116), and A. tumefaciens (48) recA analogues were found to significantly restore these processes. In contrast, T. ferrooxidans (151), B. fragilis (58), and H. influenzae (10, 166) recA genes could only partially complement RecA dependent activities, or could complement only a subset

of the activities examined. The B. fragilis recA analogue does not increase its own expression in an E. coli host in response to DNA damaging treatments. Results of similar experiments have not yet been reported for the remainder of the strains.

Other stress responsive or DNA repair genes which have been identified or cloned include B. subtilis damage inducible (din) genes believed to be involved in competence development (57, 105, 106), a Streptococcus sanguis DNAse which is apparently required for the repair of methyl methanesulfonate or UVC generated DNA damage (101), the bacteriocin carotovoricin and pectinolytic enzyme pectin lyase of Erwinia carotovora subsp. carotovora (122, 218), the extracellular nuclease gene nucA of Serratia marcescens (8), and the recP gene encoding a product of about the same size as E. coli RecQ protein which is involved in recombination in Streptococcus pneumoniae (153). The S. marcescens nucA gene and the E. carotovora pnlA gene encoding pectin lyase are interesting in that they are regulated by the RecA protein present in the native species, but are not stress responsive in RecA$^+$ E. coli (8, 121).

Additional putative SOS-type genes have been identified in bacteria through an examination of the phenotype they impart when mutated. For example, the Streptomyces fradiae mcr-6 mutation appears to disrupt recA analogue gene sequences since it renders the host repair deficient, and since it can be complemented by the E. coli recA gene product (118). Similarly, mutations in Streptomyces cattleya (uvr1 and uvr2; 71) and in Streptomyces lividans (rec-46; 88) have been isolated which diminish DNA repair and recombination.

In bacterial species in which mutants and genetic clones have not yet been obtained, attempts have been made to define the recombination and repair capacity of the organism by directly assaying for these events. The purple bacteria Rhodobacter

capsulatus demonstrates UV dose- dependent mutagenesis of its chromosome, and
increases the synthesis of a protein of M_r approximately 30,000 between 2 to 3 hours
postirradiation (9). In other organisms, repair has primarily been assessed by the
ability of the irradiated bacteria to reactivate and mutagenize UV irradiated
bacteriophage. Using this approach, Weigle reactivation was demonstrated in
Acinetobacter calcoaceticus (14), Bacillus thurengiensis (5), Haemophilus influenzae
(135), and strains of the cyanobacteria Anabaena (99), but is not observed for P.
aeruginosa (175), and Streptococcus pneumoniae (56). Salmonella typhimurium shows
only a very low level of Weigle reactivation (139). While Weigle mutagenesis was
not examined in the cyanobacteria, A. calcoaceticus (14), H. influenzae (135), P.
aeruginosa (175), and S. pneumoniae (56) do not demonstrate Weigle mutagenesis,
and B. thuringiensis (5) and S. typhimurium (139) exhibit only a very weak mutagenic
response (5). It is generally believed that the UVC induced ability to mutate
irradiated bacteriophage reflects chromosomal possession of sequences analogous to
umuDC. Direct examination for chromosomal sequences capable of hybridizing to
E. coli umuDC genes revealed the presence of these sequences in 3 out of 11 related
species of enterobacteria examined (165). However, it is unclear whether the
umuDC-like sequences of these strains encode functional proteins, since S.
typhimurium was recently demonstrated to encode a defective umuDC-like operon
(179).

A Search for SOS-Like Genes and Responses in P. aeruginosa

Investigation of SOS-like genes and responses in bacteria other than E. coli
clearly indicates that RecA is conserved (125). In contrast, UV inducible error free

and mutagenic repair do not appear to be present in every bacterial species. Potential LexA binding sites have been observed in some but not all recA promoters, and stress responsive genes which are damage inducible in the native species may or may not demonstrate stress responsive expression in E. coli. These experiments question the conservation of a RecA and LexA regulated SOS network of damage inducible genes outside of the genus Escherichia. Four different scenarios can be envisioned regarding SOS repair and recombination gene analogues: (i) these genes may not be conserved; (ii) SOS-like genes may be present in other species of bacteria, but may not be regulated; (iii) SOS-like genes may be regulated by RecA and LexA analogues; (iv) SOS-like genes may be regulated by gene products which are dissimilar to RecA and LexA. This question of conservation of SOS-like repair and recombination genes is important, since the ability of microorganisms to tolerate stresses including DNA damage may determine species survival in certain environments, and since a capacity for recombination may affect genetic diversity and evolution of bacterial populations (126). This question becomes even more relevant when considering microorganisms such as the autochthonous soil and water bacterium Pseudomonas aeruginosa, which has tremendous potential for use in environmental remediation, and as a genetically engineered microorganism (GEM).

A search has been conducted for SOS-like responses and genes in P. aeruginosa. Initially this search was concentrated upon the identification of repair events in this species. The high sensitivity of P. aeruginosa as compared to E. coli to UVC radiation lead early investigators to question whether P. aeruginosa was even capable of DNA repair (94, 97). However, the isolation and phenotypic analysis of UV radiation and methylmethanesulfonate sensitive mutants of P. aeruginosa

suggested the presence of a repair system(s). Recombination deficient mutants were also identified which altered the ability of P. aeruginosa to recombine DNA received through the processes of conjugation and transduction, and to establish and maintain a lysogenic relationship with temperate bacteriophages (54, 127).

In subsequent experiments, host cell reactivation (or the ability of an unirradiated host to repair UVC irradiated bacteriophages), Weigle reactivation, Weigle mutagenesis, and chromosomal mutagenesis were investigated following exposure of P. aeruginosa to different stress agents. While a slight increase in chromosomal mutation frequency has been reported in some experiments (98, 175), compared to E. coli, P. aeruginosa appears to be relatively nonmutable by UVC radiation. P. aeruginosa also does not demonstrate Weigle reactivation and Weigle mutagenesis in response to UVC (175) and quinolone exposure (12). These results suggest that P. aeruginosa may lack functional genes analogous to umuDC required for the observation of DNA damage inducible repair and mutagenesis. In contrast, this species does manifest a norfloxacin inducible response which appears analogous to the induced replisome reactivation phenomenon observed in E. coli. Namely, treatment of P. aeruginosa with an intermediate concentration of norfloxacin results in inhibition of DNA replication followed by secondary or recovery DNA synthesis (12). Recovery synthesis is abolished by the inhibition of translation with chloramphenicol, which indicates this is an induced function. Similar to results reported for E. coli, P. aeruginosa recovery synthesis can be observed in the presence of a recA mutation, recA908 (91), which appears to result in constituitive RecA proteolytic activity. P. aeruginosa encodes a recA gene which has been cloned by two independent laboratories (89, 137). Aside from E. coli recA, the P. aeruginosa recA gene appears

to be the best studied of the <u>recA</u> gene analogues (126). The <u>P. aeruginosa recA</u> gene is capable of interspecific complementation of deficiencies present in RecA⁻ <u>E. coli</u> cells, as indicated by the ability of the analogue to restore recombinational proficiency, DNA repair, and ability to induce temperate prophage to host cells (89, 90, 137). Unlike <u>E. coli</u>, <u>P. aeruginosa recA</u> is transcribed as two distinct mRNAs. These mRNAs appear to be stress inducible, and differ in the length of their 3' termini (69). However, the following properties of the <u>P. aeruginosa recA</u> gene and its encoded protein suggest it may act analogously to <u>E. coli recA</u>, as an effector of stress responsive gene expression: (i) the proteins demonstrate structural conservation (158); (ii) RecA synthesis in <u>P. aeruginosa</u> is increased in response to UVC and norfloxacin exposure (124); (iii) <u>E. coli din::lacZYA</u> gene fusions are induced by DNA damage in a <u>recA</u> mutant of <u>E. coli</u> when the cloned <u>P. aeruginosa recA</u> gene is present on a plasmid (90); (iv) introduction of the truncated <u>recA</u> gene or <u>recA::lacZYA</u> gene fusions from <u>P. aeruginosa</u> sensitizes RecA⁺ <u>E. coli</u> to UVC radiation (124).

There is also some evidence which suggests that the <u>P. aeruginosa recA</u> gene product may be capable of interacting with a LexA-like repressor. Specifically, <u>P. aeruginosa</u> RecA protein mediates the lytic growth of lambda prophage in <u>E. coli</u> (89), and D3 and F116 prophages in <u>P. aeruginosa</u> (90) in response to DNA damaging agents, presumably via a cleavage type reaction analogous to that demonstrated for <u>E. coli</u> RecA protein against lambda and LexA repressors. Also, <u>P. aeruginosa</u> RecA protein complements <u>recA</u> deficiencies and maintains regulation of <u>recA</u>-dependent functions in a RecA⁻ <u>E. coli</u> host (89, 90, 137), which suggests that the <u>P. aeruginosa</u> RecA protein is capable of interacting with the <u>E. coli</u> LexA repressor. Experiments

have not yet directly addressed whether a <u>lexA</u> gene analogue is present in <u>P.aeruginosa</u>.

CHAPTER III

ISOLATION OF Tn3-lacZYA FUSIONS WITH NOVEL STRESS INDUCIBLE (sin) PROMOTERS OF PSEUDOMONAS AERUGINOSA

There has been considerable recent interest in the use of P. aeruginosa for agricultural purposes (43), and for the biodegradation of environmental pollutants (43, 55). The application of P. aeruginosa in open environments requires knowledge of the ability of this species to receive DNA from indigenous bacterial strains through the processes of transduction, conjugation, and transformation, to recombine this exogenous DNA into its genome, and to repair its cellular DNA when subjected to environmental stressors.

In the enteric organism E. coli, many recombination and repair genes are regulated by the RecA and LexA proteins. P. aeruginosa encodes a recA gene which structurally and functionally resembles E. coli recA. The P. aeruginosa RecA protein is induced in response to exposure to UVC radiation and quinolones (124), is required for UV induction of P. aeruginosa temperate prophages (90), and mediates induction of lambda prophage (89) and din gene transcription in E. coli recA mutants (90). Howeverr, as discussed in a recent review by Miller et al. (126), it is not clear whether this gene regulates a response network analogous to the SOS network of E. coli.

In an attempt to determine whether P. aeruginosa encodes SOS-like stress response genes, a promoterless lacZYA reporter gene was inserted into a P. aeruginosa

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chromosomal library (190) using the Tn3-lacZYA transposon Tn3-HoHoI (183). Tn3--HoHoI is a very effective single-site transposon mutagen since it preferentially and randomly transposes into extrachromosomal elements. Furthermore, it can be used to generate either transcriptional or translational gene fusions, and it produces gene fusions which are inherently stable. The promoterless lacZYA reporter gene serves as an indicator for transcription of the sequences into which it has inserted in that expression of the chromosomal gene can be monitored by following changes in the specific activity of the lacZ gene product, β-galactosidase.

In the present chapter, the isolation of Tn3-lacZYA fusions with novel, stress responsive P. aeruginosa promoters is described. These fusions were identified by treating cells with quinolones and UVC radiation. These stress conditions were selected for investigation since they result in SOS gene induction in E. coli.

Materials and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are indicated in Table 2.

Culture conditions

E. coli and P. aeruginosa strains were routinely grown in Luria broth (LB; Gibco, Paisley, Scotland). Growth of liquid cultures was monitored using a Klett-Summerson Photoelectric Colorimeter (Klett Mfg. Co., New York, NY). Solid media contained 1.2% Bacto-agar (Difco, Detroit, MI). Unless otherwise indicated, all cultures were incubated at 37°C. Concentrations (ug/ml) of antibiotics used for E. coli selection were as follows: ampicillin, 75; carbenicillin, 150 (Pfizer, New York, NY); tetracycline, 12.5; kanamycin, 75; nalidixic acid, 60; nitrofurantoin, 2.0;

Table 2.--Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
P. aeruginosa		
PAO25	argF10 leu-10	54
RM265	leu-10 recA102	PAO25
E. coli		
HB101	proA2 leuB6 thi-1 galK lacY1 hsdR hsdM recA13 supE44 rpsL20 xyl mtl	19
C2110	his rha polA Rif^r, Nal^r	183
JM103	thi-1 Δ(lac pro) sbcB15 endA hpsR4 supE [F;traD36 proAB lacI^{q}Z Am-15]	S. Kaplan
Plasmids		
pCP13	IncPI replicon cosmid; Tc^r Km^r	K. Farrand
pCP16	IncPI replicon cosmid derived from pCP13 by inverting the EcoRI polylinker and deleting the BamHI Km^r fragment; Tc^r	K. Farrand
pHoHoI	pMB8 replicon plasmid carrying the Tn3-lacZYA transposon Tn3-HoHoI; tnpA, Ap^r	183
pSShe	p15A replicon plasmid carrying the tnpA coding region of Tn3; Cm^r	K. Farrand
pRK2013	pBR322 cointegrate plasmid carrying the mob and tra plasmid transfer functions of RK2; Km^r	49
pKML2	pBR322 containing the P. aeruginosa recA gene on a 9.2 kb BamHI fragment	89
pKML2003	pBR322 containing the P. aeruginosa recA gene on a 2.2 kb BamHI-HindIII fragment	90
pBML2	pCP16::Tn3-lacZYA fusion constituitive for β-galactosidase expression; Tc^r, Ap^{r}r	This study
pBML7	pCP16::Tn3-lacZYA fusion expressing a low level of β-galactosidase activity; Tc^r, Ap^r	This study / This study

(Table 2:--Continued)

Strain or plasmid	Relevant characteristics[a]	Source or reference
Plasmid		
pBML400	<u>sinA::lacZYA</u> fusion present in pCP16; Tc^r, Ap^r	This study
pBML700	<u>sinB::lacZYA</u> fusion present in pCP16; Tc^r, Ap^r	This study
pBML900	<u>sinC::lacZYA</u> fusion present in pCP16; Tc^r, Ap^r	This study
pBML100	uncharacterized <u>sin::lacZYA</u> fusion present in pCP16; Tc^r,Ap^r	This study
pBML200	uncharacterized <u>sin::lacZYA</u> fusion present in pCP16; Tc^r,Ap^r	This study
pBML300	uncharacterized <u>sin::lacZYA</u> fusion present in pCP16; Tc^r,Ap^r	This study
pBML600	uncharacterized <u>sin::lacZYA</u> fusion present in pCP16; Tc^r,Ap^r	This study
pBML800	uncharacterized <u>sin::lacZYA</u> fusion present in pCP16; Tc^r, Ap^r	This study
pBML1200	may contain the <u>sinC::lacZYA</u> fusion present in a larger insert than pBML900	This study

[a]Nomenclature and abbreviations are essentially those of Demerec et al. (45). Rif^r, Nal^r, Tc^r, Km^r, Ap^r and Cm^r refer to resistance to rifampicin, nalidixic acid, tetracycline, kanamycin, ampicillin/carbenicillin, and chloramphenicol, respectively.

chloramphenicol, 50. Nalidixic acid was prepared by suspending in 0.01 N NaOH, and adding 10 N NaOH until the antibiotic dissolved. Norfloxacin (Merck Sharp and Dohme Research Lab, Rahway, NJ) used in screening the transposon mutagenized library was dissolved in 0.01 N NaOH.

P. aeruginosa exconjugants were selected on Pseudomonas Isolation agar (Difco) supplemented with tetracycline (200 ug/ml) and/or carbenicillin (500 ug/ml), and containing 2% glycerol as a carbohydrate source. When used with P. aeruginosa strains, kanamycin was employed at a concentration of 600-800 ug/ml. 56/2 agar plates utilized in the construction of pCP16::Tn3-lacZYA controls were supplemented with tetracycline, carbenicillin, 0.2% glucose, and 0.03 mM thiamine. 56/2 medium contained 16.2 mM $Na_2HPO_4 \cdot 7H_2O$, 72.6 mM KH_2PO_4, 0.01% $MgSO_4$, 0.005% $(NH_4)_2SO_4$, 0.0005% $Ca(NO_3)_2$, and 0.000025% $FeSO_4$. Pseudomonas Minimal Medium (PMM) used in triparental matings consisted of 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.05% sodium citrate, 0.1% $MgSO_4 \cdot 7H_2O$, and 0.1% $(NH_4)_2SO_4$.

In screening the transposon mutagenized library, expression of β-galactosidase was detected on lactose MacConkey agar (Difco). To verify results of MacConkey agar screening, MacConkey agar was replaced with LB agar containing 40 ug/ml 5-bromo-4-chloro-3-indolyl-β-galactosidase (X-gal), or cultures on LB or MacConkey agar plates were sprayed with a 1:10 dilution of a 10 mg/ml suspension of 4-methylumbelliferyl-β-D-galactoside (MUG) in dimethylsulfoxide and visualized after 10 minutes for fluorescence using a long wave UV source (217).

Restriction enzymes and chemicals

Most of the restriction enzymes used were purchased from Boehringer-Mannheim (Indianapolis, IN). Less frequently the enzymes SalI, BglII,

BamHI, HindIII, and KpnI were purchased from the Amersham Corp. (Arlington Heights, IL) and New England Biolabs (Beverly, MA). For restriction enzyme incubations, low salt buffer was 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, and 1 mM dithiothreitol. Medium salt buffer contained 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl and 1 mM dithiothreitol. High salt buffer consisted of 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, and 1 mM dithiothreitol. BamHI buffer used for single digestion with BamHI was 10 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM 2-mercaptoethanol, and EcoRI buffer for single digestion with EcoRI contained 100 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, and 50 mM NaCl. Digestions were allowed to proceed for at least 1 hour using the temperature and salt buffer recommended by Maniatis et al. (114), and containing a final DNA concentration of between 10 and 50 ug/ml digestion volume.

X-gal was purchased from 5 Prime --> 3 Prime, Inc. (Paoli, Pennsylvania), and LE agarose from the FMC Corporation (Rockland, ME). Formamide was obtained from International Biotechnologies, Inc. (New Haven, CT). All other chemicals and antibiotics were purchased from the Sigma Chemical Co. (St. Louis, MO).

Tn3-HoHoI mutagenesis

The target sequences for Tn3-HoHoI transposition consisted of a P. aeruginosa chromosomal library enriched for DNA fragments of 18 to 25 kilobase pairs (kb) in size cloned into the HindIII site of the tetracycline resistant cosmid pCP16 (190). Vector pCP16 (Figure 1) is a derivative of pCP13 (40) which possesses the EcoRI polylinker in the reverse orientation, and is deleted for the BamHI fragment containing the kanamycin resistance gene. The cosmid library packaged into phage lambda heads was infected into E. coli HB101 also containing the plasmids pHoHoI and pSShe
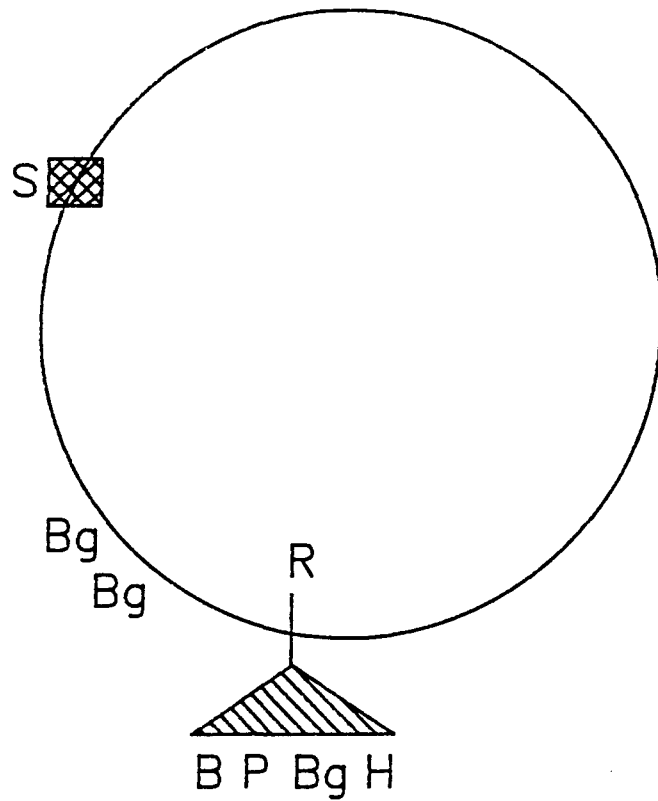
Fig. 1. Restriction endonuclease map of pCP16: box = tetracycline resistance gene; triangle = polylinker; S = SalI; Bg = BglII; B = BamHI; P = PstI; H = HindIII; R = EcoRI.

(183). Briefly, 20 ml of an overnight culture of HB101 (pHoHoI, pSShe) grown in
LB supplemented with carbenicillin, chloramphenicol, and 0.2% maltose was pelleted
and resuspended in 10 ml of 10 mM $MgSO_4$. A volume of 200 ul of the resuspended
cells was incubated with 100 ul of the cosmid infection mixture consisting of the
cosmid library packaged into phage lambda heads for 15 min at 37°C. To this
mixture, 3 ml of LB containing 10 mM $MgSO_4$ was added, and the culture was
incubated an additional hour at 37°C. All incubations were performed without shaking
to prevent disruption of adsorbed phage particles.   The culture was plated directly
onto LB agar containing carbenicillin, chloramphenicol, and tetracycline. Colonies
resulting from the infection were resuspended in a minimal volume of LB supple-
mented with the   same antibiotics.   This mixed culture became the donor in a
triparental mating also involving HB101 (pRK2013) and the   polA recipient strain
C2110. The mixture resulting from this triparental mating was plated onto LB agar
containing nalidixic acid, carbenicillin, and tetracycline to select for C2110 containing
Tn3-HoHoI insertions into pCP16 vector and chromosomal insert sequences.   The
plasmids pHoHoI, pSShe, and pRK2013 are incapable of being stably maintained within
C2110 since all depend upon the polA gene product DNA polymerase I for their
replication.   The resulting colonies were resuspended, and the transposon mutagenized
library was mobilized into the RecA⁺ P. aeruginosa strain PAO25 in a triparental
mating.

Triparental matings

Triparental matings were conducted using a modification of the method of
Ruvkun and Ausubel (156). E. coli donor cultures and HB101 containing pRK2013
were grown at 37°C to 40-60 $Klett_{660}$ units (10^7-10^8 CFU/ml) in appropriately

supplemented LB. One milliliter each of donor and conjugation helper were mixed with the same volume of recipient maintained for 10-20 hours preceding the experiment at either 37°C (E. coli recipients) or 43°C (P. aeruginosa) recipients. To ensure inactivation of the restriction system of P. aeruginosa (154), these cultures were maintained at 43°C under low oxygenation conditions (i.e. 15 to 20 ml of culture was grown without shaking in a 30 ml capacity test tube). Mating mixtures were filtered through a 0.45 um pore size cellulose nitrate filter (Nalge Company, Rochester, N.Y.). The filter was placed on LB agar plates and incubated at 37 °C for 4 to 24 h. Following incubation, filters were recovered and confluent growth was resuspended in 2-4 ml of LB or a 1:1 mixture of LB and PMM. The resuspended mixture was plated onto selective medium and incubated at 37 °C for one to two days.

Transformations

E. coli was made competent for transformation using a modified $CaCl^2$ protocol (42). Specifically, a 40 ml culture LB was grown to 40-50 $Klett_{660}$ units (approximately 10^8 CFU/ml) was pelleted at 5,000 rpm, 4 °C for 5 min using a SS34 rotor in a Sorvall RC-5B centrifuge. The cell pellet was resuspended in 20 ml of ice cold 50 mM $CaCl_2$, and maintained on ice for 20 min. Cells were pelleted again under the same conditions, and resuspended in 2 ml of ice cold 100 mM $CaCl_2$ for 5 min. Competent cells to which glycerol had been added to a final concentration of 10% were routinely aliquotted into microfuge tubes, frozen quickly in a dry ice/ethanol bath, and thawed when required for transformation. This treatment appeared to result in an increased efficiency of transformation.

At the time of transformation, 0.1 ml cells was added to DNA in a prechilled 5 ml polystyrene tube. The amount of DNA used was typically less than 50 ng in a volume of water or TE (10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA) between 10 and 100 ul. Cells and DNA were gently mixed and incubated on ice for 10 min. The cultures were heat treated either by incubating at room temperature for 10 min, or placing cultures in a 42 °C water bath for 1.5 min. Following heat treatment, 0.3-1.0 ml LB was added, and the cultures were incubated at 37 °C with gentle shaking for 1 h. Cells were plated directly onto selective medium.

Ultraviolet irradiation

Bacteria were irradiated using a General Electric germicidal lamp with a λ_{max} of 254 nm. The flux was measured with a UVX radiometer (Ultra-Violet Products, San Gabriel, CA). Irradiation and all subsequent manipulations were carried out under amber light. Liquid cultures and plates not maintained under amber light were wrapped in aluminum foil to prevent photoreactivation. In screening the transposon mutagenized library, colonies replicated onto MacConkey agar were allowed to grow 4-6 h before irradiating. This treatment resulted in a greater number of cells exposed to UVC radiation, and facilitated detection of induction.

Isolation of plasmid DNA

Two approaches were employed in the isolation of small quantities of extrachromosomal DNA. The alkaline lysis method of Ish-Horowitz (114) was routinely used to isolate better quality DNA which could conceivably be subjected to short term storage in the refrigerator. A modified alkaline lysis method (129) was used less frequently with only E. coli strains when isolated DNA was not to be stored. Protocols were followed as indicated, with the exception that in the alkaline

lysis method, phenol:chloroform extraction of DNA isolated from P. aeruginosa was performed two to three times. Furthermore, using both approaches, DNA yields were increased by doubling the time of the last two centrifugations.

A large scale alkaline lysis protocol (114) was followed for the isolation of relatively pure DNA from culture volumes of 250 ml or more. This protocol was modified in that the recommended final isopropanol precipitation step was not performed. Instead, the supernatant resulting from potassium acetate precipitation was transferred to separate 50 ml tubes, and an equal volume of a 1:1 mixture of phenol and chloroform was intermixed by vortexing. The phases were separated by centrifugation for 15 min at 6,000 rpm in a Sorvall GLC-2B centrifuge. Phenol:chloroform extraction was repeated at least one more time. Following the last extraction, one to two volumes of ethanol were added to the supernatant, and the mixture was centrifuged for at least 30 min at 6,000 rpm in a Sorvall GLC-2B centrifuge. The resulting pellet was rinsed twice with 1.0 ml 70% ethanol, and dried at 65 °C. Up to 1.0 ml of water containing Ribonuclease A was added, and the pellet was allowed to hydrate at least 30 minutes before transferring to a different tube.

When necessary, extrachromosomal DNA was purified on a cesium chloride gradient. DNA to be cesium purified was isolated using the unmodified large scale alkaline lysis protocol (114). Cesium chloride and a 10 mg/ml solution of ethidium bromide were added to the isolated DNA in the amounts of 1 gram/ml DNA solution and 80 ul/ml cesium solution, respectively. This solution was centrifuged at 20°C either for 40-60 h at 40,000 rpm using a Ti50 rotor in a Beckman L5-65 ultrac-entrifuge, or for 16 h at 100,000 rpm using a TL100.2 rotor in a Beckman TL-100

ultracentrifuge. The gradient was examined under long wave UV light to identify the closed circular plasmid DNA band. This band was collected and ethidium bromide was removed through repeated extraction of the DNA solution with isopropanol saturated with 20X SSC buffer (3 M NaCl, 0.3 M sodium citrate, pH 7). The sample was diluted one to four with 50 mM Tris (pH 8.0), and a one-tenth volume of 3 M potassium acetate was added to facilitate precipitation. The plasmid DNA was precipitated by the addition of one volume of 95% ethanol and overnight incubation at -20°C. Centrifugation of this mixture at 7,000 rpm for 45 minutes using a HB4 rotor allowed the recovery of a DNA pellet which was washed with a small volume of 70% ethanol and dried at 65 °C. This pellet was resuspended in TE or water.

Gel electrophoresis and fragment electroelution

DNA fragments were separated by horizontal agarose gel electrophoresis using TBE buffer consisting of 89.2 mM Tris base pH 8.0, 90 mM boric acid, and 2.5 mM EDTA. Unless otherwise specified, gels contained 0.7% agarose and 0.7 ug ethidium bromide/ml TBE buffer. DNA samples were loaded into gel wells with 0.25 volumes of tracking dye consisting of 30% glycerol, 7% sodium lauryl sulfate, and 0.07% bromophenol blue. Lambda DNA along with HindIII and HindIII-EcoRI digests of lambda were used as molecular weight standards. Electrophoresis was conducted by applying between 15 and 100 volts for periods of 2.5 to 15 h. DNA patterns were visualized by examining under short wave UV light, and recorded by photographing the gel.

Gel bands containing DNA fragments to be electroeluted were carefully excised from the rest of the gel while visualizing using a long wave UV source to minimize

DNA shearing. Electroelution was carried out in 0.5X TBE buffer at 100 volts for about an hour using an Analytical Electroeluter model 46000 (International Biotechnologies, Inc., New Haven, CT). DNA fragments were recovered in conductive salt bridges consisting of about 150 ul of 7.5 M ammonium acetate to which bromophenol blue had been added to facilitate visualization of the salt bridge. The ammonium acetate-DNA solution was transferred to a microfuge tube also containing 1 ml of 95% ethanol. To this tube, 1 ul of a solution of 5 mg phenol:chloroform extracted tRNA/ml was added to facilitate precipitation. DNA precipitation was carried out by immersing the microfuge tube in crushed dry ice for 10-15 min, and centrifuging an equal length of time. The pellet was carefully washed with 70% ethanol, recentrifuged 10 min and dried at 65 $^{\circ}$C. A total of 5 to 50 ul of water was added and the pellet was allowed to hydrate at least 15 min before quantitating the yield and purity of electroeluted fragment on an agarose gel.

<u>Preparation of radioactive probes and blot hybridization</u>

Restriction endonuclease digests of plasmid (0.1-1.0 ug/lane) or chromosomal (about 10 ug/lane) DNA separated on agarose gels were transferred to a BA85 nitrocellulose filter (Schleicher and Schuell, Inc., Keene, NH) essentially according to the method of Southern (181). Processing of gels before transfer was carried out at room temperature using a rotary shaker to ensure mixture agitation. DNA in gels was fragmented to facilitate transfer of large fragments by continual exposure of gels for 15 min to short wave UV, followed by immersing the gels in 0.25 N HCL (2.5 ml acid solution/ml gel) for an equal length of time. Denaturation of fragments was achieved by soaking the gels 30 min in an alkaline solution (2 ml solution/ml gel) of 1.5 M NaCl and 0.5 M NaOH. Gels were neutralized in a solution of 1.5 M

NaCl, 1.0 M Tris pH 7.4 (2 ml buffer/ml gel) for one hour. Capillary transfer of DNA to nitrocellulose using a 10X SSC buffer (1.5 M NaCl, 150 mM sodium citrate) was allowed to proceed about 15 h. DNA was adhered to the filters by incubating under vacuum at 65-70°C for 2 to 3 hours.

DNA was labelled with ^{32}P-dCTP (3000 Ci/mmol, New England Nuclear Medicine, Boston, MA) using a nick translation kit purchased from either the Amersham Corporation or Boehringer-Mannheim Biochemicals according to the recommendations of the manufacturer. A spun column procedure (114) was used to separate the labelled probes from unincorporated nucleotides. Prehybridization of filters was performed for at least 4 h at 42°C in a reaction volume of 150 ul prehybridization solution (M. Kelley, personal communication) per square centimeter of filter. The prehybridization solution was filtered through a 0.45 um pore size membrane filter, and contained 20% sodium phosphate buffer, 5X Denhardt's solution, 200 ug/ml sheared and denatured calf thymus DNA, 1% SDS, and 50% formamide. Whereas the sodium phosphate buffer consisted of 5 M NaCl, 50 mM NaPO$_4$ pH 6.5, and 0.5% tetrasodium pyrophosphate, 100X Denhardt's was 0.02% each of polyvinylpyrrolidone, bovine serum albumin, and Ficoll 400. Formamide was deionized prior to use by refrigerating overnight with a gram of Amberlite MB-3 for every 10 ml of formamide, and filtering at least twice through a Whatman #1 filter (Whatman, Maidstone, England). Either one half or the entire volume of labelled, denatured probe was added to the filter in prehybridization solution, and hybridization was allowed to proceed approximately 15 h at 42 °C. Radioactive filters were washed with a solution of 0.2X SSC and 1.0% SDS one time for 15 min at room temperature, and four times for 30 min at 65°C. Autoradiographs were taken at -80°C

using Kodak XAR-5 film and when necessary, intensifying screens. Filters were stripped by boiling one to three times for 20 min in a mixture containing 0.01X SSC and 0.1% sodium lauryl sulfate.

Investigation of the ability of sin::lacZYA fusions to

complement a P. aeruginosa recA mutation

For complementation tests, sin::lacZYA plasmids were mobilized into the P. aeruginosa recA102 host RM265. These strains along with RM265 were replica plated and UVC irradiated. UV sensitivity was compared to the RecA⁺ strain PAO25, and PAO25 also containing the inducible plasmids.

Results

Construction of a transposon mutagenized chromosomal library and

establishment in a RecA⁺ P. aeruginosa genetic background

Tn3-lacZYA insertions in a P. aeruginosa chromosomal library were generated as described in Materials and Methods, and as outlined in Figure 2. Infection of HB101 (pHoHoI, pSShe) with the cosmid library packaged into phage lambda heads yielded at least 5,000 independent colonies, which corresponds to a 99% probability of representing the entire P. aeruginosa genome five to six times (114). To increase the chances of obtaining insertions at each chromosomal site, and of establishing the entire mutagenized library in P. aeruginosa, the resuspended library present in HB101 (pHoHoI, pSShe) was divided into five separate pools designated L4A, L4B, L4C, L4D, and L4E. Transposon mutagenized plasmids represented in these pools were mobilized en masse into E.coli strain C2110 to isolate these plasmids from pHoHoI and pSShe. At least 30,000 colonies were obtained from each mating
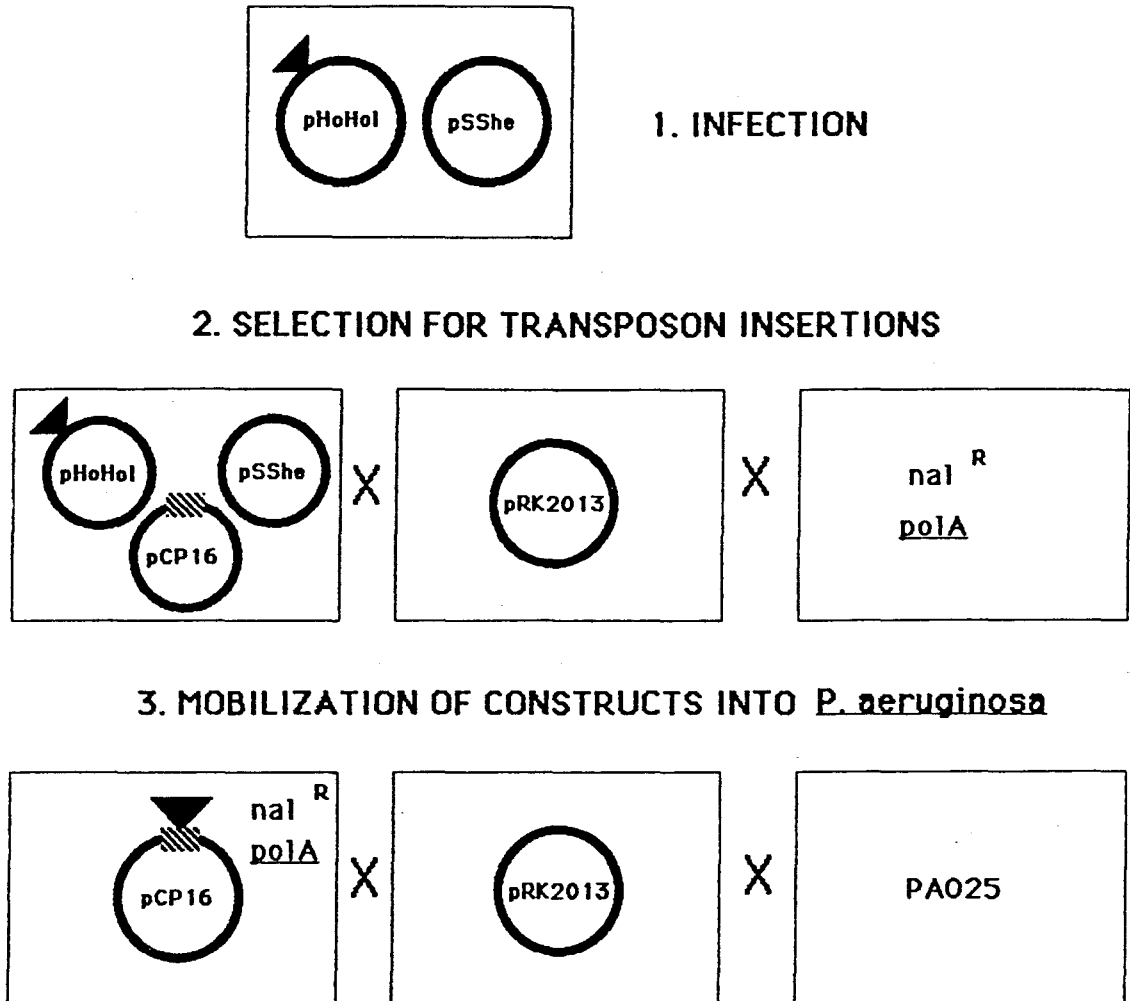
Fig. 2. Construction of a transposon mutagenized P. aeruginosa chromosomal library and mobilization into RecA⁺ PAO25: triangle = Tn3-HoHoI; rectangle = cloned chromosomal insert; thin line = vector sequences. The procedure followed is described in Materials and Methods.

involving a separate library pool. Transconjugants were again resuspended in separate library pools designated L5A, L5B, L5C, L5D, and L5E, and the library was mobilized into RecA$^+$ P. aeruginosa PAO25. Successful mobilization was achieved for pools L5A and L5E, which yielded about 8,000 transconjugants each. These transposon mutagenized libraries in PAO25 were designated L6A and L6E, respectively.

<div align="center">Construction of Tn3-lacZYA controls</div>

Tn3-lacZYA insertions into pCP16 were obtained in a similar fashion as the transposon mutagenized libraries. Specifically, HB101 (pHoHoI, pSShe) was transformed with pCP16, and the resulting mixed culture was mated with HB101 (pRK2013) and C2110 to select for transposon insertions into pCP16. The transconjugants produced in this mating represent a library of pCP16 into which single insertions of Tn3-HoHoI have occurred at various sites. This pCP16::Tn3-HoHoI gene fusion library was mobilized into the Lac$^-$ strain JM103 using a triparental mating. Plasmids expressing high (pBML2) and low (pBML7) levels of β-galactosidase were identified and mobilized by triparental mating into PAO25. Restriction maps of pBML2 and pBML7 are presented in Figure 3.

<div align="center">Screening the transposon mutagenized library for stress</div>

<div align="center">inducible gene expression</div>

Before the L6A or L6E libraries could be screened for stress responsive behavior, parameters for screening conditions needed to be determined since the relationship between gene induction and cell killing in response to different stress treatments is not known for P. aeruginosa. This was accomplished by making use of the Tn3-lacZYA positive control pBML2, and negative control pBML7. These
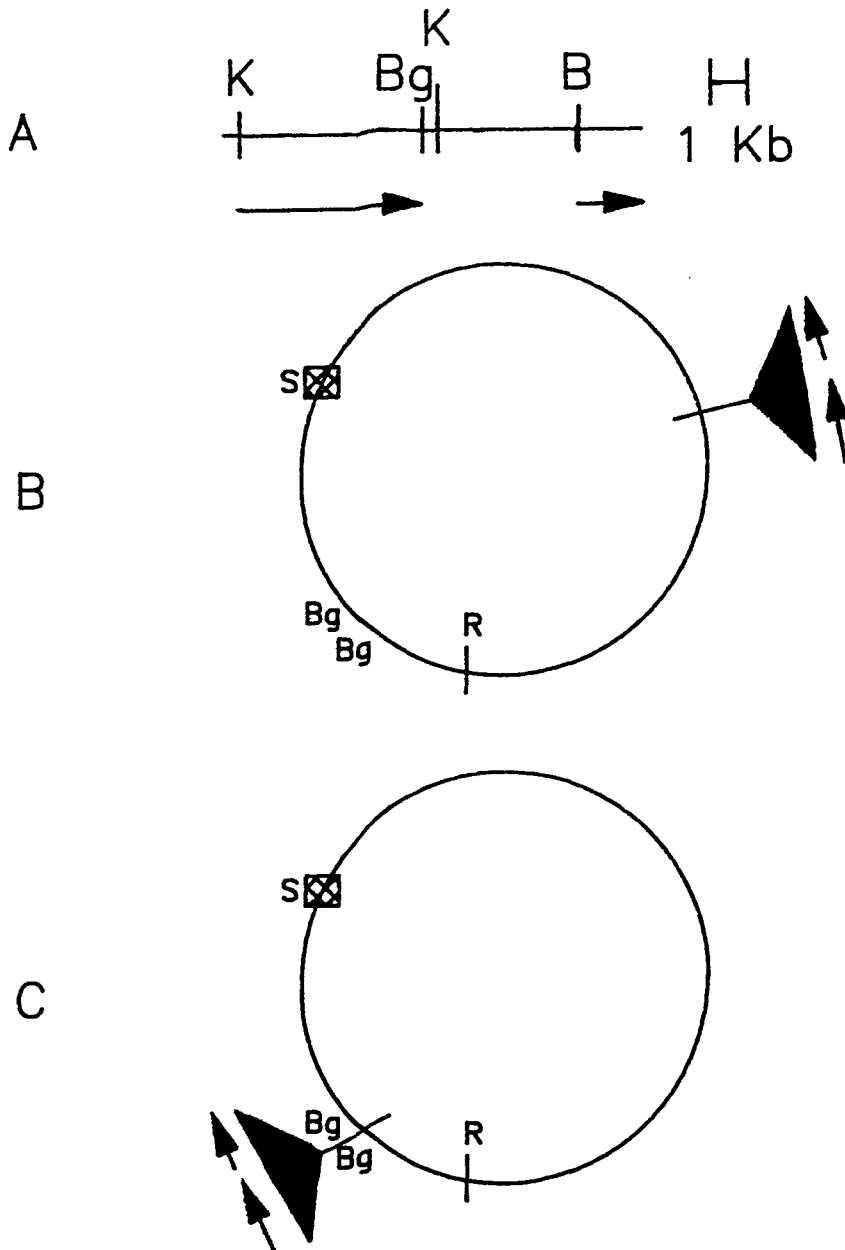
Fig. 3. Restriction endonuclease map of Tn3-HoHoI and Tn3::lacZYA controls pBML2 and pBML7. (A) Tn3-HoHoI, (B) pBML2, and (C) pBML7: triangle = Tn3-HoHoI; large arrow = direction of transcription of lacZYA; small arrow = direction of transcription of bla; S = SalI; Bg = BglII; K = KpnI; B = BamHI; R = EcoRI polylinker of pCP16.

controls and a random sample of the L6A library were replicated onto a series of MacConkey agar plates which contained or were subsequently exposed to different concentrations of inducing agents. In this manner, the level of each stress agent resulting in cell killing was determined. Results of these experiments (Table 3) indicated that cell killing of strain PAO25 on MacConkey agar becomes significant at UV exposures greater than 20 J/m^2, and at nalidixic acid concentrations between 100 and 150 ug/ml. While PAO25 appears capable of growth in the presence of higher concentrations of norfloxacin, at a concentration greater than 0.40 ug/ml, buffering of the medium occurs such that differences in β-galactosidase expression cannot be detected.

In the actual screen of the transposon mutagenized library, nalidixic acid concentrations of 75 and 125 ug/ml, norfloxacin concentrations of 0, 0.25, and 0.37 ug/ml, and UV exposures of 5, 10, 15 and 30 J/m^2 were employed. The screens were carried out by spreading the L6A library onto supplemented Luria agar, and replicating onto a series of master plates. These master plates were then used to replicate control or experimental MacConkey agar plates, with the experimental plates being those containing or subjected to stress agents. A total of greater than 25,000 colonies were screened in this fashion. In the initial screens, 327 putative stress inducible colonies were obtained, with 215 and 73 being detected in the nalidixic acid and norfloxacin screens respectively, and 39 identified in the UV screen. Repeat testing narrowed these inducible colonies down to about 50 colonies which appeared to increase their production of β-galactosidase in response to UV and quinolone exposure. These colonies were purified by streaking out cultures for isolated colonies two to three times, and checking isolates for inducible behavior.

Table 3.--Determination of parameters for screening conditions

MacConkey agar* containing:	PAO25(pBML2)	PAO25(pBML7)	Random Library sample
0 ug/ml Nal	-	-	-
50 ug/ml Nal	+	-	-
100 ug/ml Nal	+	+/-	+/-
150 ug/ml Nal	+/-	PG	PG
200 ug/ml Nal	PG	PG	PG
250 ug/ml Nal	NG	NG	NG
0 J/m² UV	+	-	-
2.5 J/m² UV	+	-	-
5.0 J/m² UV	+	-	-
10.0 J/m² UV	+	-	-
15.0 J/m² UV	+	PG	-
20.0 J/m² UV	PG	PG	PG
0 ug/ml Nor	+	-	-
0.25 ug/ml Nor	+	-	-
0.30 ug/ml Nor	+	-	-
0.40 ug/ml Nor	+/-	+/-	+/-
0.50 ug/ml Nor	+/-	+/-	+/-

Symbols: + indicates red on MacConkey agar
- indicates white on MacConkey agar
+/- indicates a light pink color on MacConkey agar
PG indicates poor growth
NG indicates no growth

* Plates also contained carbenicillin and tetracycline.

Comparison of restriction digests allows the recognition of eight different gene fusions

Analysis of the BamHI, PstI, BamHI-HindIII, BglII- PstI, and BglII-HindIII restriction patterns of the isolated inducible plasmids revealed that many were identical. Eight different digestion patterns were recognized among nonidentical cosmids. However, certain restriction pattern similarities apparent between some of these nonidentical cosmids indicated that they may be comprised of overlapping regions of chromosomal DNA, or may even contain the reporter gene linked to the same promoter. Relationships between restriction patterns of isolated plasmids are given in Table 4.

Determination of restriction sites preceding the reporter

gene in isolated inducible plasmids

Restriction sites located within 12 kb upstream of the inserted transposon were mapped in each of the stress responsive plasmids to determine whether similarities in obtained restriction patterns reflected reporter gene fusions with the same promoter. In this approach, the KpnI-BglII lacZYA-containing fragment of pHoHoI (Figure 3, Panel A) was radioactively labelled. The enzymes BglII, BamHI, PstI, HindIII, and SalI all lack restriction sites within this Tn3-HoHoI fragment. In a series of experiments, the probe was hybridized to BglII, BglII-BamHI, BglII-PstI, BglII-HindIII, and BglII-SalI digests of the eight inducible plasmids. Results of a representative hybridization experiment are presented in Figure 4. The size of the fragments detected by the lacZYA probe was used to determine the distance from the transposon bglII site to upstream restriction sites. Figure 5 summarizes results of the Southern hybridization experiments which were analyzed in this fashion. These data clearly demonstrate that

Table 4.--Comparison of restriction patterns of plasmids possessing stress responsive gene fusions

Cosmid	Description	Isolated constructs
pBML800	characteristic BamHI, BamHI-HindIII, PstI, BglII-PstI, and BglII-HindIII restriction patterns	36 or 4, 10, 18, 20, 22, 31, 45
pBML100	restriction patterns are similar to pBML800 although extra PstI bands are present and a different BglII-PstI pattern is obtained	1 or 16, 19, 21, 37, 46
pBML200	characteristic BamHI, BamHI-HindIII, PstI, BglII-PstI, and BglII-HindIII restriction patterns	2 or 394, 469, 524
pBML300	restriction patterns are similar to pBML200 although extra BamHI, PstI, and BamHI-HindIII bands are present	3
pBML400	characteristic BamHI, BamHI-HindIII, PstI, BglII-PstI, and BglII-HindIII restriction patterns	35 or 42, 44, 48
pBML600	restriction patterns are similar to pBML400 although extra BamHI, PstI, and BamHI-HindIII bands are present and the BglII-PstI pattern is different	9
pBML700	restriction patterns are similar to pBML400 although different BglII-PstI and BglII-HindIII patterns are obtained	11 or 5, 7, 8, 13, 14, 15, 17, 32, 33, 34, 38, 39, 41, 43
pBML900	characteristic BamHI, BamHI-HindIII, PstI, BglII-PstI, and BglII-HindIII restriction patterns	28 or 29*

*Isolated cosmid 29 (pBML1200) may possess the sinC fusion in a larger insert as discussed in Appendix B
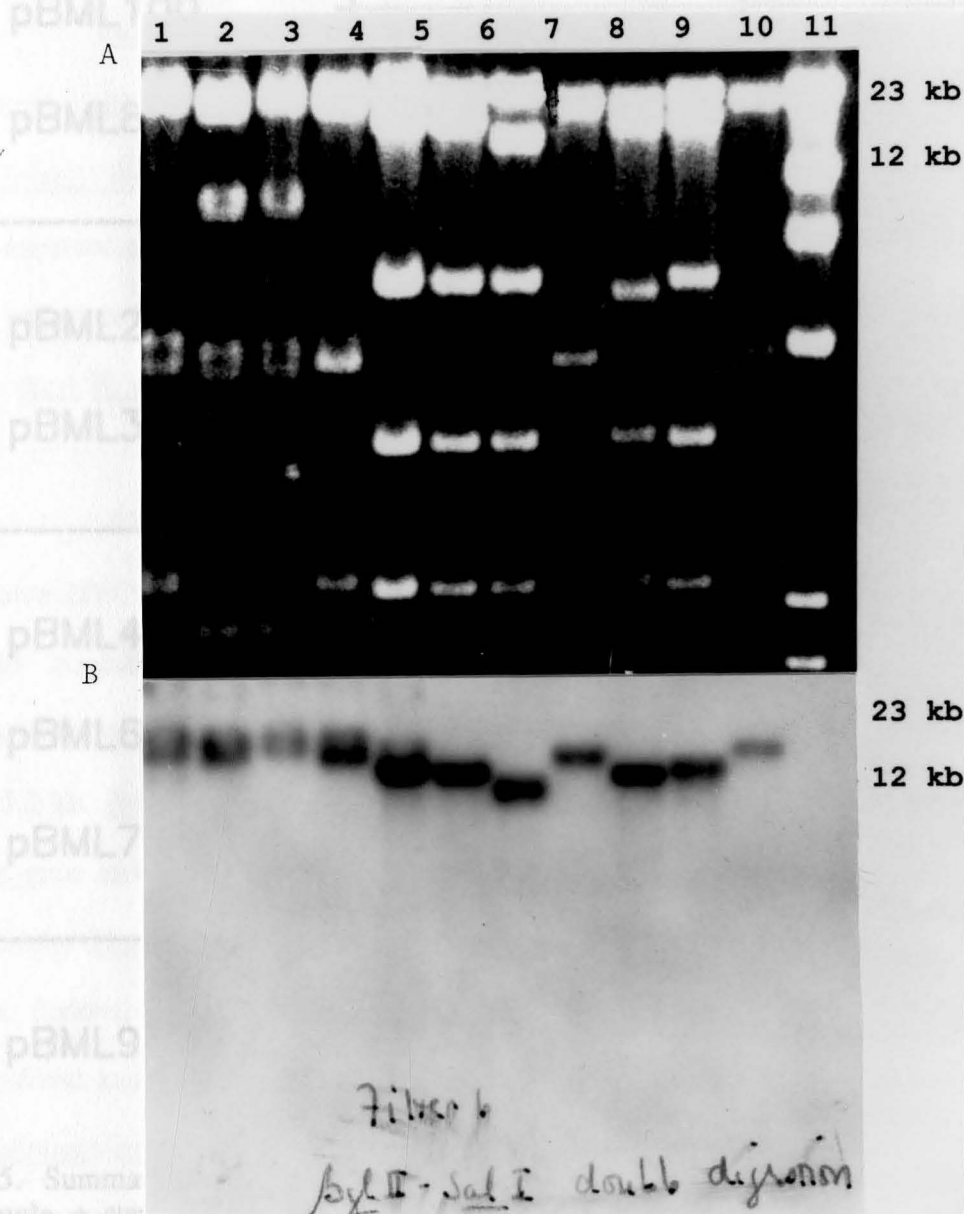
Fig. 4.   Southern analysis performed to determine   restriction sites preceding Tn3-HoHoI in isolated   inducible constructs. (A) Ethidium bromide stained gel, (B) autoradiogram.   Lanes 1-11: BglII-SalI   digests pBML100, pBML200, pBML300, pBML800, pBML400, pBML600, pBML700, pBML800, pBML900, pBML400, and pBML800, repectively; lane 13, lambda HindIII fragments in low salt buffer.
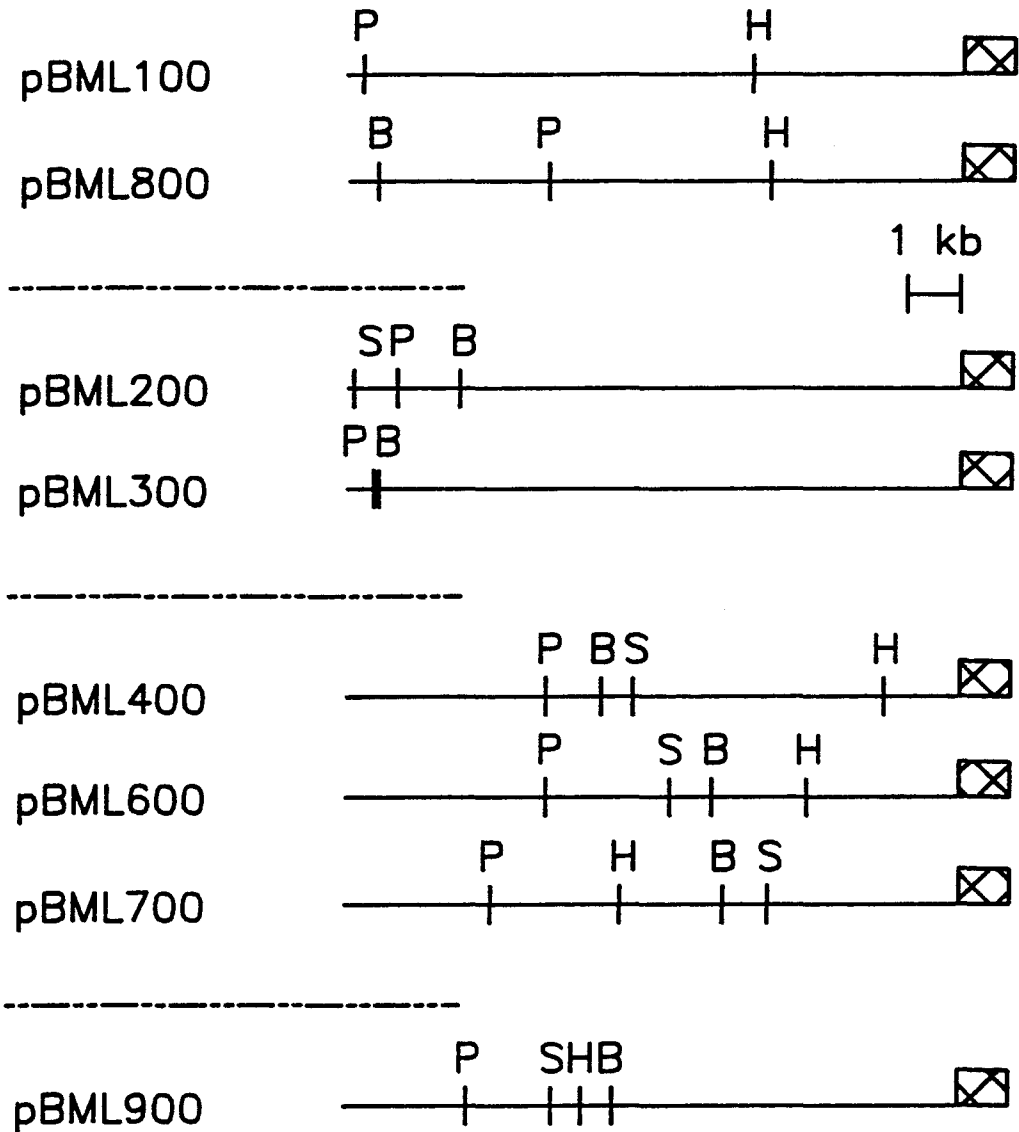
Fig. 5. Summary of restriction sites within 12 kb upstream of the inserted transposon: rectangle = start of Tn3-lacZYA sequences; P = PstI; B = BamHI; S = SalI; H = HindIII. Because of the manner in which the restriction maps were constructed, the locations of restriction sites farther upstream of the transposon insertion are subject to greater error.

the eight gene fusions differ in restriction sites preceding the reporter gene, and nost

likely represent fusions with unique P. aeruginosa promoters.

Out of the eight inducible plasmids identified, the sinA::lacZYA-containing

plasmid pBML400, the sinB::lacZYA-containing plasmid pBML700, and the

sinC::lacZYA-containing plasmid pBML900 were selected for further study based upon

the apparent strongly inducible behavior of pBML400 and pBML700, and based upon

the unique restriction patterns exhibited by pBML900. The plasmids were established

in a recA E. coli background to minimize recombination.

<u>Isolated gene fusions are not recA::lacZYA fusions</u>

Since the recA gene of P. aeruginosa has been demonstrated to mediate

its own UVC and quinolone inducible gene expression (124), isolated gene fusions

could conceivably represent the reporter gene linked to the recA promoter. This

possibility was investigated by performing Southern analysis of all gene fusions using

the 2.2 kb BamHI-HindIII fragment of pKML2003 (90) containing the P. aeruginosa

recA gene as a probe. The recA probe failed to hybridize to the isolated inducible

plasmids. HindIII-KpnI promoter-containing probes generated from the sinA and sinC

gene fusions, and a SalI-KpnI probe generated from sinB::lacZYA were also

hybridized to a BamHI-HindIII digest of plasmid pKML2 (89), which contains the P.

aeruginosa recA gene in a larger, 9 kb chromosomal insert (89). Each sin probe

failed to hybridize to recA, but did hybridize to a 5.5 kb fragment which occurs

immediately upstream of the recA promoter. This hybridization may have been due

to the presence of about 200-500 basepairs of the upstream end of Tn3-HoHoI in

each of the sin probes, since the labelled BglII lacZYA fragment of pBML7 (Figure

3, Panel A) also detected the same fragment. While it was not possible to establish

sinC::lacZYA in a recA P. aeruginosa host, both sinA::lacZYA and sinB::lacZYA containing plasmids failed to complement the UV sensitivity of a P. aeruginosa recA102 mutant.

Discussion

A mutagenized P. aeruginosa chromosomal library containing Tn3-HoHoI insertions at different chromosomal sites was successfully constructed and mobilized into the RecA⁺ P. aeruginosa strain PAO25 to allow the isolation of genes which respond to various stresses by increasing their level of expression. This library was screened under conditions of UVC and quinolone exposure, and fusions linking a β-galactosidase reporter gene to stress responsive promoters of P. aeruginosa were identified. These sin fusions do not represent the reporter gene linked to recA or contain this gene within their insert, since all fusions failed to hybridize to P. aeruginosa recA. These results are significant since they clearly indicate that P. aeruginosa possesses genes other than recA whose expression increases in response to stress agents, and that fusion of a reporter gene to these novel stress responsive genes have been obtained.

Comparison of restriction digests of isolated plasmids suggested the existence of eight distinct stress inducible gene fusions. Some of the plasmids demonstrated similar restriction digestion patterns which may reflect their possession of overlapping regions of chromosomal DNA, or organization of sin genes in operons. Restriction sites up to 12 kb upstream of the inserted reporter gene were different in the eight gene fusions. These results suggest that the eight sin::lacZYA plasmids most likely represent unique gene fusions. Three gene fusions, sinA::lacZYA, sinB::lacZYA,

and <u>sinC::lacZYA</u> were selected for further investigation of stress responsive behavior, which is described in the next Chapter.

CHAPTER IV

CHARACTERIZATION OF THE STRESS RESPONSIVE BEHAVIOR

OF sinA, sinB AND sinC GENE FUSIONS

Bacteria respond to environmental and chemical stressors by inducing characteristic sets of proteins specific for each stress (Table 1). Some networks are capable of responding to alternative forms of stress. For example, the heat shock response can be precipitated by exposure of E. coli to quinolones and UVC radiation, which primarily induce an SOS response (194). From an ecophysiological point of view, heat shock may be a common environmental stressor of soil and water micro-organisms. Based upon these considerations, the ability of sin::lacZYA gene fusions to respond to stresses including heat shock, UVC radiation, and norfloxacin exposure was investigated. This study was carried out to determine whether sin genes correspond to P. aeruginosa heat shock genes, or potentially represent analogues of E. coli SOS recombination and repair genes.

Materials and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are indicated in Table 5.

Table 5.--Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
P. aeruginosa		
PAO25	argF10 leu-10	54
OT684	leu-1 lys-1 res-4	150
E. coli		
HB101	proA2 leuB6 thi-1 galK lacY1 hsdR hsdM recA13 supE44 rpsL20 xyl mtl	19
AB1157	argE3 his-4 leuB6 proA2 thr-1 thi-1 galK2 lacY1 Sm-31 supE44 ara-14 xyl-5 mtl-1 tsx-33	37
Plasmids		
pRK2013	pBR322 cointegrate plasmid carrying the mob and tra plasmid transfer functions of RK2; Km^r	49
pBML400	sinA::lacZYA fusion present in pCP16; Tc^r, Ap^r	This study
pBML700	sinB::lacZYA fusion present in pCP16; Tc^r, Ap^r	This study
pBML900	sinC::lacZYA fusion present in pCP16; Tc^r, Ap^r	This study
pRK2013:501	pRK2013 containing an insertion of Tn501; Km^r, Hg^r	M.Davidson
pBluescript	ColEI replicon phagemid derived from pUC19; Tc^r, Ap^r	M.Kelley
sin::lacZYA subclones		
pBML4001	BglII-PstI religation product of pBML400; Tc^r,	This study
pBML4010	HindIII religation product of pBML400; Tc^r, Ap^r	This study
pBML4300	SalI religation product of pBML400; Tc^r, Ap^r	This study
pBML7019	BamHI religation product of pBML700; Tc^r, Ap^r	This study
pBML7070	HindIII religation product of pBML700; Tc^r, Ap^r	This study
pBML7300	SalI religation product of pBML700; Tc^r, Ap^r	This study
pBML9001	BglII-PstI religation product of pBML900; Tc^r	This study

Culture conditions and chemicals

Unless indicated otherwise, all strains were incubated at 37°C. HgCl^2 was used for selection in E. coli and P. aeruginosa at final concentrations of 15-35 ug/ml. For the assay of β-galactosidase expression, bacteria were grown in PMM supplemented with 0.4% glucose, 0.2% casamino acids (Difco) and either 500 ug carbenicillin/ml or 200 ug tetracycline/ml. Z buffer used in the β-galactosidase assay contained 60 mM Na$_2$HPO$_4$·7H$_2$O, 40 mM NaH$_2$PO$_4$·H$_2$O, 10 mM KCl, 1 mM MgSO$_4$·H$_2$O, 50 mM 2-mercaptoethanol, and had a final adjusted pH of 7.0. O-rtho-nitrophenyl-β-D-galactoside (ONPG) used as substrate in the assay reaction was solubilized in a 0.1 M sodium phosphate buffer of pH 7.0. 2-mercaptoethanol was obtained from Mallinckrodt (Saint Louis, MO). Exonuclease III and Nuclease SI were purchased from Promega (Madison, WI), whereas Klenow DNA polymerase, calf intestinal alkaline phosphatase (CIAP), and T4 DNA ligase were purchased from Boehringer Mannheim. Ultrapure nucleotides used in Exonuclease III/Nuclease SI digestion were obtained from Pharmacia (Pleasant Hill, CA).

Determination of cellular β-galactosidase levels

β-galactosidase activity was determined essentially according to the method of Miller (123). Cultures grown at 37°C to about 40 Klett$_{660}$ units were divided. Some fractions were exposed to different inducing treatments including incubation at 43°C, the addition of the quinolone norfloxacin, and exposure to various doses of UVC radiation. The control fraction did not receive any inducing treatment. At various intervals, duplicate 1.5 ml samples were removed and placed on ice for a minimum of 20 min. β-galactosidase was assayed by withdrawing 0.2 ml or 0.5 ml cells (depending upon the anticipated level of β-galactosidase to be measured) to 0.8 ml or

(Table 5.--Continued)

Strain or plasmid	Relevant characteristics[a]	Source or reference
sin::lacZYA subclones		
pBML9011	BamHI religation product of pBML900; Tc[r]	This study
pBML9090	HindIII religation product of pBML900; Tc[r], Ap[r]	This study
Tn501 mutagenized plasmids		
pBML4011	inducible pBML4010::Tn501 construction; Tc[r], Ap[r], Hg[r]	This study
pBML4012	inducible pBML4010::Tn501 construction; Tc[r], Ap[r], Hg[r]	This study
pBML4013	inducible pBML4010::Tn501 construction; Tc[r], Ap[r], Hg[r]	This study
pBML4017	noninducible pBML4010::Tn501 construction; Tc[r], Ap[r], Hg[r]	This study
pBML4018	noninducible pBML4010::Tn501 construction; Tc[r], Ap[r], Hg[r]	This study
pBML4019	noninducible pBML4010::Tn501 construction; Tc[r], Ap[r], Hg[r]	This study
pBluescript subclones		
pBML4200	HindIII-BamHI fragment of pBML400 containing sinA::lacZYA subcloned into pBluescript SK; Ap[r]	This study
pBML4240	HindIII-BglII fragment of pBML4200 containing sinA::lacZYA subcloned into the HindIII and BamHI sites of pBluescript SK; Ap[r]	This study
pBML4211	deletion derivative of pBML4240; Ap[r]	This study
pBML4212	deletion derivative of pBML4240; Ap[r]	This study
pBML4213	deletion derivative of pBML4240; Ap[r]	This study

[a]Nomenclature and abbreviations are essentially those of Demerec et al. (45). Tc[r], Km[r], Ap[r], and Hg[r] refer to resistance to tetracycline, kanamycin, ampicillin/carbenicillin and mercury, respectively.

0.5 ml Z buffer in a 2.0 ml microfuge tube. Cells were lysed by the addition of three to four drops of 0.1% sodium lauryl sulfate and about 5 drops chloroform, followed by a 10 min incubation at room temperature. The assay reaction mixture was warmed by incubating in a 28-29°C water bath for 5 min. The reaction was initiated through the addition of 0.2 ml of a 4 mg/ml solution of ONPG, and was allowed to proceed 15 min at the same temperature. Termination of the reaction was accomplished by shifting the pH to 11 through the addition of 0.5 ml of a 1 M solution of Na_2CO_3. Cell debris was removed from the assay solution by centrifuging in an Eppendorf centrifuge for 2 min. The absorbance of this solution at 420 nm was determined, taking care not to disturb the cell pellet. The remainder of the withdrawn sample was used to determine cell density by recording the absorbance at 600 nm. A Gilford Response UV-Vis spectrophotometer was used to measure absorbance. All aliquots were assayed within 4.5 h following removal from culture. β-galactosidase activity was determined according to the formula:

$$\text{Units of } \beta\text{-galactosidase} = A_{420} \times 1000 \: / \: A_{600} \times T \times V$$

where A_{420} is the absorbance at 420 nm,
A_{600} is the absorbance at 600 nm,
T is the reaction time (15 min), and
V is the volume of cells assayed (0.2 or 0.5 ml cells).

A simple computer program was written to facilitate calculation of β-galactosidase activity (Appendix A).

<u>Subcloning</u>

Deletion subclones in pCP16 were constructed by digesting pBML400, pBML700, and pBML900 with single restriction enzymes, and then religating digestion products obtained. The ligation reaction was incubated between 15-20 h at 14°C,

and contained up to 300 ug total DNA per ml ligation reaction, up to 1 unit of T4 DNA ligase, 20 mM Tris·HCl pH 7.6, 10 mM MgCl$_2$, 10 mM dithiothreitol, and 0.6 mM adenosine triphosphate.

The subclone pBML4200 was constructed by ligating the HindIII-BamHI sinA::lacZYA fragment of pBML400 into the HindIII and BamHI sites of pBluescript SK. The ApaI site present in the downstream Tn3-HoHoI end of pBML4200 was removed by subcloning the HindIII-BglII lacZYA containing fragment of pBML4200 into the HindIII and BamHI sites of pBluescript SK to generate pBML4240. Using the same ligation buffer and reaction conditions as used for construction of pCP16 deletion subclones, 0.2 ug of vector was incubated with a 3- to 5-fold molar excess of insert. The 5' termini of the vector were dephosphorylated by the addition of CIAP to the digestion reaction. Dephoshorylation was allowed to proceed about 1 h at 37°C. CIAP was removed through repeated phenol:chloroform extraction, or by gel purification followed by electroelution of the DNA fragment. The digested insert DNA was treated in an identical fashion to remove excess protein.

Transformations

Transformation of E. coli strains with ligation mixtures requires a high efficiency of transformation. In these cases, a rubidium chloride transformation protocol (62) was used to maximize chances of successful subcloning.

Tn501 mutagenesis

Tn501 mutagenesis (13, 74) was carried out using a modified conjugation. Recipient strains PAO25 (pBML4010), PAO25 (pBML7019) and OT684 (pBML9090) were grown at 43°C under selection for 24 h preceding the experiment. The donor strain RM1079 was grown at 37°C in the presence of mercury to approximately 10^8

CFU/ml. One milliliter each of donor and recipient were mixed and filtered through a 0.45 um pore size filter. The filters were placed on LB agar plates and incubated about 15 h at 37°C. The following day, confluent growth on filters was resuspended in 3 ml LB and plated onto Pseudomonas Isolation Agar containing 500 ug carbenicillin/ml and 35 ug HgCl₂/ml. Transposition of Tn<u>501</u> into recipient strains as compared to rescue of pRK2013::Tn<u>501</u> through recombination with pCP16 sequences was verified by replica plating transconjugants onto LB agar containing kanamycin.

Exonuclease III/Nuclease SI deletions

Deletions proceeding into the <u>sinA</u> promoter region were obtained by Exonuclease III/nuclease SI digestion (63) of pBML4240. Nine micrograms of cesium purified pBML4240 DNA was sequentially digested with <u>Apa</u>I and <u>Hind</u>III. The digested DNA was extracted first with a 1:1 mixture of phenol and chloroform, and then with chloroform alone. Two volumes of 95% ethanol and a 1/10 volume of 2 M NaCl were added, and the DNA was precipitated by maintaining in a dry ice bath 15 min and then centrifuging for 15 min. Precipitation was repeated to increase yield of DNA. The obtained pellet was washed with 70% ethanol, recentrifuged, dried at 65°C, and resuspended in 30 ul of a 1X Exonuclease III buffer containing 66 mM Tris-HCl, pH 8.0 and 1 mM EDTA. The resuspended DNA was warmed to 30°C in a water bath, and 250 units of Exonuclease III was added. Following a 30 second lag, 2.5 ul aliquots were removed at 60 second intervals to microtiter plate wells containing 7.5 ul Nuclease SI mix, and maintained on ice. Nuclease SI mix consisted of 0.3 units SI nuclease/ul, 40 mM potassium acetate pH 4.6, 340 mM NaCl, 1.35 mM ZnSO₄, and 6.8% glycerol. Upon removal of the last aliquot, 1 ul

of Nuclease SI stop buffer containing 0.3 M Tris base and 50 mM EDTA was added to each well, and aliquots were incubated at 70°C for 10 min to terminate the reaction. The microtiter plate was placed in a 37°C water bath, and 1 ul Klenow mix containing 0.1 units Klenow DNA polymerase/ul, 20 mM Tris-HCl, pH 8.0, and 100 mM MgCl$_2$ was added to each well. This mixture was incubated for 3 min at 37°C before adding 1 ul of a dNTP mixture (0.125 mM each of dATP, dCTP, dGTP, and dTTP) and incubating an additional 5 min. The microtiter plate was removed to room temperature, and 40 ul of a ligation mixture containing 0.005 units T4 DNA ligase/ul, 5% PEG, 1 mM dithiothreitol, 50 mM Tris-HCl pH 7.6, 10 mM MgCl$_2$, and 1 mM ATP was added to each well. Ligation was allowed to proceed 1 h. Ten microliters of each ligation reaction was used to transform competent HB101.

Since pBluescript is a high copy number plasmid, deleted plasmids were purified by streaking out transformants and isolating DNA from single colonies. This DNA was diluted in water and used to retransform HB101 and/or AB1157. In some cases this purification was repeated.

<div align="center">Other methods</div>

Restriction endonuclease digestion, ultraviolet irradiation, triparental matings, Southern analysis, and isolation of plasmid DNA were carried out as described in Chapter III.

Results

Heat stress does not promote expression from sinA,

sinB, and sinC promoters

The E. coli heat shock genes groEL and dnaK can be induced in response to both UV and quinolone exposure (93). To investigate whether sin reporter gene fusions are controlled by heat shock promoters, the sinA, sinB, and sinC gene fusions were tested for heat shock responsive behavior (Figure 6). For these experiments, cultures of the RecA$^+$ P. aeruginosa strain PAO25 containing either pBML400, pBML700, or pBML900 grown at 37°C were heat stressed by shifting the culture temperature to 45°C. The temperature shift failed to result in increased β-galactosidase activity within a period of time during which P. aeruginosa heat shock genes are normally induced (2). Heat shock induction was not observed for any of the cultures within four hours of the temperature increase.

Kinetics of the ultraviolet light response of sin::lacZYA gene fusions

Different E. coli SOS genes have been shown to be induced with varying kinetics following stress treatment (200). The inducible response of the pBML400, pBML700, and pBML900 gene fusions following exposure to UVC radiation was investigated in the RecA$^+$ P. aeruginosa strain PAO25 (Figure 7). The sinA::lacZYA fusion was induced with the fastest kinetics, and exhibited a bimodal induction profile. Twenty five- to 30-fold induction of this fusion occurred between 120-160 min following UV exposure. Induction of sinB::lacZYA of about 22-fold occurred at 160 min after UV treatment. This gene fusion appeared to demonstrate a second 2.5-fold inductive peak at 210 min after exposure. The sinC gene fusion was
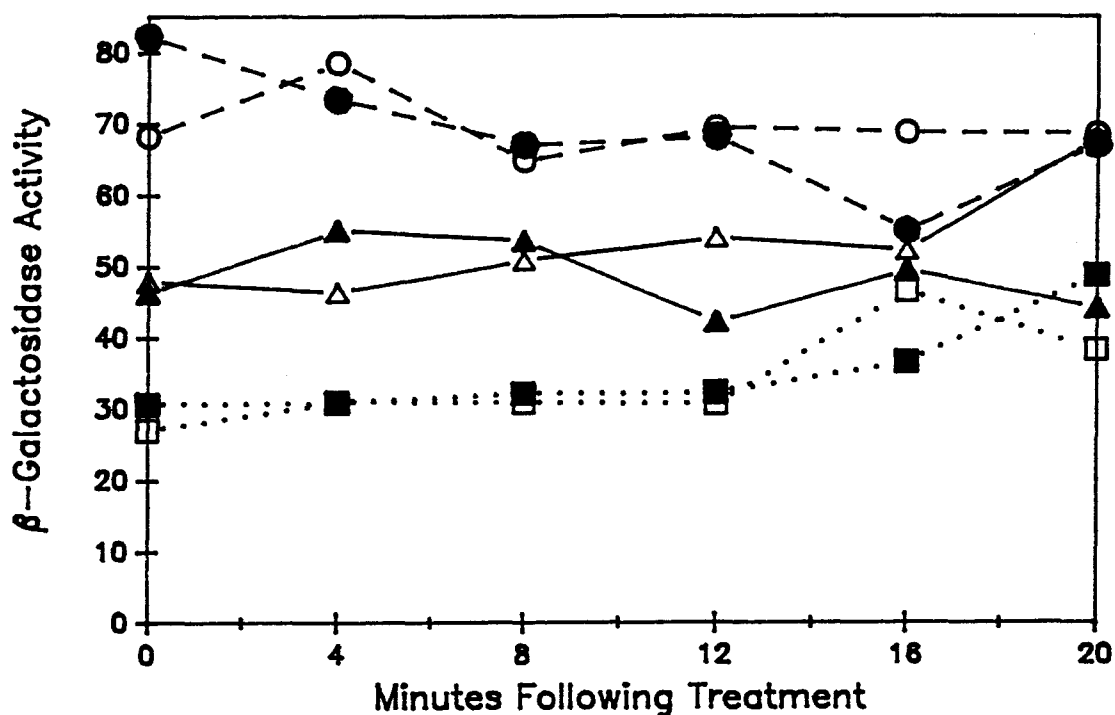
Fig. 6. Heat shock assay of PAO25 (pBML400), PAO25 (pBML700) and PAO25 (pBML900): (O) PAO25 (pBML400) 37°C; (●) PAO25 (pBML400) 45°C; (Δ) PAO25 (pBML700) 37°C; (▲) PAO25 (pBML700) 45°C; (□) PAO25 (pBML900) 37°C; (■) PAO25 (pBML900) 45°C. Cultures of PAO25 containing the plasmids were grown at 37°C, divided, and incubated at either 37°C or 45°C. Heat treated cultures attained a temperature of 45°C in less than 3 minutes. In a separate experiment, gene induction was not observed within 4 hours of the temperature increase.
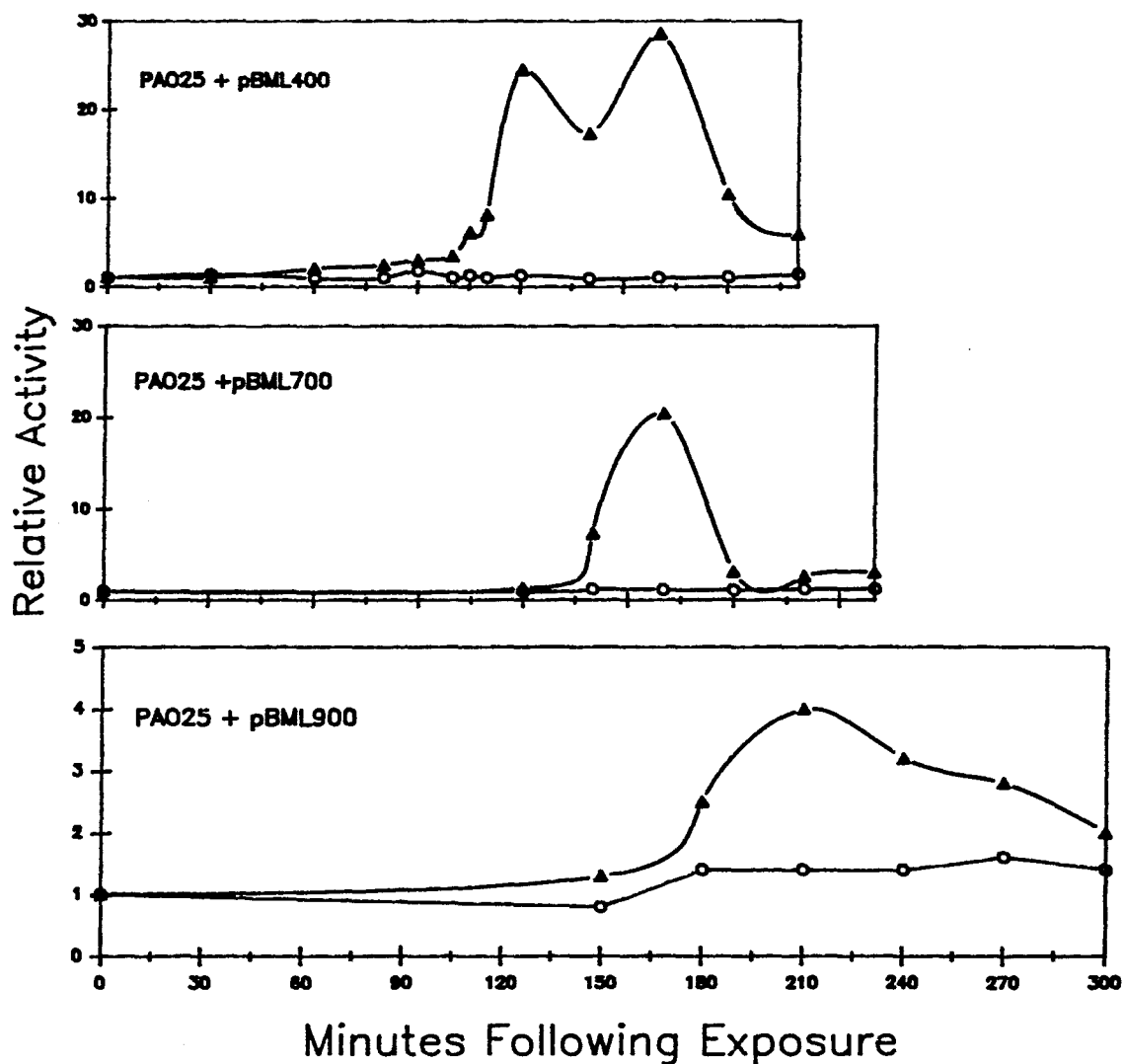
Fig. 7. UV inducible behavior of PAO25 (pBML400), PAO25 (pBML700), and PAO25 (pBML900): (O) untreated; (▲) 20 J/m². Relative activity was calculated by dividing β-galactosidase units present at each sampling time by those present in the same culture at time zero. Untreated cultures contained 40-60 units of β-galactosidase. Maximal activity values ranged from 100 to 1600 units. The plots represent the average of at least two independent experiments. Assays were performed in duplicate.

induced only about 4-fold at 210 min subsequent to stress treatment. The induction profile of sinC::lacZYA also appeared to be multimodal, with a second peak occurring at about 270 min. For all three sin gene fusions, induction was not prolonged, and β-galactosidase levels returned to approximately baseline levels relatively quickly following UV treatment. This recovery phenomenon, along with the observed multimodal induction profiles were due to alterations in β-galactosidase activity and not changes is cell density, since cell density quickly reached a stable plateau following stress treatment (Figure 8).

Dose dependency of the inductive response to ultraviolet irradiation

The relatively low level of induction of PAO25 (pBML900) might have reflected irradiation with a suboptimal dose of UVC. This possibility was investigated for the pBML400, pBML700 and pBML900 gene fusions by exposing the RecA$^+$ P. aeruginosa strain PAO25 containing these plasmids to different doses of UVC radiation (Figure 9). Experimental results indicated that maximal induction occurred following a UV dose of 20 J/m^2. Exposure of sinA::lacZYA and sinC::lacZYA to greater or lesser UV doses resulted in submaximal induction. The response of sinB::lacZYA to UV doses above 20 J/m^2 was not investigated.

Stress responsive promoters can be induced upon exposure to norfloxacin

In screening the transposon mutagenized library, sinA, sinB, and sinC gene fusions were identified as both UVC and quinolone inducible. In the case of sinA::lacZYA, this norfloxacin inducible behavior was investigated by exposing cultures of the RecA$^+$ P. aeruginosa strain PAO25 containing pBML400 to different doses of norfloxacin (Figure 10). Induction was maximal following exposure to a norfloxacin concentration between 15-19 ug/ml. Similar to the UV dose dependent
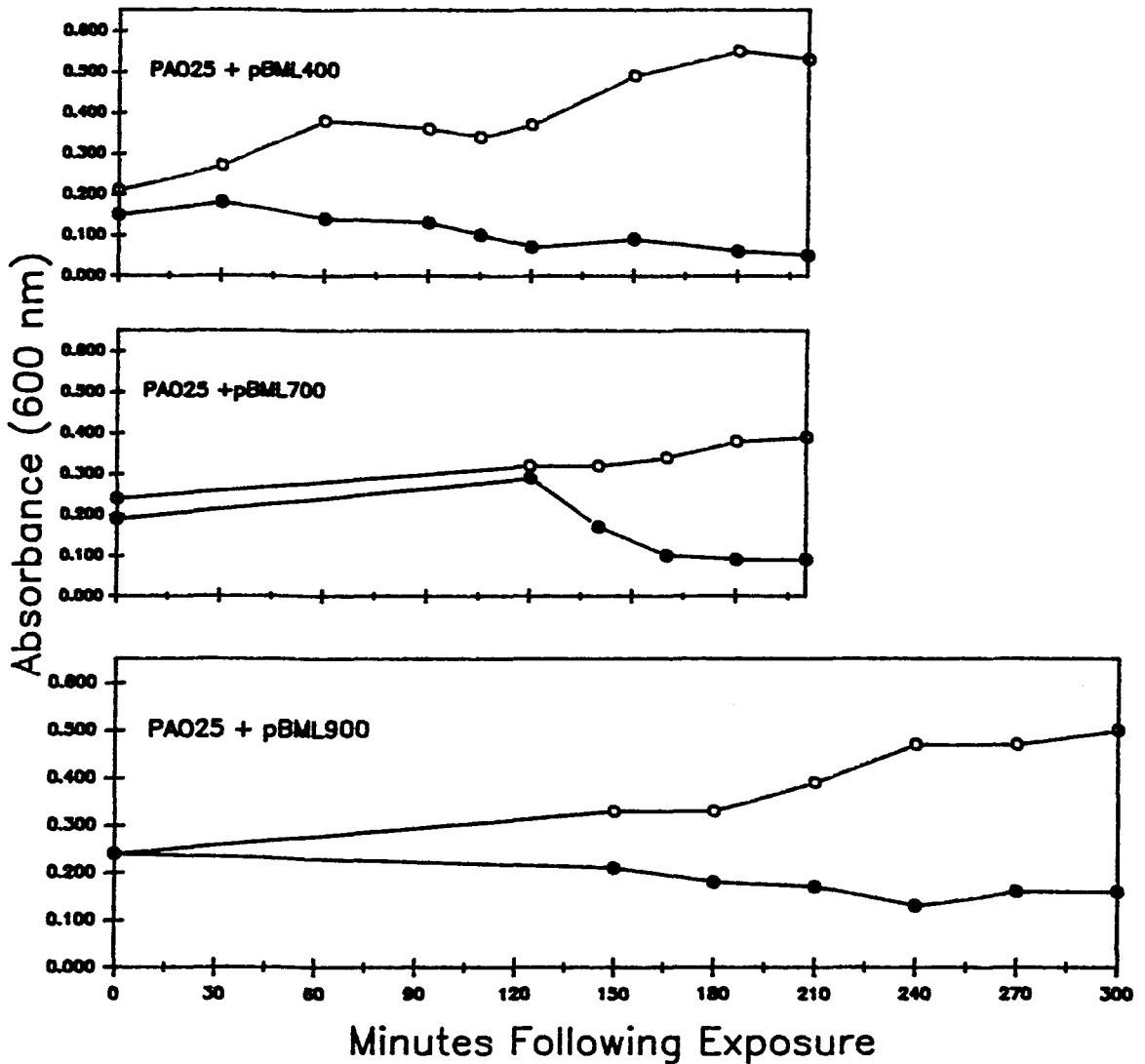
Fig. 8. Measurement of cell density of UVC irradiated cultures of PAO25 (pBML400), PAO25 (pBML700), and PAO25 (pBML900): (O) untreated; (●) 20 J/m². The plots represent the average of two or more experiments.
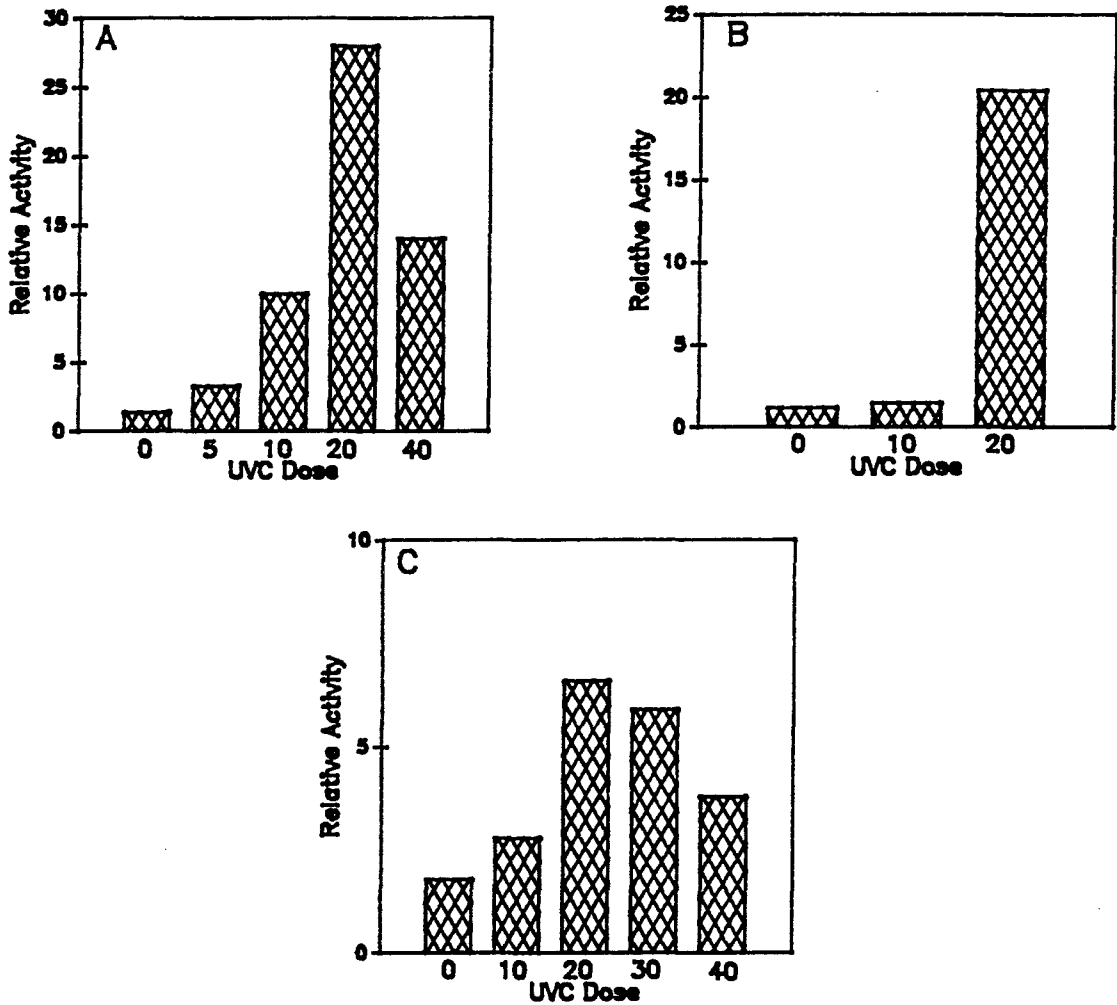
Fig. 9. UVC dose dependent response of PAO25 sin::lacZYA gene fusion-containing strains. (A) PAO25 (pBML400), (B) PAO25 (pBML700), (C) PAO25 (pBML900). Plotted values reflect maximum induction values observed within 4 hours of UVC exposure. Each bar in Panel A represents the average of two independent experiments. Most assays depicted in Panels B and C were repeated. All assays were performed in duplicate.
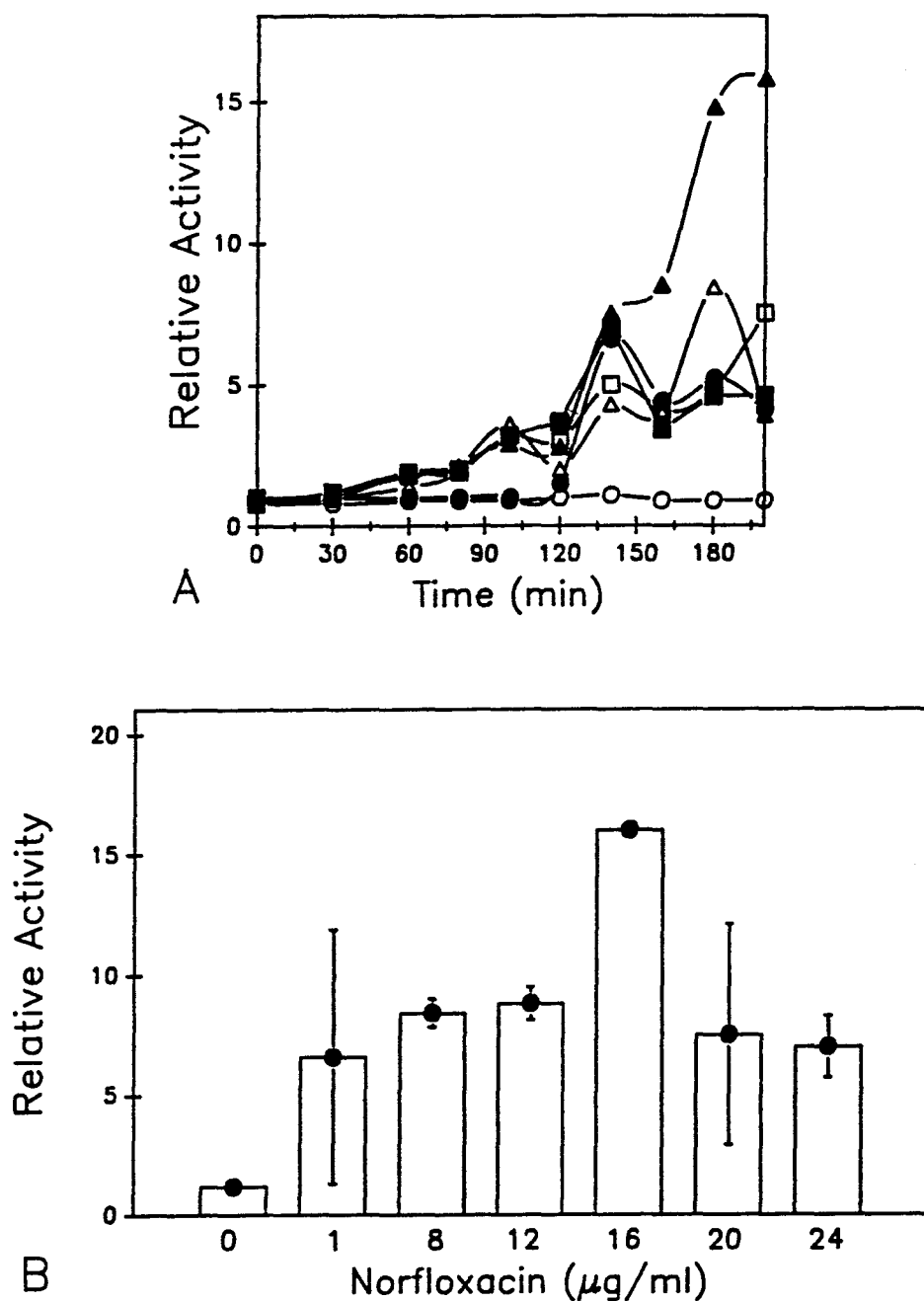
Fig. 10. Norfloxacin inducible behavior of PAO25 (pBML400). (A) Induction profile of quinolone treated cultures: (O) 0 ug/ml; (●) 1 ug/ml; (□) 8 ug/ml; (■) 12 ug/ml; (▲) 16 ug/ml; (△) 20 ug/ml. The untreated culture contained around 60 units of β-galactosidase, and the highest level of activity observed was 1100 units. (B) Dose response of quinolone treated cultures. Plotted values reflect maximum induction observed within 3 h following addition of norfloxacin. All assays were carried out in duplicate, and with the exception of the 16 ug/ml dose, were repeated.

response of sinA::lacZYA, induction was submaximal at norfloxacin concentrations greater than or less than the optimal dose. The induction profile of a norfloxacin treated culture of PAO25 (pBML400) was very similar to a UVC irradiated culture. However, the sinA promoter appeared to respond slightly less strongly to a norfloxacin as compared to UVC challenge, and a maximum induction value of about 16-fold was obtained as a consequence of norfloxacin exposure.

Construction of sin::lacZYA deletion subclones and analysis of inducibility

An attempt was made to localize cis-acting sequences important in the regulation of sin promoters. This attempt entailed subcloning sin::lacZYA fusions to obtain the smallest gene fusion containing fragments. The response of subclones to UVC irradiation was monitored using MacConkey agar, or by direct assay of β-galactosidase expression (Figure 11). pBML4010 was the smallest inducible sinA::lacZYA subclone obtained. The sinA promoter is located in pBML4010 in a 2.0 kb span of DNA immediately preceding the reporter gene. The sinB promoter is located in the smallest obtained inducible subclone pBML7300 in an approximate 5 kb span of DNA upstream of the Tn3-HoHoI insertion. Comparison of the pBML900 subclones pBML9090 and pBML9011 initially suggested that the sinC promoter was contained within a BamHI-HindIII fragment less than 1 kb in size located about 7 kb upstream of Tn3-lacZYA coding sequences. However, further examination of pBML9011 (as discussed in Appendix B) revealed the presence of a deletion in the region preceding the reporter gene. Based upon examination of this deletion, the sinC promoter appears to be located between the upstream HindIII and BglII sites, in a span of DNA less than 2 kb in size.
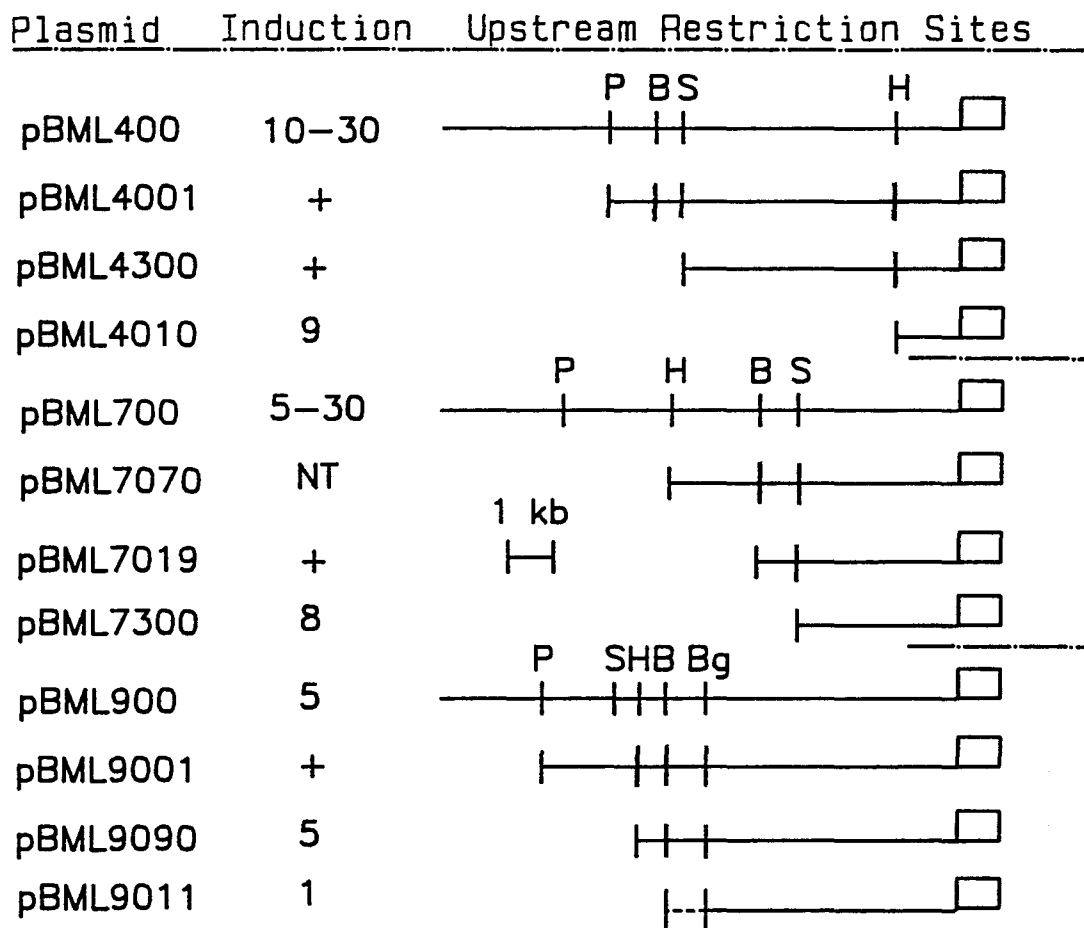
Fig. 11. Localization of <u>sin</u> promoter regions through subcloning <u>sin::lacZYA</u> fusions. Subclones were tested on MacConkey agar or assayed for UVC-mediated increases in β-galactosidase expression: + = UV induction observed by agar screening; NT = induction not tested; P = <u>Pst</u>I; B = <u>Bam</u>HI; H = <u>Hind</u>III; S = <u>Sal</u>I; Bg = <u>Bgl</u>II; rectangle = start of Tn<u>3</u>-HoHoI sequences; stippled line = approximate boundaries of pBML9011 deletion. Numbers indicate assay fold induction values. Only restriction sites up to 12 kb upstream of Tn<u>3</u>-HoHoI are presented. <u>Bgl</u>II sites are indicated only for pBML900 and its derivatives. pBML9090 was examined in strain OT684, and all other plasmids were tested in PAO25. pBML9011 (and possibly pBML4001) appears to be unstable.

Tn501 mutagenesis of pBML4010, pBML7019, and pBML9090

Since pBML4010, pBML7019, and pBML9090 represented the smallest, best

mapped subclones, these plasmids were Tn501 mutagenized (13, 74) to further localize

sin promoter regions. Greater than 100 colonies each of PAO25 (pBML4010), PAO25

(pBML7019), and OT684 (pBML9090) containing Tn501 insertions were obtained and

tested on MacConkey agar. Insertions were scored as Ind⁻ if they abolished UV

responsive behavior, and Ind⁺ if they resulted in no change in UVC-mediated

induction. For pBML7019, Ind⁻ insertions identified were all located in lacZYA

coding sequences, and Ind⁺ insertions were not examined. In the case of pBML9090,

Ind⁺ insertions in the promoter region were not obtained. Four different types of Ind⁻

insertions were identified outside of lacZYA sequences. These Tn501 insertions

subsequently excised from pBML9090. Tn5 has been demonstrated to undergo

excision with a frequency dependent upon the location of the insertion, the type of

extrachromosomal replicon, and the host cell genotype (15).

In contrast to results obtained with pBML7019 and pBML9090, pBML4010

was successfully Tn501 mutagenized. Both Ind⁻ and Ind⁺ insertions in the region of

the promoter were obtained (Figure 12). All insertions were clustered within 500-800

bp upstream of Tn3-lacZYA coding sequences. While Ind⁻ insertions lie close to the

Tn3-HoHoI end, Ind⁺ insertions are clustered slightly farther upstream. These

experiments localize the sinA promoter to a span of DNA less than 300 bp.

Deletion of the sinA promoter region

Exonuclease III/nuclease SI digestion of the sinA subclone pBML4240 yielded

deletions from 0.2 to 2 kb in size extending into or completely removing the sinA

promoter. A few of the deletions generated are diagrammed in Figure 13. Expression
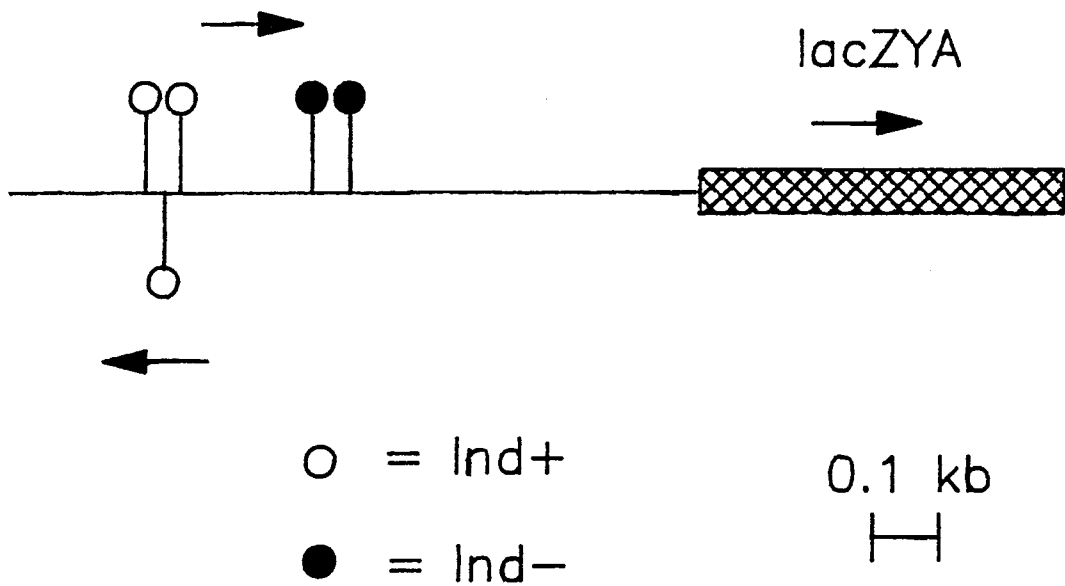
Fig. 12. Tn501 insertion mutagenesis of pBML4010. The locations of Ind⁻ and Ind⁺ insertions of Tn501 are indicated: arrow = direction of Tn501 transcription. Ind⁺ insertions demonstrating the same direction of transcription as sinA::lacZYA are in the plasmids designated pBML4012 and pBML4013. The Ind⁺ insertion demonstrating the opposite orientation is in pBML4011. Ind⁻ insertions are in plasmids pBML4018 and pBML4019.
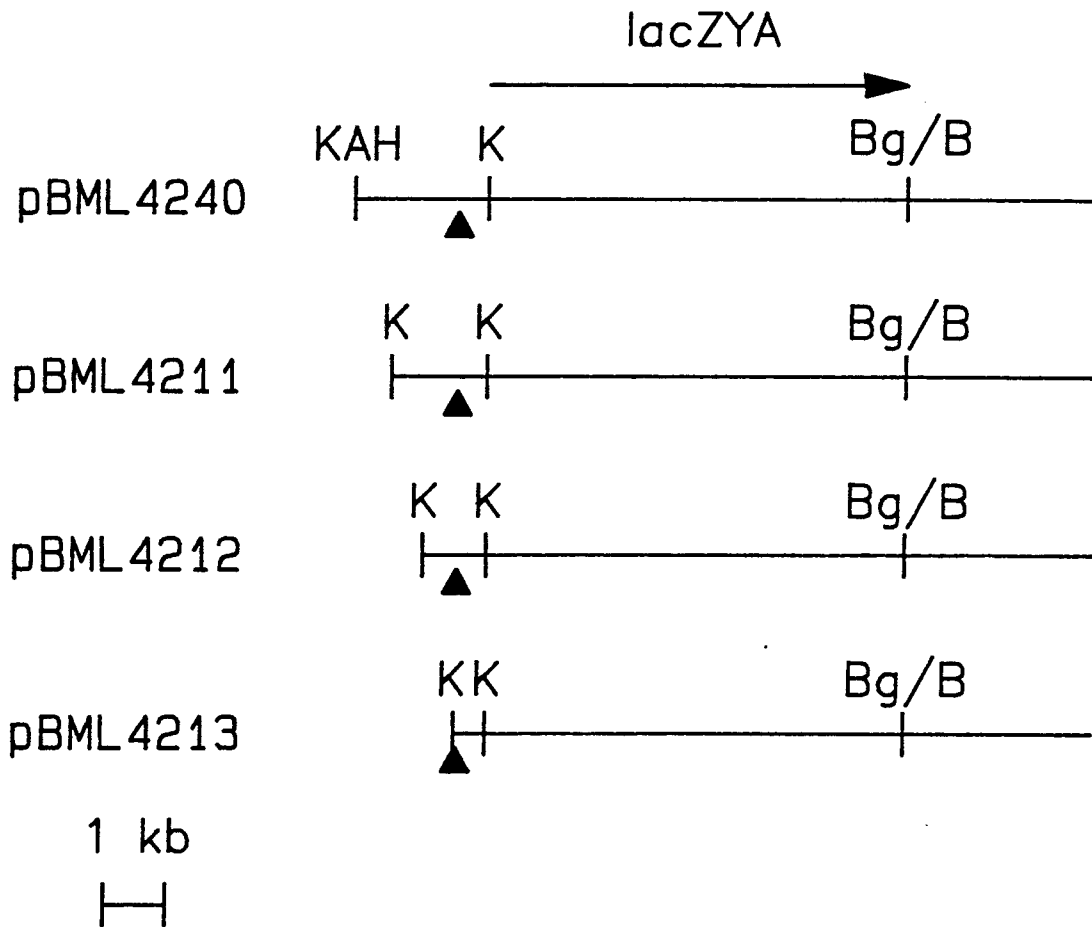
Fig. 13. Restriction maps of some deletion derivatives of pBML4240. Subclones obtained by Exonuclease III/Nuclease SI digestion followed by religation of pBML4240 are diagrammed: filled triangle = approximate start of the Tn3-HoHoI sequences; A = ApaI; H = HindIII; K = KpnI; Bg = BglII; B = BamHI. The sinA::lacZYA subclones possess identical 3' termini, but differ in their 5' termini.

of some of the deleted subclones was examined on MacConkey agar in the RecA⁺ E. coli strain AB1157. While pBluescript SK, pBML4212 and pBML4213 did not appear to express β-galactosidase, colonies containing pBML4240 and pBML4211 demonstrated red colony color on MacConkey agar. These results suggest that the sinA promoter is able to drive lacZYA expression in pBML4240 and pBML4211, and that the deletions present in pBML4212 and pBML4213 have removed portions of the sinA promoter critical for expression. The results are in agreement with localization of the sinA promoter by Tn501 mutagenesis.

Discussion

When present in RecA⁺ P. aeruginosa strain PAO25, β-galactosidase expression of sinA, sinB, and sinC gene fusions does not increase when the cultures are abruptly transferred from 37°C to 45°C incubation. Since P. aeruginosa heat shock proteins have been shown to increase within 15 minutes following such a temperature shift (2), these experiments suggest that sinA, sinB, and sinC are not regulated as part of the heat shock network.

In contrast, all three sin fusions are clearly induced upon exposure to UVC radiation. The sinA, sinB, and sinC gene fusions all appear to exhibit their maximal inductive response at about the same level of UVC exposure. For sinA::lacZYA and sinC::lacZYA, induction is maximal following exposure to 20 J/m², and is decreased following exposure to higher or lower UV doses. For sinB::lacZYA, exposure to a UVC dose less than 20 J/m² resulted in submaximal induction, and exposure to higher UVC doses was not investigated. This optimal dose of 20 J/m² is comparable to UV doses required for stress responsive gene expression in E. coli. E. coli SOS genes

have been shown to be maximally induced upon exposure to UVC doses ranging from 5 to 60 J/m^2 (160, 203). It is not clear how the optimal dose for P. aeruginosa sin gene expression relates to the optimal dose for P. aeruginosa recA induction since experiments demonstrating recA induction in response to UVC radiation have used doses of 0, 5, 10, 30, but not 20 J/m^2 (69, 124). However, fivefold induction of recA is observed following doses of 10 and 30 J/m^2, and is not sustained after a lower UVC dose (69).

While the three fusions demonstrate their maximal inductive response at about the same dose of UVC radiation, they differ in the kinetics and magnitude of the inductive response. In this respect, P. aeruginosa sin genes are similar to E. coli SOS genes (200), which demonstrate magnitudes of induction ranging from less than 5-fold (160) to greater than 150-fold (157). The sinA and sinB gene fusions exhibit 20 to 30-fold induction following UVC exposure, whereas sinC::lacZYA is induced only about 4-fold. Stress responsive expression of the sinA, sinB, and sinC gene fusions occurs between 120-160 minutes, between 160-210 minutes, and between 210-270 minutes, respectively, subsequent to UVC exposure.

The multimodal induction profiles observed for the three gene fusions may not be unusual for P. aeruginosa gene expression. Several genes including lasB, toxA (72), and possibly recA (69) exhibit biphasic expression patterns. Expression of some E. coli SOS genes including umuD (117) returns to baseline relatively quickly after stress exposure, and it has not been shown whether this is in fact followed by a second inductive peak. Furthermore, it is possible that the induction profile of sin gene fusions may be altered due to the expression of wild type sin genes. Such a situation has in fact been reported for stress responsive expression of uvrB (160).

It is interesting that the <u>sin</u> inductive peaks occur with about the same periodicity as the DNA replication cycle. Further experiments are needed to verify whether <u>sin</u> genes are possibly involved in the recovery of DNA synthesis, or whether <u>sin</u> induction is related to the DNA replication cycle.

Induction of <u>sinA::lacZYA</u> in response to the stress agent norfloxacin was investigated. Quinolones such as norfloxacin exert a very different type of stress upon the cell than UVC radiation. Whereas UVC radiation primarily results in pyrimidine dimers, norfloxacin inhibits DNA synthesis and causes DNA double stranded breaks (167). Despite this difference in mechanism of action, significant induction was observed with norfloxacin doses ranging from 1-25 ug/ml. While the magnitude of induction in response to norfloxacin treatment was slightly reduced when compared to the UVC-mediated induction profile, the kinetics of induction were almost identical. Induction was maximal at a norfloxacin concentration between 15-19 ug/ml. This concentration corresponds to the minimum bactericidal concentration (MBC) and is about 40 times greater than the minimal inhibitory concentration (MIC) for a norfloxacin sensitive strain of <u>P. aeruginosa</u> (12). This concentration-dependence is similar to <u>E. coli</u>, as <u>E. coli</u> SOS genes exhibit their maximal inductive response upon exposure to a norfloxacin concentration corresponding to 20-40 times the MIC (146, 147).

In order to initiate a study of <u>P. aeruginosa</u> stress responsive promoters, an attempt was made to localize <u>sin</u> promoters by subcloning successively smaller <u>sin::lacZYA</u> containing fragments, and testing subclones for UVC responsive behavior. Using this approach, the <u>sinB</u> promoter was roughly localized to within 5 kb upstream of the Tn<u>3</u>-HoHoI insertion. The <u>sinC</u> promoter was found to reside within a region

of DNA less than 2 kb occurring about 7 kb upstream of the reporter gene. This rather large span of DNA separating the promoter from the reporter gene may suggest sinC::lacZYA is a translational fusion, or part of an operon. The sinA promoter was localized to within 2 kb upstream of the reporter gene. Exonuclease III/nuclease SI deletion analysis and Tn501 mutagenesis of sinA::lacZYA identified the promoter within a 300 bp region of DNA occurring 500-800 bp upstream of Tn3-lacZYA.

These results clearly demonstrate that sinA, sinB, and sinC resemble E. coli SOS genes in some aspects of their response to UVC radiation and quinolones. The results are significant because they suggest sin genes could potentially represent analogues of E. coli SOS recombination and repair genes. To further investigate this issue, studies determining whether the chromosomal location of sin genes correspond to the known location of mutations altering P. aeruginosa recombination and repair were performed and are described in the following Chapter.

CHAPTER V

DEFINING THE CHROMOSOMAL LOCATION OF sinA, sinB, and sinC

Combined investigative efforts have resulted in a relatively well defined map of the P.aeruginosa chromosome. Mutations which affect repair and recombination in P. aeruginosa have been studied (94, 127, 158), and several of the genes involved have been mapped on the bacterial chromosome (Figure 14). Therefore the chromosomal map locations of sinA, sinB and sinC could provide clues to the function of the corresponding stress responsive gene products. Approximate map locations of the stress inducible genes were obtained by hybridizing sin promoter-containing probes to very large chromosomal fragments separated by pulsed field gel electrophoresis. Using this approach, the sinA, sinB, and sinC genes were localized to a 200 kb region within 34.6 and 37.4 minutes on the Holloway map (67) of the P. aeruginosa chromosome (U. Romling and B. Tummler, personal communication).

The chromosomal region to which the sin genes have been mapped also contains a cluster of bacteriocin genes encoding pyocin R2. These bacteriocin genes are inserted between the chromosomal markers trpGDC and trpE (173). Bacteriocins are conventionally defined as proteins or protein complexes which kill sensitive bacteria of the same or closely related species as the bacteriocin producing strain (80). While producing strains are normally immune to the effect of their own bacteriocins, expression of bacteriocin is lethal for the producer. Bacteriocidal activity requires
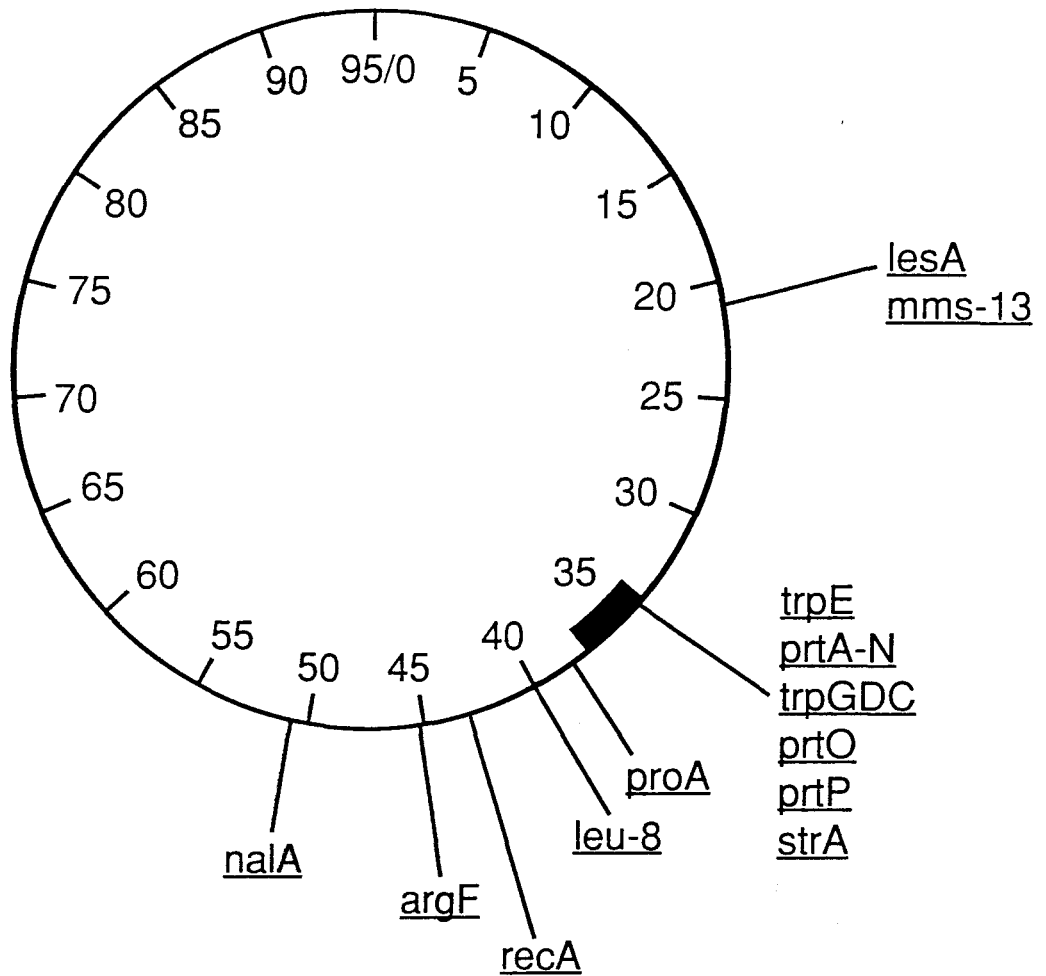
Fig. 14. Chromosomal map of P. aeruginosa PAO. The locations of markers relevant to this study are shown: rectangle = chromosomal region to which sinA, sinB, and sinC have been mapped.

adsorption of the bacteriocin to specific receptors present on the outer membrane of susceptible cells.

The term bacteriocin has been used to describe bacteriocidal proteins or complexes observed in a variety of bacteria. In contrast, pyocins are bacteriocins which are produced by P. aeruginosa. Most strains of P. aeruginosa produce pyocins which can be categorized into one of three classes: types S, F, or R (79). While S-type pyocins are smaller, simpler, and proteinase susceptible protein complexes, the latter two pyocins are much larger protein complexes with a structure similar to bacteriophage contractile tails (170, 172). One R-type pyocin has been shown to consist of more than 400 molecules with about 20 different types of protein (171). At least five R-type pyocins have been discovered in P. aeruginosa which are morphologically very similar, but can be distinguished based upon range of sensitive strains (79). It is conceivable that sin gene fusions could be Tn3-HoHoI insertions into pyocin R2 genes, as pyocin R2 is induced upon exposure to the stress agents UVC radiation and mitomycin C (79). This stress responsive behavior of pyocins is not surprising since analogous E. coli bacteriocin genes are also stress inducible and have been demonstrated to be under recA and lexA control (157).

Genetic techniques, complementation experiments, and hybridization studies were used in an attempt to more closely define the chromosomal location of sin genes, and to determine whether sin::lacZYA fusions contain the reporter gene linked to pyocin R2 promoters.

Materials and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 6.

Culture conditions and chemicals

Unless indicated otherwise, bacteria were incubated at 37⁰C. Lambda top agar used in detection of pyocin expression contained 1% Bacto-tryptone, 1% NaCl, 0.5% Bacto-yeast extract, and 0.7% Bacto-agar. Bacto-tryptone, Bacto-yeast extract and Bacto-agar were purchased from Difco. When required for selection in P. aeruginosa, streptomycin and rifampicin were used at concentrations of 500 and 200 ug/ml, respectively. PMM plates used to test chromosomal markers were solidified with 1.2% Bacto-agar, and were supplemented with 0.4% glucose and final amino acid concentrations of 25-50 ug/ml. All other media, antibiotics, and chemicals were as described in previous Chapters.

Isolation of chromosomal DNA

Chromosomal DNA was isolated according to the method of Marmur (115). Yield of chromosomal DNA was quantitated spectrophotometrically using a Gilford Response UV-Vis Spectrophotometer.

Triparental matings

Triparental matings were performed as indicated in Chapter III with the exception that in cases where transconjugation was not initially obtained, the effect of mating different volumes of donor, conjugation helper, and recipient in ratios other than 1:1:1 was investigated.

Table 6.--Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
P. aeruginosa		
PAO1	prototroph	66
PAO25	argF10 leu-10	54
PAO125	argB22 ilvD230 leu-8 proB77 trpA61 pur-136	B.Holloway
PAO3602	met-28 trpC6 nal-302 rec-2	T.Shinomiya
OT684	leu-1 lys-1 res-4	150
RM6	his lys met trpC	R.V.Miller
RM8	lesB908 argB21	126
PAO303	argB21	126
RM14	his-1 lys-12 met-28 trpC6 strA	R.V.Miller
RM43	trpD	R.V.Miller
RM174	argB22 ilvD230 leu-8 proB77 trpA61 pur-136 nal-950	Nal[r] PAO125
RM203	his-4 ilv-226 lys-12 met-28 proA82 trp-6	41
RM241	argF10 nalA2	153
RM297	str-990 isolate of PAO25	This study
E. coli		
HB101	proA2 leuB6 thi-1 galK lacY1 hsdR hsdM recA13 supE44 rpsL20 xyl mtl	19
Plasmids		
pRK2013	pBR322 cointegrate plasmid carrying the mob and tra plasmid transfer functions of RK2; Km[r]	49
pKT279	ColEI replicon plasmid derived from deletion of a portion of the bla gene of pBR322; Ap[r], Tc[r]	184

(Table 6.--Continued)

Strain or plasmid	Relevant characteristics[a]	Source or reference
pBR325	ColEI replicon plasmid derived from the insertion of the Cm^r gene into pBR322 Ap^r, Tc^r, Cm^r	19
pNM16	R-prime plasmid containing argC trpC trpD prtA-J prtK-N and trpE; Km^r	T.Shinomiya
pBML400	sinA::lacZYA fusion present in pCP16; Tc^r, Ap^r	This study
pBML700	sinB::lacZYA fusion present in pCP16; Tc^r, Ap^r	This study
pBML900	sinC::lacZYA fusion present in pCP16; Tc^r, Ap^r	This study
pBML4200	13-14 kb HindIII-BamHI sinA::lacZYA fragment subcloned into pBluescript SK; Ap^r	This study
pBML4399	8-9 kb HindIII-BglII sinA::lacZYA fragment subcloned into the BamHI-HindIII sites of pBR325; Ap^r, Cm^r, Tc^s	This study
pBML7346	3.5 kb BglII-SalI fragment flanking the sinB::lacZYA gene fusion subcloned into the BamHI-SalI sites of pBR325; Ap^r, Cm^r, Tc^s	This study
pBML9090	18-23 kb HindIII sinC::lacZYA fragment ligated into pCP16	This study
pBML9323	at least 15 kb pBML9090 BglII-HindIII fragment downstream of sinC::lacZYA ligated into the BamHI-HindIII sites of pBR325	This study

[a]Nomenclature and abbreviations are essentially those of Demerec et al. (45). Tc^r, Km^r, Ap^r and Cm^r refer to resistance to tetracycline, kanamycin, ampicillin/carbenicillin and chloramphenicol, respectively.

Isolation of a strA mutant

A spontaneous str-990 mutant of strain PAO25 was isolated by growing up PAO25 to a relatively high cell density (at least 10^9 CFU/ml) and plating onto LB agar plates containing streptomycin. Mutants were streaked out twice for isolated colonies onto the same type of selective plates, and sensitivity to other antibiotics was verified. str mutations appear to be recessive (174) to wild-type strA.

Subcloning

Plasmid pKT279 is an ampicillin-sensitive ColEI vector derived from pBR322 by a deletion near the beginning of the β-lactamase gene (184). An attempt was made to subclone the HindIII and SalI transposon-containing fragments of inducible constructs into the HindIII or AvaI sites respectively of pKT279 using the ligation conditions described in Chapter IV. Similarly, the BglII-HindIII transposon--containing fragment of pBML4200, and the BglII- HindIII fragment of subclone pBML9090 lying downstream of sinC::lacZYA were each ligated into the BamHI-HindIII sites of pBR325 to yield pBML4399 (Figure 15) and pBM9323 (Figure 16), respectively. A 3.5 kb BglII-SalI fragment flanking the transposon in pBML700 was subcloned into the BamHI-SalI sites of pBR325 to generate pBML7346. Construction of subclones pBML9090 and pBML4200 is described in Chapter IV.

Pyocin Detection

Expression of pyocins was detected by inoculating a putative pyocin-producing colony into 5 ml LB, and incubating overnight at 37° C. The culture was filtered through a 0.45 um pore size filter, and successive dilutions in LB of the filtered culture were prepared. The diluent along with 10 ul of each of the dilutions was spotted onto LB agar plates overlaid with a lawn of strain PAO1 or PAO25. Lawns
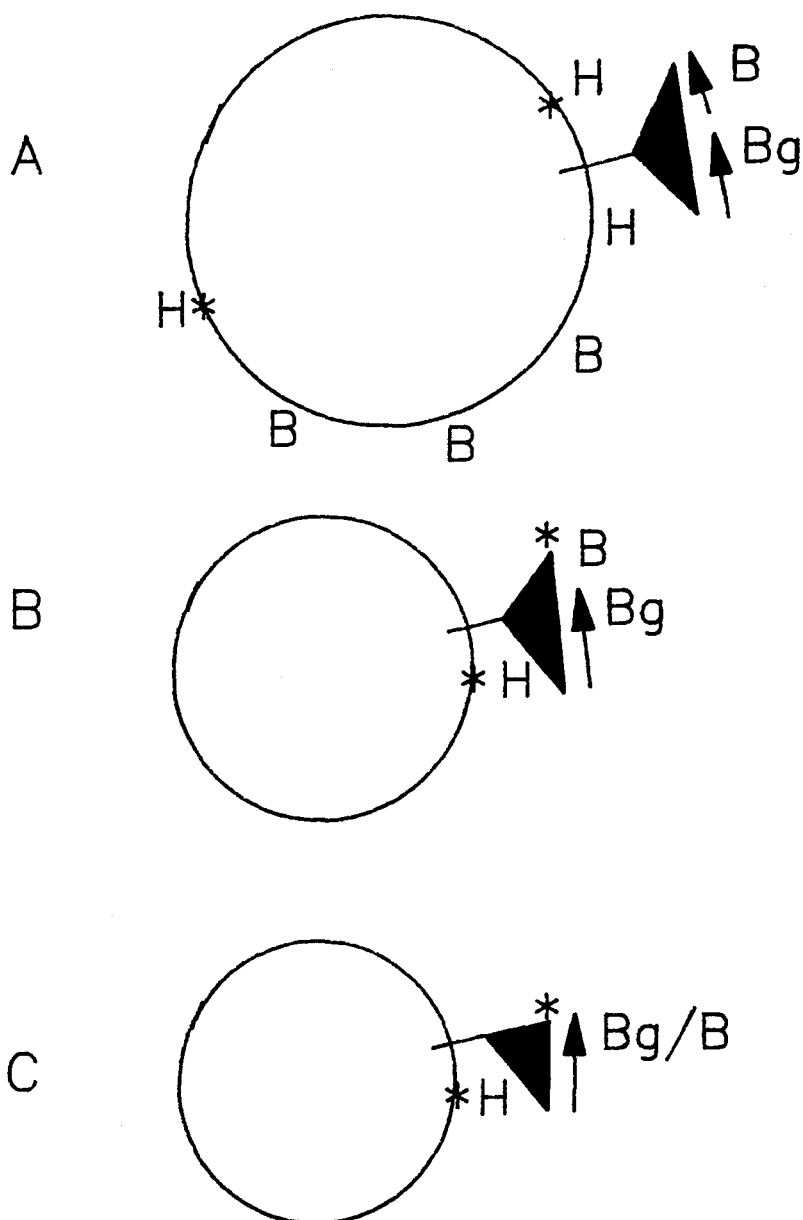
Fig. 15. Construction of pBML4399. (A) Restriction map of pBML400 (55-60 kb). (B) The 14-15 kb HindIII-BamHI sinA::lacZYA fragment of pBML400 was subcloned into the HindIII and BamHI sites of pBluescript SK to generate pBML4200 (17-18 kb). (C) The 8-9 kb HindIII-BglII sinA::lacZYA fragment of pBML4200 was subcloned into the HindIII and BamHI sites of pBR325 to generate pBML4399 (13-14 kb). Symbols: triangle = Tn3-lacZYA; asterisks = chromosomal insert; large arrow = direction of transciption of lacZYA; small arrow = direction of transcription of bla; H = HindIII; B = BamHI; Bg = BglII.
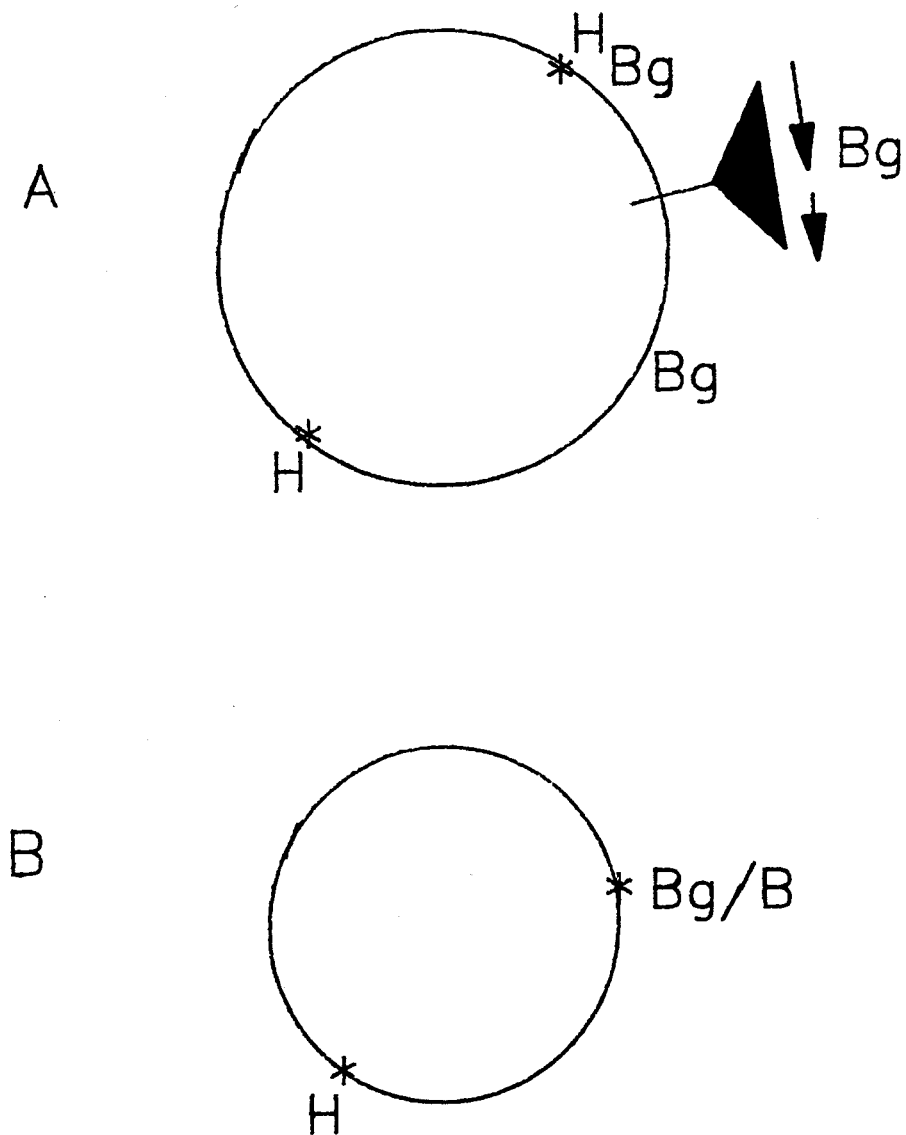
Fig. 16. Construction of pBML9323. (A) pBML9090 was generated by HindIII digestion of pBML900 followed by religation. (B) pBML9323 was constructed by ligating the 17-18 kb BglII-HindIII fragment located downstream of sinC::lacZYA into the BamHI and HindIII sites of pBR325. Symbols: triangle = Tn3-lacZYA; asterisks = chromosomal insert; H = HindIII; B = BamHI; Bg = BglI; S = SalI. The only BamHI site shown is the pBR325 BamHI site in B.

were prepared by inoculating 0.1-0.2 ml of an early exponential phase culture (less than 30 Klett$_{660}$ units) into 2.5 ml of lambda top agar and pouring over an LB agar plate. Cultures were considered to express pyocins or a pyocin-like activity if a visible clearing not containing plaques was evident in the spotted dilutions and not the spotted diluent.

Transformations

Transformation of P. aeruginosa with linear DNA was performed according to the method of Mercer and Loutit (122). Briefly, a total of 10 ml of a fresh LB culture inoculated with an overnight culture of res⁻ strain OT684 grown at 43°C, was incubated with shaking to a cell density of at least 10^8 cells/ml, and maintained on ice for 10 min. Cells were harvested by centrifugation at 5,000 rpm for 10 min at 4°C. The obtained pellet was resuspended in 5 ml of ice cold 150 mM $MgCl_2$ and recentrifuged under the same conditions. The cell pellet resulting from this second centrifugation was resuspended in 5 ml of 150 mM $MgCl_2$ and maintained on ice for 20 min. Centrifugation of this mixture yielded a pellet which was resuspended in 1 ml 150 mM $MgCl_2$. A volume of 0.1 ml competent cells was gently mixed with DNA in a prechilled tube and maintained on ice for 1 h. A variety of different DNA quantities ranging from 20 to 400 ng in a total volume less than 10 ul were used for transformation. DNA was linearized through single digestion with HindIII or sequential digestion with HindIII and SalI, since both enzymes cleave within vector and insert sequences of inducible constructs and yet lack restriction sites within Tn3-lacZYA coding sequences. CIAP was added in the last half hour of the digestion reaction, and dephosphorylation of 5'termini was allowed to proceed an additional 30 min at 37°C. CIAP was removed prior to transformation through

repeated phenol:chloroform extraction or inactivated by heating the reaction mixture to 70°C for 10 min. Following the 1 h incubation of cells and DNA, the mixture was heat shocked by placing in a 37°C water bath for 2 min. The mixture was then chilled on ice for 5 min and 1 ml LB was added. The culture was incubated with extremely gentle shaking at 37°C for 2 h, and plated onto LB agar containing 500 ug/ml carbenicillin to select for chromosomal integration of the gene fusions.

For E. coli strains, the rubidium chloride transformation protocol was used.

<u>Other methods</u>

Restriction endonuclease digestions, isolation of plasmid DNA, preparation of radioactive probes, and hybridizations were performed as described in previous Chapters.

Results

<u>Hybridization of three sin promoters to P. aeruginosa chromosomal fragments</u>

In E. coli, some damage inducible genes including umuDC, ruvAB, recA and recN either constitute operons or are clustered on the bacterial chromosome (200). The possibility of a similar organization for sin genes was investigated by determining the size of the HindIII and PstI-HindIII chromosomal fragments to which sinA, sinB, and sinC promoter probes hybridize (Figure 17) and by investigating ability to cross hybridize. While sinA and sinC hybridized to a HindIII fragment of about 6 to 6.5 kb in size, sinB detected a slightly larger chromosomal fragment of about 7 to 7.5 kb. The size difference of the sinB hybridizing fragment was verified by hybridizing freshly prepared sinA and sinB probes to HindIII chromosomal fragments in a completely independent experiment. The HindIII fragment detected by
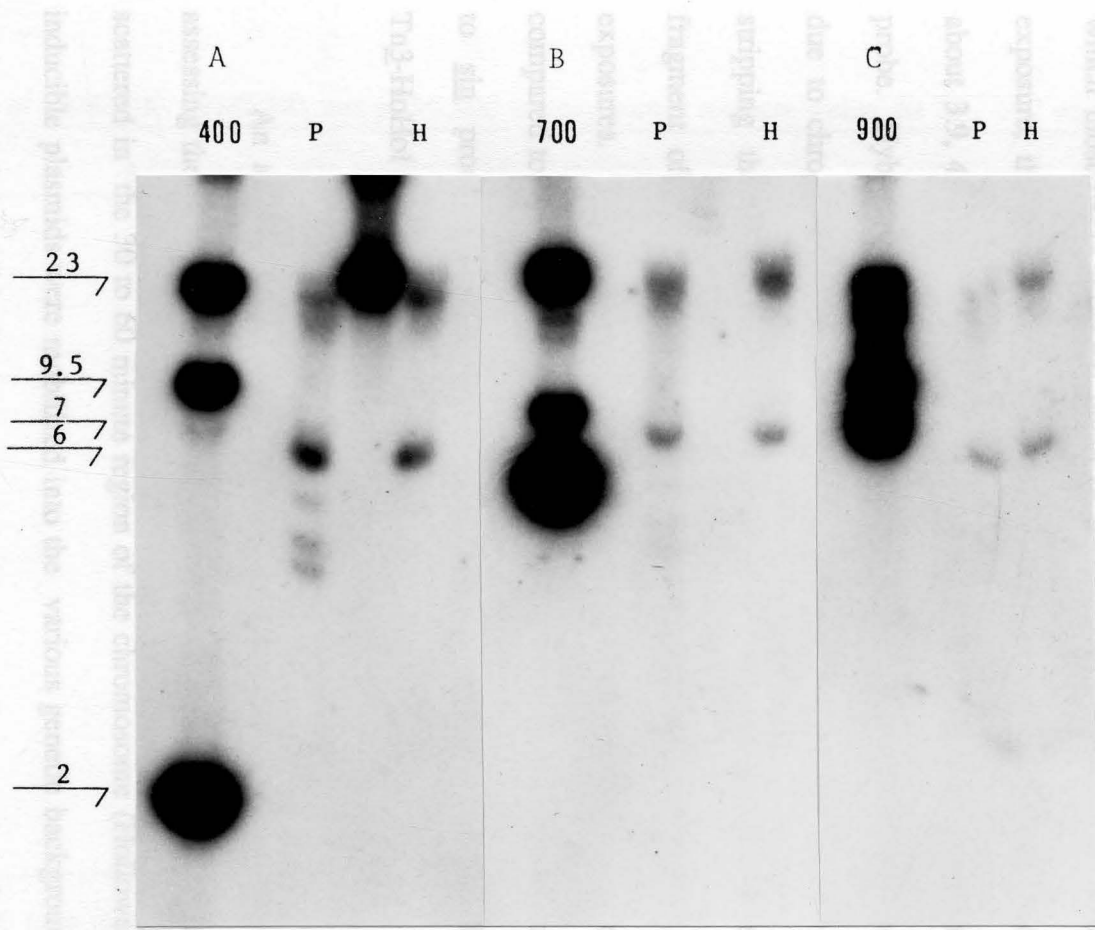
Fig. 17. Hybridization of sinA, sinB, and sinC promoter-containing fragments to the P. aeruginosa chromosome. Autoradiograms of Southern blots following hybridization to sin probes are shown. (A) sinA probed blot, (B) sinB probed blot, (C) sinC probed blot. Symbols: 400 = pBML400; 700 = pBML700; 900 = pBML900; P = PstI-HindIII digest of RM9 chromosomal DNA; H = HindIII digest of RM9 chromosomal DNA. The 5.0 kb SalI-KpnI fragment of pBML700, and the 2.1 kb and 8.0 kb HindIII-KpnI fragments of pBML400 and pBML900, respectively, were used as probes.

the sinC probe appeared to possess an internal PstI site located about 500 bp from the downstream HindIII site, since the HindIII-PstI fragment detected by the sinC probe was about 500 bp smaller than the hybridizing HindIII fragment. The probes hybridized less strongly to a higher molecular weight fragment(s) of about 18 - 23 kb which most likely reflected uncut or partially cut chromosomal DNA. With longer exposure, the sinA probe additionally detected 3 PstI or PstI-HindIII fragments of about 3.9, 4.2, and 5.0 kb which may share some sequence homology with the sinA probe. Hybridization of all sin probes to the chromosomal fragments appeared to be due to chromosomal and not transposon elements present within the probes, since stripping the filters and reprobing with the labeled BglII transposon-containing fragment of pBML7 failed to yield genomic hybridization after even very long exposures. Hybridization of the same probes to sin::lacZYA containing plasmids as compared to hybridization of the BglII pBML7 transposon probe revealed hybridization to sin promoter-containing sequences which appeared to be due to upstream Tn3-HoHoI sequences present in each sin probe.

Complementation studies

An attempt was made to further define the map locations of sin genes by assessing the ability of pBML400, pBML700, and pBML900 to complement mutations scattered in the 30 to 60 minute region of the chromosome (Holloway map;67). The inducible plasmids were mobilized into the various genetic backgrounds by triparental matings, and established strains were plated onto different types of marker test media. Despite repeated attempts, it was not possible to establish pBML900 in some of the different genetic backgrounds. Chromosomal inserts present in pBML400, pBML700, and pBML900 were incapable of complementing the markers tested (Table 7). These

Table 7.--Inability of pBML400, pBML700, and pBML900 to complement markers within the 30 - 60 ' region of the chromosome

Marker	Approximate map location[a] (minutes)	Constructs tested[b]
trpC	33-36	pBML400, pBML700
trpD	33-36	pBML400, pBML700, pBML900
strA	33-36	pBML400, pBML700, pBML900
proA	36-40	pBML400, pBML700
leu-8	40-43	pBML400, pBML700
argF	43-45	pBML400, pBML700
nalA	50-55	pBML400, pBML700

[a] Holloway genetic map (67).
[b] For pBML400 and pBML700, approximately 20 colonies of each of the different transconjugants were tested for complementation. For matings involving pBML900, less than 20 transconjugants were obtained and tested.

results could indicate that the complementing genes are not present within the cloned inserts of pBML400, pBML700, and pBML900, or that Tn3-HoHoI has inserted within the complementing genes. Alternatively, the initial maintenance of sin constructs in a RecA⁺ P. aeruginosa host may have caused rearrangements, deletions or other recombinational events resulting in an impaired complementation ability.

Lack of specific hybridization of the three sin promoters to R'
plasmids bearing pyocin R2 genes

R' plasmids containing large PAO25 chromosomal inserts spanning the 33-36 minute region of the chromosome were isolated by Shinomiya et al. to facilitate analysis of pyocin R2 genes located between trpGDC and trpC (174). To verify whether sin genes are also found within this region, sin promoter-containing probes were hybridized to the R' plasmid pNM16 (Figure 18). The three sin probes hybridized faintly to the top four bands of HindIII digested pNM16. These bands corresponded to noncontiguous chromosomal fragments of 24, 16, and 14 kb in size, and to a 60-70 kb vector band. To investigate the possibility of the observed pNM16 hybridization being mediated by transposon sequences present in sin probes, one of the filters was stripped of all radioactivity and reprobed with the pBML7 BglII transposon probe. The pBML7 probe detected the same pNM16 chromosomal fragments as did the three sin probes.

ColEI constructs for replacement of intact chromosomal genes
with sin::lacZYA fusions are unstable

In gene mapping studies, chromosomal genes can be replaced by transposon inactivated versions and gene fusions can be integrated into the bacterial chromosome to provide markers for chromosomal mapping. Replacement of chromosomal sin

A

HindIII sites

2.4   24   6.1   16   8   9.9 2.5 14

BamHI sites

4.5   21   24   11   19   3

B

Chromosomal Genes

strA   argC trpGCD prtA-J trpE
rifA   prtO   prtK-N
prtP

C

Chromosomal Location

36'   33'

Fig. 18. Chromosomal region contained within the R' plasmid pNM16. (A) pNM16 HindIII and BamHI restriction sites. Fragment sizes (kb) are indicated. (B) Chromosomal genes contained within pNM16. (C) Approximate map locations (min) of chromosomal genes. This figure was modified from Shinomiya et al. (174).

genes with sin::lacZYA fusions, or integration of the reporter gene fusions into the chromosome can be accomplished by subcloning gene fusions into a ColEI vector such as pKT279, and mobilizing the resulting plasmids into P. aeruginosa. Since ColEI plasmids are incapable of replicating in P. aeruginosa, selection for transposon-encoded carbenicillin resistance allows selection for single recombinational events forming a chromosome-plasmid cointegrate, or double recombinational events replacing the intact chromosomal gene with the transposon-inactivated version. While single crossover events occur more frequently within a cell, techniques are available which allow direct selection for the loss of vector sequences and resolution of the cointegrate structure by a second recombinational event (59).

Several attempts were made to subclone gene fusions into the ampi-cillin-sensitive vector pKT279 to generate plasmids appropriate for gene replacement. For homologous recombination to occur at both ends of the transposon, these subclones needed to contain the gene fusions as well as sequences occurring both upstream and downstream of Tn3-lacZYA. The enzymes SalI and HindIII lack restriction sites within transposon coding sequences, and cleave gene fusion--containing fragments of at least 18 kb in size from pBML400, pBML700, and pBML900. SalI and HindIII digests of pBML400, pBML700, and PBML900 were ligated to alkaline phosphatase treated AvaI and HindIII digests of pKT279 in five independent experiments. Initial selection for transformants was made using either ampicillin, which allows selection for the transposon-encoded β-lactamase, tetra-cycline, which selects for vector pKT279, or both ampicillin-containing and tetracycline-containing plates, since HindIII subcloning into pKT279 results in variable

tetracycline sensitivity (18). Putative subclones were then replica plated or patched onto plates containing the appropriate antibiotic.

Transformants obtained ranged between 20 to over 1000 in the separate experiments. Three distinct types of transformants could be recognized: colonies which had been transformed with undigested pBML400, pBML700, or pBML900 DNA; subclones in which transposon-containing fragments had ligated back into pCP16 instead of pKT279; and transformants possessing pKT279 ligated to a HindIII or SalI fragment which did not correspond to any HindIII or SalI fragments of pBML400, pBML700, or pBML900. During the course of analyzing this third class of transformants, it became apparent that antibiotic resistance and digestion patterns had changed and were continuing to change with repeated subculturing. The most likely explanation of these experimental results is that large HindIII and SalI gene fusion-containing fragments are unstable in E. coli HB101 when subcloned into the pKT279 vector.

Attempts to integrate the sinA::lacZYA gene fusion
into the chromosome appear to result in pyocin induction

While replacement of sinA, sinB, and sinC chromosomal genes requires that sequences including and flanking the reporter gene be subcloned into a ColEI vector, integration of the gene fusions can be accomplished through recombination with a single homologous chromosomal site, and requires subcloning of sequences either upstream or downstream of Tn3-lacZYA. An attempt was made to subclone promoter-containing fragments of pBML400, pBML700, and pBML900 including the beginning portion of Tn3-lacZYA into plasmid pBR325. Only in the case of pBML400 was this attempt successful. The plasmid pBML4399 (Figure 15) is a

carbenicillin resistant, tetracycline sensitive pBR325 subclone which contains the sinA:lacZYA gene fusion.

In several different experiments, HB101 (pBML4399) along with the negative and positive controls HB101 (pBR325) and HB101 (pCP16) were mated with the P. aeruginosa recipients PAO1, PAO25, and PAO303 in triparental matings. The frequency of mobilization of pCP16 into the various strains was at least 10 transconjugants/10^6 CFU, whereas transconjugants were not obtained for HB101 (pBR325). In matings involving HB101 (pBML4399), colonies were obtained at about the frequency expected for single crossover events (0.1-1.0 trans-conjugants/10^8 CFU; 41, 190). These transconjugants were viable when patched onto LB agar, but were incapable of growth in the presence of carbenicillin, despite the fact that the transconjugants were obtained following overnight growth on carbeni-cillin-containing plates. Many of the colonies appeared to be surrounded by a halo of clearing. When tested, these transconjugants were found to express a pyocin or pyocin-like activity absent from overnight cultures of PAO303, PAO25, and PAO25 (pBML400), and effective against strains PAO25 and PAO1. In three independent experiments, pyocin activity was detected in transconjugants resulting from pBML4399 mobilization. Since pyocin expression is lethal for the producing strain, an attempt was made to select for a compensatory tol mutation conferring tolerance to this expression by allowing triparental mating mixtures an additional 15 h of growth under nonselective conditions before plating onto selective medium. This attempt failed to yield transconjugants.

The possibility that the Tn3-HoHoI upstream end present in pBML4399 might interfere with recombination, or somehow mediate the observed pyocin induction was

investigated by including the additional donor strains HB101 (pBML7346) and HB101 (pBML9323) in mating experiments. These donors possessed random chromosomal segments of pBML700 and pBML900 not associated with transposon sequences ligated into pBR325. The mating involving HB101 (pBML7346), but not the mating involving HB101 (pBML9323) resulted in transconjugant expression of a pyocin-like activity.

Gene replacement is not observed upon transformation of a mutant P.aeruginosa strain with linearized sin::lacZYA cosmids

The P. aeruginosa strain OT684 possesses a res mutation which renders it unable to degrade linear duplex DNA (149). The res mutation abolishes expression of a deoxyribonuclease, and also allows OT684 to uptake and recombine linear, double stranded DNA with a frequency of 2-3 transformants /10^7 CFU (122). Two separate attempts were made to transform OT684 with HindIII or HindIII-SalI digested pBML400, pBML700 and pBML900 DNA. While competent OT684 was transformed with closed circular duplex DNA with an efficiency of about 14 transformants/10^7 CFU, transformation with linear duplex DNA resulting in replacement of the chromosomal gene with the inactive reporter gene fusion was not observed. In fact, the only colonies obtained following transformation with the linearized DNA contained closed circular cosmid DNA which had escaped restriction enzyme cleavage.

Discussion

Pulsed field gel electrophoresis mapping indicates that sinA, sinB, and sinC are located within a 200 kb region at 34.6 to 37.4 minutes on the P. aeruginosa chromosome. These results are in agreement with experimental results from Chapter III, which indicate that sin gene fusions are not recA fusions. The results also suggest a reason why pBML400 restriction patterns resemble those of pBML700. Most likely, sinA and sinB are separated by a large HindIII fragment of about 20 kb. This intervening fragment could be present in both pBML400 and pBML700, along with the adjoining sinA-containing fragment in pBML400, and with the contiguous sinB-containing fragment in pBML700.

Probes generated from sinA and sinB hybridize to different size HindIII chromosomal fragments. The sinC probe hybridizes to a HindIII fragment of the same size as the sinA hybridizing fragment, but which differs from this fragment in that it appears to possess an internal PstI site. These results suggest that the sinA, sinB, and sinC gene fusions are Tn3-HoHoI insertions in different HindIII chromosomal fragments. Furthermore, promoter regions of the three sin genes have been found to occur downstream of a HindIII site (Chapter IV), and restriction sites present upstream of the Tn3-HoHoI insertion are different in the sinA, sinB, and sinC plasmids. The only explanation compatible with these results is that sinA::lacZYA, sinB::lacZYA, and sinC::lacZYA are Tn3::lacZYA insertions in independent stress responsive genes. In cross hybridization studies, sin probes do not appear to hybridize to fragments upstream or downstream of the fragments used as probes. This

result indicates that sin genes are ≈ not located within less than 2 kb of each other on the bacterial chromosome.

An attempt was made to further define the map location of sin genes by analyzing the ability of sinA-, ≈ sinB- and sinC-containing plasmids to complement mutations in the 30 to 60 minmute region of the chromosome. The sin gene containing plasmids were incapatible of complementing the mutations, which most likely indicates that the wild- typpe alleles of the genes examined are not present in the chromosomal insert of the plz_asmids.

The chromosomal region tc⊃ which the three sin genes have been mapped also contains a cluster of pyocin R2 g≈enes located between the trpGDC and trpE genes (173). R2 pyocins as well as other pyocin genes are inducible by UVC and mitomycin C exposure (79). The ≈ possibility that sin::lacZYA constructs contain the reporter gene fused to pyocin R2 promoters, or contain pyocin R2 genes within their cloned inserts was investigated by ▾ hybridizing sin probes to a R' plasmid containing pyocin R2 genes. Faint hybridiza▪tion of the three sin probes to R' HindIII fragments of 24, 16, and 14 kb in size was observed. This hybridization was probably due to homology with transposon seque⊃nces present in each probe, since a BglII pBML7 transposon probe also hybridized f≈aintly with the same fragments. This interpretation is supported by additional evide⊃nce. First of all, the 14 kb hybridizing fragment appears to be located earlier upon the chromosome than 34 minutes (Figure 18, Panel C), and is outside of the region to which sinA, sinB and sinC have been mapped. Second, the genomic hybridizatio●n of sin probes (Figure 17) suggests that sin genes are located on HindIII chromosormal fragments which are considerably smaller than the large hybridizing pNM16 fra≈ments. Third, BamHI restriction patterns of the 24

and 16 kb fragments and flanking HindIII segments (Figure 18, Panel A) are inconsistent with any of the sin gene fusions representing insertions into these regions. Fourth, gene fusions do not appear to represent Tn3-HoHoI insertions into the 24 kb HindIII fragment since this fragment contains trpGDC, and sin gene fusion-containing constructs fail to complement either trpD or trpC mutations.

These arguments also indicate that sin gene fusions do not represent Tn3-lacZYA insertions into the UV inducible pyocin R2 genes prtO and prtA-J, which are contained within the 24 and 16 kb HindIII fragments, respectively. Similarly, sin probe failure to hybridize to an 8 kb HindIII fragment of pNM16 indicates that gene fusions are not insertions into pyocin R2 genes prtK-N. Unfortunately, the pyocin R2 gene prtP is not contained within the R' plasmid pNM16. This gene is thought to be involved in regulation of pyocin synthesis, and mapping studies indicate is flanked by the genes strA and rifA (173). In this context, the inability of pBML400, pBML700 and pBML900 to complement a str-990 mutation may indicate an absence of prtP from these constructs.   However, these results are difficult to evaluate since the distance separating prtP and strA is unknown.

An effort was made to provide markers for gene mapping studies by mobilizing different types of ColEI   sin::lacZYA subclones into P. aeruginosa. Since ColEI plasmids are unable to replicate in P. aeruginosa, a double crossover recombination event should result in replacement of the chromosomal gene with the transposon inactivated version, whereas recombination at a single homologous site should result in integration of the reporter gene into the chromosome. It was not possible use ColEI subclones for gene replacement, since the necessary gene fusion-containing fragments are apparently too large to be stably subcloned into a ColEI vector. Use

of the appropriate ColEI subclones to integrate sinA::lacZYA and chromosomal DNA flanking sinB::lacZYA into the chromosome had the unusual and unexpected effect of inducing pyocin synthesis in transconjugants. In contrast, attempts to integrate sequences flanking sinC::lacZYA did not result in the recovery of transconjugants. These experiments suggest that the observed transconjugant expression of a pyocin-like activity is not mediated by Tn3-HoHoI sequences, but results from the chromosomal integration of the sinA::lacZYA gene fusion and sequences flanking sinB.

Since pyocin production is a lethal event, pyocin expression in transconjugants is reminiscent of 'dominant negative' and 'dominant' mutations discussed by Herskowitz (64). Dominant negative mutations are described as an alteration of a cloned gene such that it produces a mutated product which inhibits the wild-type gene product. Alternatively, a mutant phenotype can be created by the increased expression of a wild-type gene. In this latter example of a dominant mutation, an imbalance of subunit concentration of a multimeric protein can cause a disruption of function. Both types of mutation can be invoked to explain transconjugant production of pyocins, which are normally multi-subunit structures, and are regulated in a fashion which is complex, most likely stringent, and certainly poorly understood (173, 174). For example, integration of sinA::lacZYA and DNA flanking sinB::lacZYA could result in pyocin induction if these sequences encoded a transcriptional activator of pyocins, or were capable of titrating a cellular inhibitor of pyocins. Similarly, the Tn3-HoHoI insertion in sinA could represent a translational gene fusion which produces a mutated protein. If sinA encoded a repressor of pyocin synthesis, it could be inactivated if the transposon insertion impaired repressor ability to bind DNA

without affecting ability to oligomerize. In this case, mixed aggregates containing mutant and wild-type subunits of a multimeric pyocin repressor protein could be formed which would be unable to bind DNA and inhibit pyocin synthesis. These situations illustrating a regulatory imbalance could conceivably result in pyocin expression.

A final attempt to provide chromosomal markers for gene mapping studies by transforming a Res⁻ P. aeruginosa strain with linear sin::lacZYA-containing DNA failed to yield gene replacement strains. It is possible in this experiment that the transformation efficiency was not high enough to obtain linear transformation. Alternatively, gene replacement strains may have been obtained, but may not be viable. Inactivation of certain genes, such as that encoding the topoisomerase gyrase, has been shown to result in lethality (119). If sin genes act in P. aeruginosa recombination or repair, then it is very likely that the function of these genes may be necessary for cell viability.

In summary, results of the chromosomal mapping studies indicate that sinA, sinB, and sinC gene fusions are not pyocin R2 structural gene fusions, and do not contain pyocin R2 structural genes within their cloned inserts. The sin::lacZYA plasmids could potentially encode or contain the reporter gene fused to the pyocin R2 regulatory gene prtP. Additional pyocin genes have not been mapped to the chromosomal region in which the sin genes are located. Therefore if inducible plasmids are Tn3-HoHoI insertions into or contain pyocin genes other than those encoding pyocin R2 subunits, these genes represent uncharacterized pyocin genes. Alternatively, an inability to inactivate chromosomal versions of sin genes by gene

replacement could indicate that these genes are involved in processes which are critical for cell viability.

Further studies are clearly required to determine whether stress responsive <u>sin</u> genes are pyocin genes, genes encoding products involved in recombination or repair, or possess some alternative function. Investigation of regulation of <u>P. aeruginosa sin</u> gene expression would clarify whether <u>P. aeruginosa sin</u> genes are regulated in a manner analogous to <u>E. coli</u> SOS genes, and would allow an additional comparison to be made between <u>P. aeruginosa</u> and <u>E. coli</u> stress responsive genes. This investigation was undertaken and is described in the next Chapter.

CHAPTER VI

INVESTIGATION OF REGULATION OF <u>sin::lacZYA</u> GENE FUSIONS

Information is rapidly accumulating concerning the regulation of gene expression in the environmentally important species <u>P. aeruginosa</u>. Many genes including <u>recA</u> have been cloned and sequenced (158), and both <u>cis</u>-acting sequences (16) and <u>trans</u>-acting factors (46, 191) important in the regulation of various genes have been identified. Mechanisms of expression including transcriptional activation and repression (136), organization of genes in operons (32), and even the occurrence of coding sequences which are transcribed but not translated (215) have been described for <u>P. aeruginosa</u>. Furthermore, experiments have been conducted investigating the ability of several <u>P. aeruginosa</u> genes to interact with, and be controlled by, regulatory factors of other bacterial species, including <u>E. coli</u> (60, 90).

To determine whether <u>sin</u> genes are regulated similar to <u>E. coli</u> SOS genes, regulation of <u>sin::lacZYA</u> expression was investigated in both <u>P. aeruginosa</u> and <u>E. coli</u> hosts. Experiments were targeted toward understanding regulation of the <u>sinA</u> promoter to allow for a more complete analysis of this representative stress responsive gene.

Materials and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are indicated in Table 8. Properties of E. coli recA and/or lexA strains used to investigate regulation of sinA::lacZYA expression are described in Table 9.

Media, growth conditions and chemicals

E. coli strains possessing the temperature-sensitive recA441 mutation were incubated at 30°C. All other strains were maintained at 37°C, unless otherwise specified. M9 medium used for growth of E. coli strains for β-galactosidase assays contained 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 0.03% $MgSO_4 \cdot H_2O$ and was supplemented with 0.4% glucose, 0.2% casamino acids, and 50 ug/ml ampicillin. In the E. coli induction assays, adenine was added to 42°C incubated cultures to a final concentration of 100 mM (28). Rifampicin used to inhibit bacterial transcription was dissolved in methanol.

Transformations

The rubidium chloride protocol described was used to construct most E. coli strains. However, this transformation protocol proved ineffective for transformation of some E. coli recA and lexA mutants with pBML400 DNA. For these transformations, competent cells were prepared using the $CaCl_2$ protocol indicated in Chapter III. Freshly prepared competent cells were not frozen, but were used immediately for transformation. Transformation involved gently mixing 0.1 ml cells with pBML400 DNA which had been modified by passage through E. coli strain DH1. Following a 10 min incubation on ice, cells were heat shocked at room temperature

Table 8.--Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
P. aeruginosa		
PAO25	argF10 leu-10	54
RM265	leu-10 recA102	PAO25
E. coli		
HB101	proA2 leuB6 thi-1 galK lacY1 hsdR hsdM recA13 supE44 rpsL20 xyl mtl	19
DHI	thi-1 supE44 recAl gyrA96 endA1 hsdR17 relA1	61
AB1157	argE3 his-4 leuB6 proA2 thr-1 thi-1 galK2 lacY1 Sm-31 supE44 ara-1 xyl-5 mtl-1 tsx-33	37
DM49	as for AB1157 except lexA3	D. Mount
DM1180	as for AB1157 except recA441 sfiAll argE+ lexA3	D. Mount
DM1187	as for AB1157 except recA441 sfiAll supE+ lexA3 spr51	D. Mount
GC3217	as for AB1157 except recA441 sfiall supE+	D. Mount
RM1084	as for AB1157 except recA13	D. Mount
Plasmids		
pRK2013	pBR322 cointegrate plasmid carrying the mob and tra plasmid transfer functions of RK2; Km^r	49
pBML400	sinA::lacZYA fusion present in pCP16; Tc^r, Ap^r	This study
pBML700	sinB::lacZYA fusion present in pCP16; Tc^r, Ap^r	This study
pBML900	sinC::lacZYA fusion present in pCP16; Tc^r, Ap^r	This study

[a]Nomenclature and abbreviations are essentially those of Demerec et al. (45). Tc^r, Km^r, and Ap^r refer to resistance to tetracycline, kanamycin and ampicillin/carbenicillin, respectively.

Table 9.--Properties of E. coli recA and/or lexA strains

Strain[a]	Relevant genotype	E. coli SOS gene expression	Reference
AB1157	recA⁺	SOS genes are induced by exposure to DNA damaging agents	213
RM1084	recA13	RecA activity is absent and SOS genes are noninducible	29
GC3217	recA441[b]	When the culture temperature is shifted from 30 to 42°C, RecA coprotease activity is activated and SOS genes are constituitively expressed	28
DM1180	recA441 lexA3	The LexA repressor is resistant to coproteolytic cleavage, and SOS genes are noninducible at any temperature	28 29
DM1187	recA441 lexA3 spr51	LexA is unable to function as a repressor, and SOS genes are constituitively expressed at any temperature	28, 29 129
DM49	lexA3	The LexA repressor is resistant to coproteolytic cleavage, and SOS genes are noninducible	29

[a] All strains are derived from AB1157.
[b] Strains possessing the recA441 mutation are also sfiA11.

for an additional 10 min. A 0.3 ml volume of LB was added, and the culture was incubated 1 h at 30°C before plating onto selective media. Plates were incubated at 30°C.

Other methods

Restriction endonuclease digestions, isolation of plasmid DNA, determination of UVC sensitivity, measurement of β-galactosidase activity, and triparental matings were performed as described in preceding Chapters.

Results

Induction of sin::lacZYA fusions in P. aeruginosa is a rifampicin-sensitive process

Rifampicin is an antibiotic capable of selectively inhibiting bacterial transcription (212). The effect of rifampicin on the UV induction profiles of PAO25 containing pBML400, pBML700, and pBML900 was investigated by adding 200 ug rifampicin/ml to cultures immediately following UVC exposure. This treatment has been shown to effectively inhibit P. aeruginosa transcription within 1 min after addition (2). Under these conditions, UV inducible behavior of gene fusions was not observed (Table 10).

The sinA and sinB promoters are dependent upon the function

of the P. aeruginosa recA gene product

The effect of the recA102 mutation on sinA::lacZYA and sinB::lacZYA expression was investigated by monitoring the UVC responsive expression of these fusions when present in a recA102 RM265 genetic background as compared to a RecA+ PAO25 background (Figure 19). The pBML400 and pBML700 gene fusions were not induced in strain RM265 when subjected to UV exposures of 10 and 20

Table 10.--Rifampicin inhibition of the UV inducible behavior of <u>sin</u> gene fusions

STRAIN	UV DOSE (20 J/m^2)	RIFAMPICIN (200 ug/ml)	*RELATIVE ACTIVITY
PAO25 (pBML400)	-	-	1.0
	-	+	1.0
	+	-	12.9
	+	+	1.0
PAO25 (pBML700)	-	-	1.3
	-	+	1.1
	+	-	33.9
	+	+	1.0
PAO25 (pBML900)	-	-	1.6
	-	+	1.0
	+	-	2.9
	+	+	1.0

* Relative activity values reflect maximum β-galactosidase levels observed within 5 hours following UV treatment. The Table indicates results of a representative assay which was carried out in duplicate.
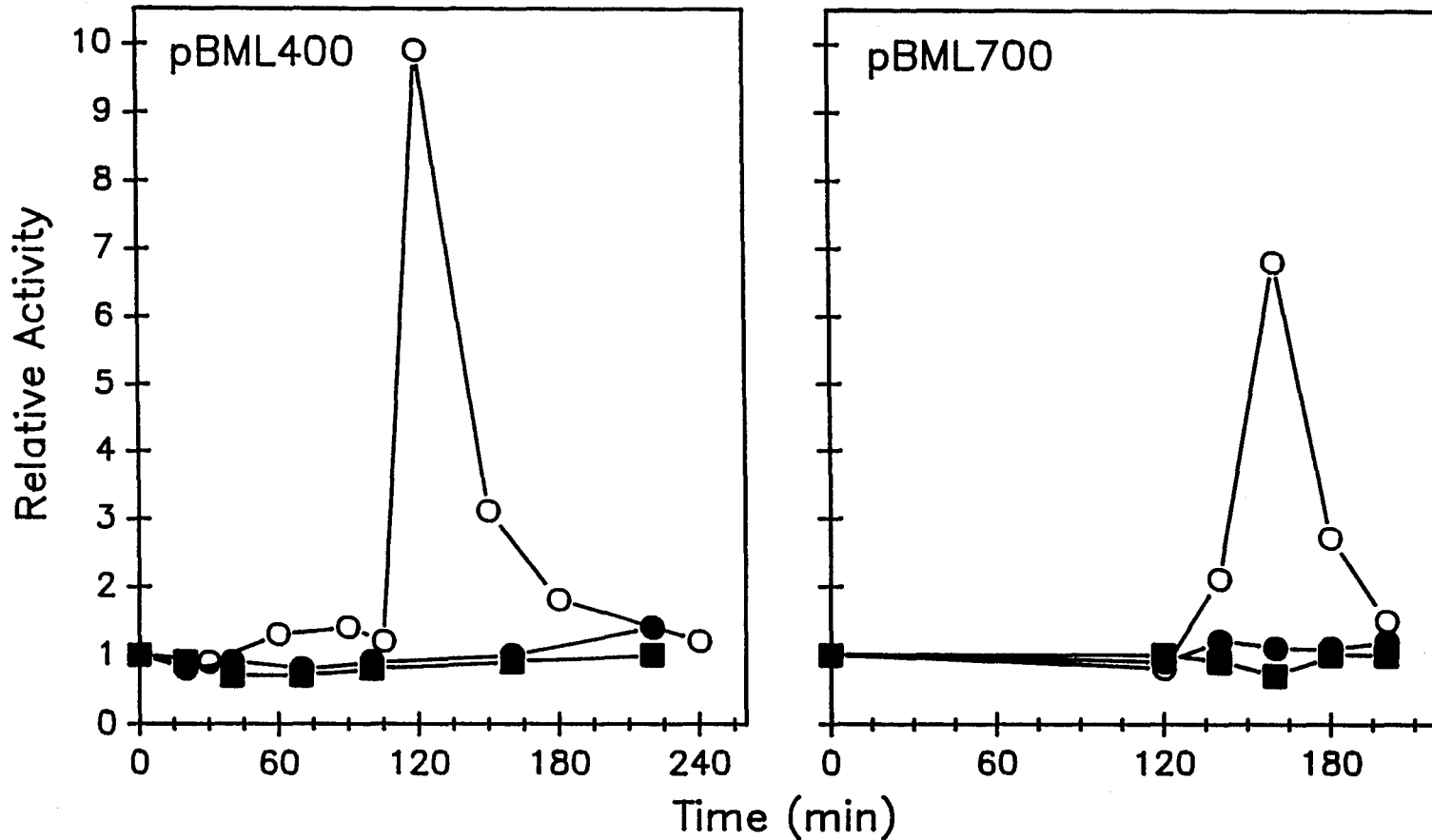
Fig. 19. RecA dependence of sinA::lacZYA and sinB::lacZYA UVC responsive expression. (A): (●) PAO25 (pBML400) untreated; (O) PAO25 (pBML400) 10 J/m²; (■) RM265 (pBML400) 10 J/m². B: (●) PAO25 (pBML700) untreated; (O) PAO25 (pBML700) 20 J/m²; (■) RM265 (pBML700) 20 J/m². Irradiated and control RM265 cultures gave identical results. Basal levels of expression were about two-thirds lower in RM265 cultures as compared to PAO25 cultures. The graphs represent the average of two independent assays which were performed in duplicate. Similar results were obtained using exposures of 5, 10 and 20 J/m².

J/m², respectively. While these data are not shown, induction of RM265 containing pBML400 or pBML700 also could not be detected following UVC exposures of 5, 10, or 20 J/m², even though the sinA gene fusion is clearly induced by these treatments in a RecA⁺ P. aeruginosa host.

The sinA::lacZYA fusion does not manifest an ultraviolet dose-dependent response when present in E. coli

The recA gene of P. aeruginosa appears to be both expressed and regulated in E. coli (90). An attempt was made to determine whether sin gene fusions are also regulated in E. coli using sinA::lacZYA as a representative sin fusion. In one approach to answering this question, the UVC-mediated response of sinA::lacZYA was monitored in the RecA⁺ E. coli host AB1157 (Figure 20). An increase in β-galactosidase activity was not observed within 3.5 h following esposure to UVC doses of 0, 10, 20, 40, and 60 J/m² (Figure 20, Panel A). Examination of the response of this strain to higher UV doses suggested a slight increase in β-galactosidase activity following exposure to UVC doses greater than 100 J/m² (Figure 20, Panel B). However, examination of relative activity (Figure 21) revealed that this slight increase was not significant, and probably reflected normal experimental variation in β-galactosidase activity.

pBML400 does not complement various E. coli recA or lexA mutations

Failure to observe a UVC-mediated response of the sinA::lacZYA gene fusion when present in the E. coli strain AB1157 may have reflected an error in selection of the UVC dose range or time frame in which β-galactosidase activity was monitored. One way to avoid this possibility is to examine sinA::lacZYA expression in the presence of E. coli recA and/or lexA mutations which result in constituitive, temperature sensitive, or noninducible expression of E. coli SOS genes. The
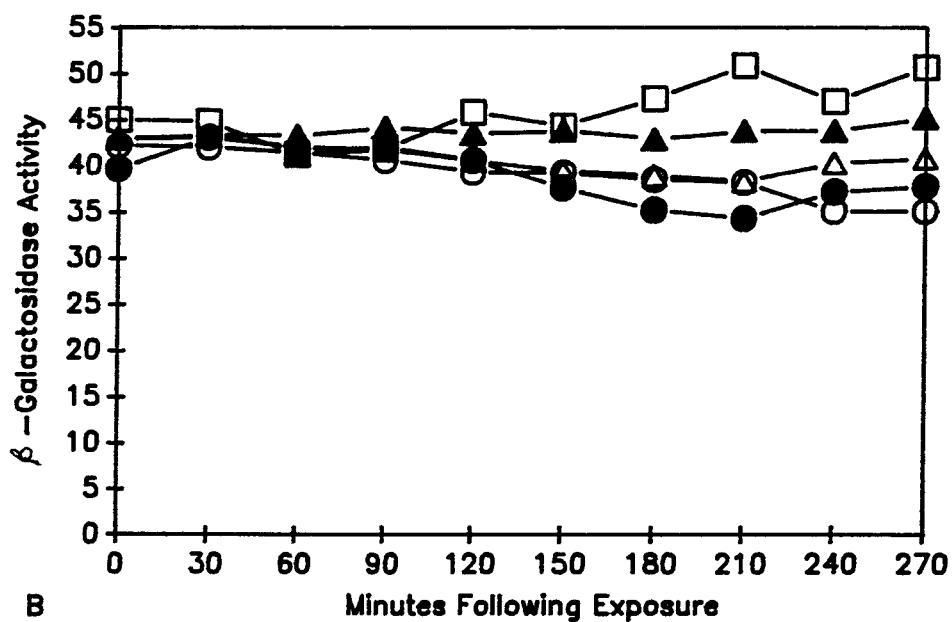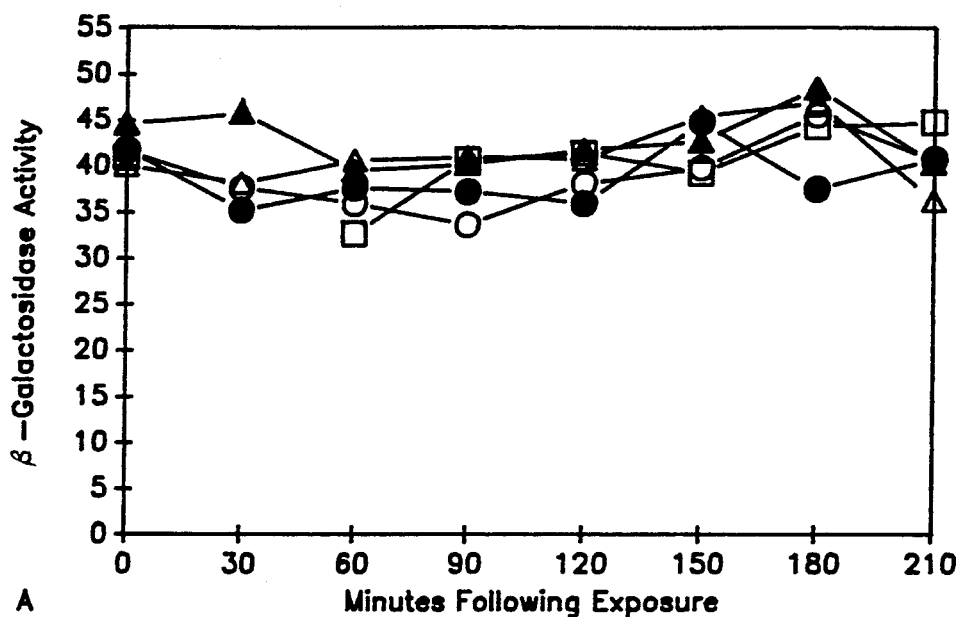
Fig. 20. Response of AB1157 (pBML400) to UVC radiation. (A) Response of AB1157 (pBML400) to relatively low UVC doses: (O) 0 J/m²; (●) 10 J/m²; (Δ) 20 J/m²; (▲) 40 J/m²; (□) 60 J/m². Results represent the average of two independent assays. (B) Response of AB1157 (pBML400) to higher UVC doses: (O) 0 J/m²; (●) 50 J/m²; (Δ) 100 J/m²; (▲) 150 J/m²; (□) 200 J/m². A representative experiment is presented.
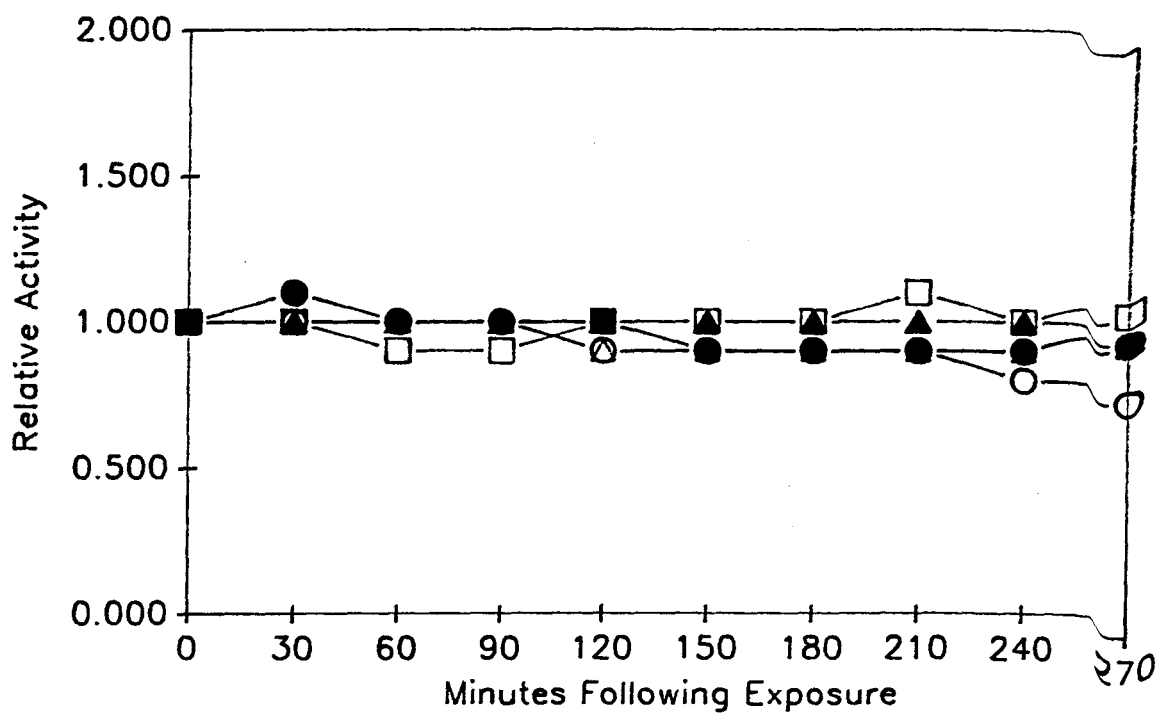
Fig. 21. Relative activity of AB1157 (pBML400) exposed to higher UV doses, (O) 0 J/m²; (●) 50 J/m²; (Δ) 100 J/m²; (▲) 150 J/m²; (□) 200 J/m². A representative assay which was performed in duplicate is presented. Relative activity was calculated as in the legend to Figure 7.

sinA::lacZYA fusion was introduced into these various genetic backgrounds through transformation of wild-type AB1157 and recA and/or lexA derivatives (Table 9) with pBML400. Before sinA::lacZYA expression could be monitored in these strains, the inability of pBML400 to complement the various recA or lexA alleles needed to be verified. Mutant strains both containing and not containing pBML400 were streaked onto agar. Portions of the streak were exposed to increasing doses of UVC irradiation. UVC sensitivity was compared to AB1157 and AB1157 (pBML400) treated in the same fashion. The pBML400 plasmid did not appear to complement any of the UVC-sensitive alleles to UVC resistance.

The sinA promoter does not respond to E. coli recA or lexA control

E. coli recA and lexA single and double mutant strains were used to directly assess whether the sinA promoter is capable of interacting with E. coli recA and lexA regulatory factors. The β-galactosidase activity of the pBML400-containing E. coli strains incubated at 30°C, and shifted to incubation at either 30 or 42°C was monitored (Figures 22 and 23).

Temperature shift assays involving recA441 GC3217 (pBML400), recA13 RM1084 (pBML400) and wild-type AB1157 (pBML400) (Figure 22) demonstrated that the recA441 allele did not result in increased sinA::lacZYA expression upon temperature shift up to 42°C. If anything, β-galactosidase expression in all E. coli hosts (Figure 22 and 23) decreased slightly following 42°C incubation. This effect may simply reflect a reduced efficiency of transcription or translation at the increased temperature. Compared to AB1157 (pBML400), basal levels of β-galactosidase expression were slightly elevated in the recA441 GC3217 (pBML400) host, and slightly
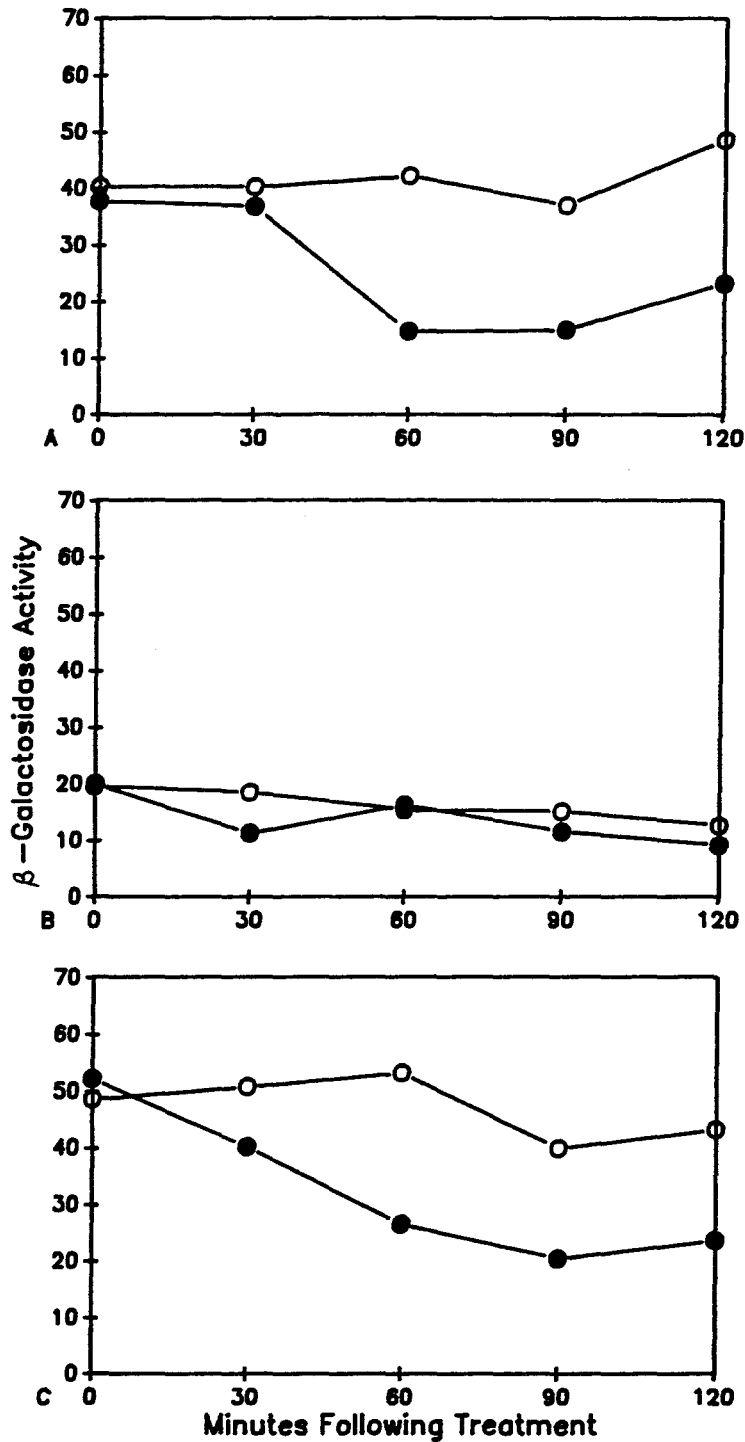
Fig. 22. Temperature shift assay of AB1157 and recA⁻ derivatives containing pBML400. (A) wild-type strain AB1157 (pBML400), (B) recA13 strain RM1084 (pBML400), (C) recA441 strain GC3217 (pBML400): (O) 30°C; (●) 42°C + adenine. Results for AB1157 and GC3217 strains represent the average of two assays.
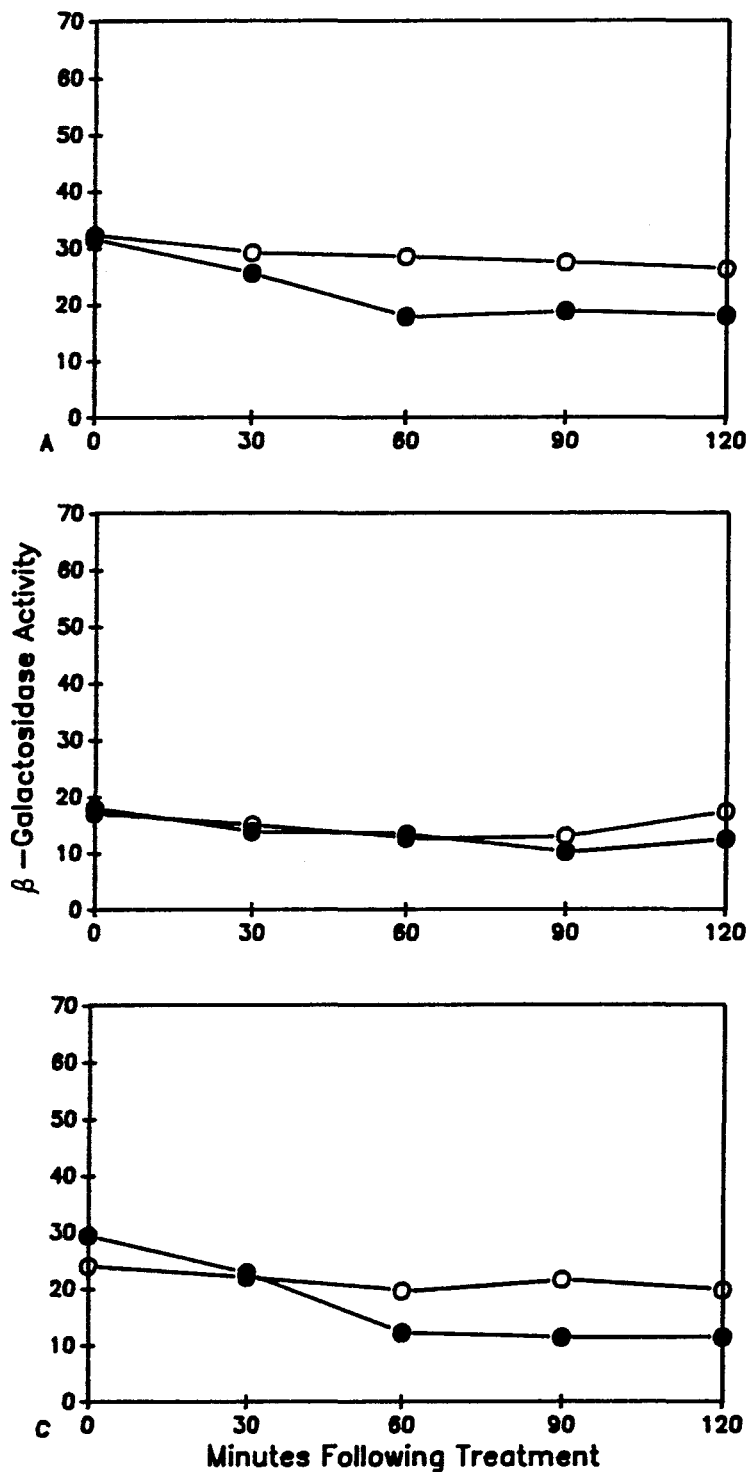
Fig. 23. Temperature shift assay of AB1157 lexA⁻ derivatives containing pBML400. (A) recA441 lexA3 strain DM1180 (pBML400), (B) recA441 lexA3 spr51 strain DM1187 (pBML400), (C) lexA3 strain DM49 (pBML400): (O) 30°C; (●) 42°C + adenine. Results for DM1180 and DM1187 strains are the average of two assays.

reduced in the recA13 RM1084 (pBML400) host. This small difference does not appear to be significant and probably reflects normal experimental variation.

Upon comparison of lexA hosts (Figure 23), it can be seen that the presence of the spr51 mutation resulting in an inactive LexA repressor in the recA441 lexA3 host DM1187 did not result in increased constituitive sinA::lacZYA expression in DM1187 (pBML400) as compared to recA441 lexA3 DM1180 (pBML400). Both strains exhibited similar levels of β-galactosidase at all temperatures as lexA3 DM49 (pBML400), and wild-type AB1157 (pBML400). The sinA::lacZYA gene fusion did appear to be expressed in the various E. coli hosts, since most pBML400-containing strains exhibited β-galactosidase levels which were approximately twice the activity observed in strains not containing pBML400 (Table 11).

Discussion

The observed rifampicin inhibition of UVC-mediated induction of sinA::lacZYA, sinB::lacZYA and sinC::lacZYA in RecA⁺ P. aeruginosa indicates that the response of these fusions is regulated at the level of transcription. Furthermore, the presence of the recA102 mutation, which alters recA activity, abolishes UVC radiation-generated increases in β-galactosidase expression in P. aeruginosa containing either sinA::lacZYA or sinB::lacZYA. Similar experiments could not be performed with sinC::lacZYA, because this fusion could not be established in RecA⁻ P. aeruginosa strains. Based on these results, it appears that both the sinA and sinB gene fusions are regulated by the P. aeruginosa recA gene product, or require the function of the recA gene product for their UVC-mediated induction. The fact that a UVC-mediated increase in P. aeruginosa recA expression occurs at an earlier time

Table 11.--Basal expression of E. coli strains containing or not containing pBML400

STRAIN	GENOTYPE	- pBML400 Basal activity[a] (Miller units)	+ pBML400 Basal activity[b] (Miller units)
AB1157	wild-type	16	39.1 (9.1)
RM1084	recA13	17	20.0 +/- 0
GC3217	recA441	15	44.0 (8.5)
DM1180	recA441 lexA3	12	37.1 (8.3)
DM1187	recA441 lexA3 spr51	14	33.2 +/- 13.2
DM49	lexA3	9	33.6 +/- 9.6

[a] Results of a representative assay carried out in duplicate are presented.

[b] Numbers indicate the average and either the deviation from the average or the the standard deviation (in parenthesis).

[ab] Strains were grown at 30°C to about 10^8 CFU/ml.

(69) than sinA::lacZYA and sinB::lacZYA induction supports this hypothesis. These findings are significant since they   clearly indicate that recA-dependent genes are conserved in P. aeruginosa.

The sinA::lacZYA-containing plasmid does not   complement UVC-sensitive E. coli recA or lexA mutations.   The ability of the sinA promoter to interact with E. coli regulatory factors was investigated using two different approaches. In the first approach, UVC-mediated induction of sinA::lacZYA was monitored in RecA$^+$ E. coli. A UV stimulated increase in sinA expression was not observed following exposure to UVC doses ranging from 0-200 J/m^2.   P. aeruginosa containing sinA::lacZYA as well as E. coli din genes normally demonstrate gene induction within this range of UVC doses, and within the time frame examined.

In the second approach to investigation of sinA promoter activity, sinA::lacZYA expression was examined in the presence of E. coli recA and lexA single or double mutations.   Somewhat surprisingly, the sinA::lacZYA fusion did not increase its expression in a RecA$^+$ E. coli host in response to UVC radiation, in mutant E. coli hosts possessing altered LexA repressor activity, or in thermally induced E. coli recA441 strains. It is possible that failure to observe responsive gene expression may have resulted from an inability of the sinA promoter to function in E. coli. However, increased constituitive β-galactosidase levels in E. coli strains containing sinA::lacZYA did not support this idea. It is conceivable but not very likely that β-galactosidase activity in the gene fusion containing strains resulted from the low level expression of another promoter present in the chromosomal insert. Results of these experiments more likely suggest that sinA::lacZYA does not respond to E. coli recA/lexA control.

The same type of result has been reported for the stress responsive genes nucA of Serratia marcescens and pnlA of Erwinia carotovora subsp. carotovora. While nucA encodes an extracellular nuclease (8), pnlA encodes a pectin degrading enzyme (121). Both genes have been shown to require the recA function of their native host for inducible gene expression (8, 218). However, nucA and pnlA are not induced in response to stress in RecA⁺ E. coli. Similar to sinA, both genes do appear to be expressed in this host (8, 121). The E. carotovora recA gene has been shown to be stress inducible in E. coli (83). Similarly, the S. marcescens recA gene responds to stress in the native host, and possesses a strongly conserved SOS consensus sequence (8). It is intriguing that the upstream region of the nuclease gene possesses a sequence that closely resembles a LexA binding site (8). In the case of both nucA and pnlA, experimental results have been interpreted as to suggest the requirement for an additional species-specific transcriptional activator for the observation of gene induction. It is conceivable that the sinA promoter may similarly require a P. aeruginosa-specific activator protein for stress responsive gene expression. In this case, sinA::lacZYA would not demonstrate gene induction in E. coli.

CHAPTER VII

GENERAL DISCUSSION

Experimental results clearly demonstrate that fusions linking a β-galactosidase reporter gene with eight novel, stress responsive promoters of P. aeruginosa have been obtained. These results are significant because they indicate that P. aeruginosa possesses genes other than recA which respond to stress agents. Similar restriction digests observed for some of the fusions suggest they may be Tn3-HoHoI insertions in the same general region of the chromosome. For example, sinA and sinB genes may be separated from each other by about 20 kb.

The sinA, sinB, and sinC gene fusions were selected for more thorough investigation as representative sin::lacZYA fusions. These three fusions apparently are Tn3-HoHoI insertions in separate genes. Neither the sinA, sinB, or sinC reporter gene fusions increased their expression following heat stress. This indicates that sin genes are not P. aeruginosa heat shock genes. In contrast, all three fusions demonstrated induction in response to both UVC radiation and norfloxacin exposure, despite the very different effects of these stressors upon the bacterial cell. While the magnitude of sinA::lacZYA induction in response to norfloxacin was slightly reduced, the general shape of the norfloxacin- as compared to the UVC-mediated induction profile was almost identical. These results indicate that P. aeruginosa sin genes

resemble E. coli SOS genes in their ability to respond to stress agents which damage DNA and impair DNA replication.

P. aeruginosa sin genes show further similarity to E. coli SOS genes in their dependence of induction on stressor dose. For both E. coli SOS genes and P. aeruginosa sin genes, maximum norfloxacin induction occurs when the concentration of quinolone is high enough to result in cell killing. The P. aeruginosa sinA, sinB, and sinC gene fusions exhibit their maximal UVC-mediated inductive response following a level of exposure which is comparable to that required to induce E. coli SOS genes. This is surprising considering that P. aeruginosa is more UV sensitive than E. coli. This result may indicate that some type of inducing signal is generated following exposure to a UVC dose of about 20 J/m^2. Alternatively, the dose-response curve of sin gene fusions may be altered due to expression of wild-type sin genes. For example, E. coli uvrB induction requires a 10-fold increase in UVC dose in a Uvr$^+$ as compared to a Uvr$^-$ host (160).

Comparison of the induction profiles of the three sin::lacZYA fusions revealed they respond with differing kinetics and magnitudes of induction to UVC radiation exposure. This is another example of a similarity between P. aeruginosa sin genes and E. coli SOS genes. These latter genes are sequentially induced in response to stress treatments due to a differing affinity of the LexA repressor for respective promoter regions (200). It is not clear whether a LexA-like repressor is required for sin gene regulation in P. aeruginosa. Differences in induction profiles of P. aeruginosa sin genes could similarly result from differences in the affinity of a LexA-like repressor, or alternatively a transcriptional activator for sin promoters.

The three gene fusions are unlike E. coli SOS genes in that they appear to demonstrate multiphasic induction profiles (69, 72). This phenomenon has been reported for additional P. aeruginosa genes and may not be unusual for P. aeruginosa gene expression. It is interesting and may prove relevant that sin inductive peaks occur with about the same periodicity as the DNA replication cycle. P. aeruginosa does demonstrate norfloxacin inducible DNA recovery synthesis (12), and it is tempting to speculate that sin genes might be involved in this process. Further experiments are clearly needed to verify whether sin gene induction is in fact related to the DNA replication cycle.

The approximate chromosomal location of sin genes was determined by analysis of large chromosomal fragments (U. Romling and B. Tummler, personal communication). The map location of sin genes does not correspond to the location of the few mapped mutations known to affect P. aeruginosa recombination and repair (94, 127, 158). An attempt was made to more closely define sin gene map locations by integrating sinA::lacZYA and chromosomal DNA flanking sinB::lacZYA in the chromosome to provide markers for gene mapping studies. This attempt resulted in pyocin synthesis in transconjugants, which may have been due to an imbalance of pyocin regulation or subunit expression. Pyocin R2 genes map to the same general location as sin genes (173). While sinA, sinB, and sinC gene fusions are not fusions to pyocin R2 structural genes, and do not contain pyocin R2 structural genes within their cloned inserts, they could potentially encode or contain the reporter gene fused to the pyocin R2 regulatory gene prtP. On the other hand, since the sinA, sinB, and sinC genes are located within the same 200 kb region of the chromosome, the gene fusions may be flanked by or contain the reporter gene inserted into novel pyocin

genes. An imbalance in pyocin regulation or subunit expression could similarly account for the difficulty in establishing and maintaining sinC::lacZYA constructs extrachromosomally in an unaltered form in P. aeruginosa (Appendix B). Namely, the sinC gene fusion or a segment of the chromosomal insert may express or regulate expression of pyocin.

A related attempt to define the location of sin genes by replacing chromosomal genes of a res⁻ P. aeruginosa strain with transposon-inactivated versions did not yield gene replacement. An inherent weakness of this type of experiment is that the frequency of crossover events may simply be too low to obtain the desired gene replacement strains. Alternatively, gene replacement strains may have been obtained, but may not have been viable. In other words, if P. aeruginosa sin genes encode pyocin regulatory components, are involved in DNA recovery synthesis, or are analogues of E. coli SOS genes mediating recombination and repair, then the function of these genes may very well be required for cell viability.

Investigation of regulation of sin::lacZYA fusions revealed that the UVC-mediated induction of sinA::lacZYA, sinB::lacZYA and sinC::lacZYA in RecA⁺ P. aeruginosa is sensitive to rifampicin. The inductive response of E. coli SOS genes is also regulated at the level of transcription (200). Both the sinA and sinB gene fusions require the function of the P. aeruginosa recA gene product for their UVC-mediated induction, which suggests that they may be regulated by recA. These findings are significant since they clearly indicate that recA-dependent genes are conserved in P. aeruginosa.

Somewhat surprisingly, the sinA::lacZYA fusion did not increase its gene expression in a RecA⁺ E. coli host in response to UVC, in mutant E. coli hosts

possessing altered LexA repressor activity, or in thermally induced E. coli recA441 strains. It is possible that failure to observe responsive gene expression may have resulted from an inability of the sinA promoter to function in E. coli. However, increased β-galactosidase levels in E. coli strains containing sinA::lacZYA did not support this idea.

Results of the E. coli induction experiments more likely indicate that sinA::lacZYA does not respond to E. coli recA/lexA control. It is possible that sinA is constituitively expressed in E. coli because this species lacks a transcriptional repressor protein required for regulation of stress responsive sinA expression. However, it is more plausible that the sinA promoter, like the S. marcescens nucA and E. carotovora pnlA promoters, requires an additional species-specific activator protein for gene induction. Regulation of P. aeruginosa sin genes would be clarified by transposon mutagenesis of the P. aeruginosa chromosome. sin::lacZYA plasmids could be mobilized into mutagenized P. aeruginosa, and insertions which alter stress responsive expression could be identified and mapped.

Miller et al. (126) have suggested that the evolutionary divergence in the RecA amino acid sequence may reflect interaction of different RecA proteins with species-specific coeffector proteins. When considered with similar results observed for the S. marcescens nucA gene (8), and the E. carotovora pnlA gene (121), the E. coli sinA::lacZYA induction experiments might support this idea. In other words, while RecA and RecA-dependent genes are conserved, certain aspects of regulation of RecA-dependent stress responsive genes may differ in bacterial species. If a LexA-like protein is involved in the regulation of gene expression in P. aeruginosa,

then the experimental results obtained indicate that it may be fundamentally different from the E. coli analogue.

Earlier experiments have clearly demonstrated that the P. aeruginosa recA gene is capable of participating in and potentially regulating stress responsive DNA repair (89, 90, 124). While some details of regulation of P. aeruginosa RecA-dependent sin genes may differ from E. coli SOS genes, sin genes are similar in many respects to SOS genes, and could potentially be involved in stress inducible repair or recovery of DNA synthesis. P. aeruginosa has been shown to possess quinolone inducible DNA recovery synthesis (12), and it is possible that sin genes may be involved in this process. In contrast, P. aeruginosa lacks Weigle reactivation in response to UVC and quinolone exposure, and is sensitive to UVC radiation (175). One explanation of this stressor-sensitive behavior of P. aeruginosa is that pyocins or possibly cryptic prophages may be induced following stress agent exposure. This would result in cell killing, which would be interpreted as a lack of inducible DNA repair in the Weigle reactivation assay.

To be of any benefit to P. aeruginosa, stress responsive repair and recombination genes would need to be differentially induced than pyocin genes. Since P. aeruginosa is a soil and water microorganism, a likely candidate for an environmental stressor potentially capable of accomplishing this feat is solar radiation. The solar UV spectrum consists of mainly UVA radiation (wavelengths >320 nm), a small amount of UVB radiation (290-320 nm wavelengths), and does not contain UVC wavelengths (<290 nm) (143). While UVC radiation primarily results in the formation of pyrimidine dimers, UVA exposure results in the generation of DNA-protein crosslinks, alkali-labile sites, and DNA strand breaks (143). The

response of <u>P. aeruginosa</u> to UVA exposure has not yet been studied. However, it is enticing to speculate that if <u>P. aeruginosa</u> does encode stress responsive DNA repair enzymes, the respective genes might be induced different from pyocin genes upon exposure to UVA radiation.

The studies conducted appear to have opened up new avenues for future experimentation in <u>P. aeruginosa</u>. The clarification of regulation of <u>sin</u> genes is of importance from an evolutionary standpoint. <u>sin</u> promoter-containing fragments could potentially be used to purify <u>trans</u>-acting regulatory proteins, and investigation of <u>sin</u> promoters would allow <u>cis</u>-acting sequences to be compared between species. In a related experiment, it would be interesting to determine whether induction of any <u>E. coli</u> genes such as the RIN genes is mediated by a transcriptional activator. Investigation of the size of encoded <u>sin</u> proteins along with studies of their function would allow a better comparison with stress responsive genes isolated from other bacteria. It may be significant to look at <u>sin</u> gene transcripts to determine if <u>sin</u> genes, like the stress responsive <u>recA</u> gene, encode more than one. Perhaps one of the most relevant studies to be performed would be an investigation of <u>sin</u> gene expression in response to naturally occurring stressors such as solar radiation. These studies will hopefully lead to exciting discoveries concerning stress responsive gene expression in the environmentally important species <u>P. aeruginosa</u>.

APPENDIX A

CALCULATION OF β-GALACTOSIDASE ACTIVITY

A computer program was written to facilitate calculation of β-galactosidase activity of a large number of samples (Figure 24).

```
5       INPUT "NUMBER OF DATA POINTS:":N
10      DIM A{N},B{N},C{N},D{N},E{N},X{N},Y{N}
20      INPUT "INDUCING CONDITIONS ARE?";H$
30      PRINT "ENTER TIMEPOINT, A420, A600, AND REACTION TIME {MIN}
40      PRINT : PRINT
50      FOR I = 1 TO N
60      INPUT A{I},B{I},C{I},D{I}
70      NEXT I
80      INPUT "ARE THERE ANY CORRECTIONS {Y/N}?";A$
90      IF A$ { } "N" THEN GOSUB 270
100     FOR I = 1 TO N
110     X{I} = B{I} * 1000
120     Y{I} = C{I} * D{I} * 0.2
130     E{I} = X{I} / Y{I}
140     NEXT I
150     PRINT CHR$ {4};"PR#1": REM ACTIVATES PRINTER
155     PRINT CHR$ {1};"80N": REM SCREEN OFF AND LINE WIDTH 80
160     PRINT H$; PRINT
170     PRINT "TP      A420      A600      RTIME{MIN}      MILLER UNITS"
180     FOR I = 1 TO N
190     PRINT SPC{3}; A{I}; SPC{9}; B{I}: SPC{11}; C{I}; SPC{13}; D{I};
        SPC{15}; E{I}
240     NEXT I
245     PRINT CHR$ {1};"I": REM SCREEN ON AND LINE WIDTH 40
250     PRINT CHR$ {4};"PR#0": REM DEACTIVATES PRINTER
260     END
270     INPUT "ENTER LINE NUMBER TO BE CORRECTED:";I
280     INPUT "ENTER NEW VALUES FOR TIMEPOINT, A420, A600, AND
        REACTION TIME:":A{I},B{I},C{I},D{I}
290     INPUT "ANY OTHER CORRECTIONS {Y/N}?;A$
300     IF A$ { } "N" THEN GOTO 270
```

Fig. 24. Simple computer program for calculation of β-galactosidase activity. The program was written to be executed on an Apple or Apple-compatible computer. Line 120 was frequently changed to accomodate different volumes of cells (i.e. 0.1, 0.2, or 0.5 ml) assayed, and the correction loop never did work.

APPENDIX B

DIFFICULTY IN THE STABLE ESTABLISHMENT OF pBML900 AND ITS DERIVATIVES IN P. AERUGINOSA

A deletion or other type of recombinational event may have been observed within pBML900. Initially pBML900 possessed restriction patterns identical to pBML1200. However, with continued maintenance in the RecA$^+$ P. aeruginosa strain PAO25, pBML900 was observed to lack 16 kb BamHI-HindIII and PstI fragments which are present in pBML1200. Carbenicillin resistance, hybridization of a labeled lacZYA probe to an appropriate size pBML900 BamHI-HindIII fragment, and tests of UVC-mediated induction of β-galactosidase expression all verified the integrity of the sinC::lacZYA gene fusion in pBML900. It is possible that pBML900 is not derived from pBML1200 by deletion or recombination. In this case, the 16 kb bands present in pBML1200 could represent some type of partial digestion products.

A deletion was clearly detected in the pBML900 BamHI subclone pBML9011 maintained in PAO25. A recombinational event was suspected when restriction digests of this subclone appeared incompatible with restriction digests of pBML900 and its HindIII subclone pBML9090. Southern analysis of pBML900, pBML9090, and pBML9011 was performed to confirm the extent of the pBML9011 deletion. Whereas a probe consisting of the Tn3-HoHoI KpnI-BglII lacZYA fragment detected the same 16-17 kb fragment present in BglII-BamHI and BglII-HindIII digests of pBML900, PBML9090, and pBML9011, a probe consisting of the 200 bp upstream end of Tn3-

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HoHoI including only the first KpnI site hybridized to KpnI-BamHI and KpnI-HindIII fragments of about 8 kb in pBML900 and pBML9090, and hybridized to a high molecular weight band present in KpnI-BamHI and KpnI-HindIII digests of pBML9011. The only explanation compatible with these results is that a BglII site is present in pBML900, pBML9011, and pBML9090 in the sinC promoter region, between the inserted transposon and BamHI and HindIII sites lying slightly further upstream. The deletion present in pBML9011 removed the upstream BamHI site, and appears to extend from the promoter region BglII site to further upstream of the BamHI site. Additional restriction digests of the three constructs were compatible with this interpretation.

Another pBML900 subclone was demonstrated to be unstable in P. aeruginosa. The inducible subclone pBML9001 was obtained through partial BglII-PstI digestion followed by religation of pBML900, and was mobilized immediately into PAO25. Growth of PAO25 (pBML9001) under nonstressed conditions resulted in the recovery of constructs possessing altered restriction patterns. The chromosomal insert present in pBML9001 did not appear to be recombining into the chromosome since the recovered altered isolates were no longer inducible. Approximately half of the colonies obtained upon streaking out a pure stock of PAO25 (pBML9001) for isolated colonies were found to be noninducible.

An attempt was made to establish the pBML900 subclone pBML9090 in various P. aeruginosa hosts. This attempt was made using pBML9090 since earlier attempts using pBML900 had failed. OT684 (PBML9090) transconjugants were obtained with a frequency of at least 10^{-6} transconjugants/recipient, which indicated that the failure to establish pBML9090 in strains PAO25, RM265, and RM522 did

not result from an inability of pBML9090 to be mobilized by pRK2013. In mobilization experiments involving pBML400 and pBML700, transconjugants of the various hosts were obtained with a relatively high frequency (10^{-6}-10^{-5} transconjugants/ recipient), which suggests that these strains represent suitable recipients in triparental matings. In this context, it is interesting that pBML9090 isolated from OT684 and transformed into E. coli HB101 could not be mobilized back into P. aeruginosa.

LITERATURE CITED

1. **Akaboshi, E., M.L.R. Yip, and P. Howard-Flanders.** 1989. Nucleotide sequence of the RecA gene of _Proteus mirabilis_. Nuc. Acids. Res. **17**:4390.

2. **Allan, B., M. Linseman, L.A. MacDonald, J.S. Lam, and A.M. Kropinski.** 1988. Heat shock response of _Pseudomonas aeruginosa_. J. Bacteriol. **170**:3668-3674.

3. **Amundsen, S.K., A.F. Taylor, A.M. Chaudhury, and G.R. Smith.** 1986. _recD_: the gene for an essential third subunit of exonuclease V. Proc. Natl. Acad. Sci. **83**:5558-5562.

4. **Armengod, M.-E.** 1982. _recF_-dependent recombination as an SOS function. Biochimie. **64**:629-632.

5. **Auffray, Y. and P. Boutibonnes.** 1987. Presence of inducible DNA repair in _Bacillus thuringiensis_. Mutat. Res. **183**:225-229.

6. **Bagg, A., C.J. Kenyon, and G.C. Walker.** 1981. Inducibility of a gene product required for UV and chemical mutagenesis in _Escherichia coli_. Proc. Natl. Acad. Sci. USA. **78**:5749-5753.

7. **Bailone, A., S. Sommer, and R. Devoret.** 1985. Mini-F plasmid-induced signal in _Escherichia coli_ is RecBC dependent. Proc. Natl. Acad. Sci. USA. **82**:5973-5977.

8. **Ball, T.K., C.R. Wasmuth, S.C. Braunagel, and M.J. Benedik.** 1990. Expression of _Serratia marcescens_ extracellular proteins requires _recA_. J. Bacteriol. **172**:342-349.

9. **Barbe, J., I. Gibert, M. Llagostera, and R. Guerrero.** 1987. DNA repair systems in the phototrophic bacterium _Rhodobacter capsulatus_. J. Gen. Microbiol. **133**:961-966.

10. **Barcak, G.J., J.-F. Tomb, C.S. Laufer, and H.O. Smith.** 1989. Two _Haemophilus influenzae_ Rd genes that complement the _recA_-like mutation _rec-1_. J. Bacteriol. **171**:2451-2457.

11. **Bates, H., S.K. Randall, C. Rayssiguier, B.A. Bridges, M.F. Goodman, and M. Radman.** 1989. Spontaneous and UV-induced mutations in Escherichia coli K-12 strains with altered or absent DNA polymerase I. J. Bacteriol. **171**:2480-2484.

12. **Benbrook, D.M. and R.V. Miller.** 1986. Effects of norfloxacin on DNA metabolism in Pseudomonas aeruginosa. Antimicro. Agents and Chemother. **29:** 1-6.

13. **Bennett, P.M., J. Grinsted, C.L. Choi, and M.H. Richmond.** 1978. Characterisation of Tn501, a transposon determining resistance to mercuric ions. Molec. Gen. Genet. **159:**101-106.

14. **Berenstein, D.** 1987. UV-inducible DNA repair in Acinetobacter calcoaceticus. Mut. Res. **183:**219-224.

15. **Berg, D.E.** 1989. Transposon Tn5. pp. 185-210. In D.E. Berg and M.M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.

16. **Berry, A., J.D. DeVault, and A.M. Chakrabarty.** 1989. High osmolarity is a signal for enhanced algD transcription in mucoid and nonmucoid Pseudomonas aeruginosa strains. J. Bacteriol. **171:**2312- 2317.

17. **Bick, D.P. and S. N. Cohen.** 1986. Identification and characterization of recD, a gene affecting plasmid maintenance and recombination in Escherichia coli. J. Bacteriol. **167:**594-603.

18. **Bolivar, F., R.L. Rodriguez, M.C. Betlach, and H.W. Boyer.** 1977. Construction and characterization of new cloning vehicles. I. Ampicillin-resistant derivatives of the plasmid pMB9. Gene. **2:**75-93.

19. **Bolivar, F., R.L. Rodriguez, P.J. Greene, M.C. Betlach, H.L. Heynecker, and H.W. Boyer.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene. **2:**95-113.

20. **Bridges, B.A.** 1988. Mutagenic DNA repair in Escherichia coli XVI. Mutagenesis by ultraviolet light plus delayed photoreversal in recA strains. Mut. Res. **198:** 343-350.

21. **Bridges, B.A., R.P. Mottershead and S.G. Sedgwick.** 1976. Mutagenic DNA repair in Escherichia coli III, requirement for a function of DNA Polymerase III in ultraviolet light mutagenesis. Mol. Gen. Genet. **144:**53-58.

22. **Bridges, B.A. and F. Sharif.** 1988. Mutagenic DNA repair in Escherichia coli, XV. Mutation frequency decline of ochre suppressor mutations in umuC and lexA bacteria occurring between ultraviolet irradiation and delayed photoreversal. Mut. Res. **197:**15-22.

23. **Bridges, B.A. and R. Woodgate.** 1984. Mutagenic repair in Escherichia coli, V. The umuC gene product may be required for replication past pyrimidine dimers but not for the coding error in UV-mutagenesis. Mol. Gen. Genetics. **196**:364-366.

24. **Bridges, B.A. and R. Woodgate.** 1985. Mutagenic repair in Escherichia coli: products of the recA gene and of the umuD and umuC genes act as different steps in UV-induced mutagenesis. Proc. Natl. Acad. Sci. USA. **82**:4193-4197.

25. **Bridges, B.A., R. Woodgate, M. Ruiz-Rubio, F. Sharif, S.G. Sedgwick, and U. Hubscher.** 1987. Current understanding of UV-induced base pair substitution mutation in E. coli with particular reference to the DNA Polymerase III complex. Mut. Res. **181**:219-226.

26. **Burckhardt, S.E., R. Woodgate, R.H. Scheuermann, and H. Echols.** 1988. UmuD mutagenesis protein of Escherichia coli: overproduction, purification, and cleavage by RecA. Proc. Natl. Acad. Sci. USA. **85**:1811-1815.

27. **Calsou, P., A. Villaverde, M. Defais. 1987.** Activated RecA protein may induce expression of a gene that is not controlled by the LexA repressor and whose function is required for mutagenesis and repair of UV-irradiated bacteriophage lambda. J. Bacteriol. **169**:4816-4821.

28. **Castellazzi, M., J. George, and G. Buttin.** 1972. Prophage induction and cell division in E. coli. I. Further characterization of the thermosensitive mutation tif-1 whose expression mimics the effect of UV irradiation. Molec. Gen. Genet. **119**: 139-152.

29. **Castellazzi, M., J. George, and G. Buttin.** 1972. Prophage induction and cell division in E. coli. II. Linked (recA, zab) and unlinked (lex) suppressors of tif-1 mediated induction and filamentation. Mol. Gen. Genet. **119**:153-174.

30. **Castilho, B.A., P. Olfson, M.J. Casadaban.** 1984. Plasmid insertion mutagenesis and lac gene fusions with mini-Mu bacteriophage transposons. J. Bacteriol. **158**: 488-495.

31. **Chandler, P.M., and V. Krishnapillai.** 1974. Isolation and properties of recombination-deficient mutants of Pseudomonas aeruginosa. Mutat. Res. **23**:15-23.

32. **Chang, M., D.W. Essar, and I.P. Crawford.** 1990. Diverse regulation of the tryptophan genes in fluorescent Pseudomonads. pp. 292-302. In Silver, S., A.M. Chakrabarty, B. Iglewski, and S. Kaplan (eds.), Pseudomonas. Biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.

33. **Chaudhury, A.M. and G.R. Smith.** 1984. A new class of Escherichia coli recBC mutants: implications for the role of RecBC enzyme in homologous recombination. Proc. Natl. Acad. Sci. **81**:7850-7854.

34. **Chaudhury, A.M. and G.R. Smith. 1984.** Escherichia coli recBC deletion mutants. J. Bacteriol. **160**:788-791.

35. **Chaudhury, A.M. and G.R. Smith. 1985.** Role of Escherichia coli RecBC enzymne in SOS induction. Mol. Gen. Genet. **201**:525-528.

36. **Clark, A.J. 1971.** Toward a metabolic interpretation of genetic recombination of E. coli and its phages. Ann. Rev. Microbiol. **25**:438-458.

37. **Clark, A.J., and A.D. Margulies. 1965.** Isolation and characterization of recombination deficient mutants of Escherichia coli. Proc. Natl. Acad. Sci. **53**:451-459.

38. **Cooper, P.K. 1982.** Characterization of long-patch excision repair of DNA in ultraviolet-irradiated Escherichia coli: an inducible function under Rec- Lex control. Mol. Gen. Genet. **185**:189-197.

39. **Cox, M.M. and I.R. Lehman.** 1987. Enzymes of general recombination. Ann. Rev. Biochem. **56**:229-62.

40. **Darzins, A. and A.M. Chakrabarty.** 1984. Cloning of genes controlling alginate biosynthesis from a mucoid cystic fibrosis isolate of Pseudomonas aeruginosa. J. Bacteriol. **159**:9-18.

41. **Darzins, A., S.-K. Wang, R.I. Vanags, and A.M. Chakrabarty.** 1985. Clustering of mutations affecting alginic acid biosynthesis in mucoid Pseudomonas aeruginosa. J. Bacteriol. **164**:516-524.

42. **Davis, R.W., D. Botstein, J.R. Roth.** 1980. A manual for genetic engineering. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

43. **Davison, J., F. Brunel, K. Kaniga, and N. Chevalier.** 1990. Recombinant DNA vectors for Pseudomonas. pp. 242-251. In Silver, S., A.M. Chakrabarty, B. Iglewski, and S. Kaplan (eds.), Pseudomonas. Biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.

44. **Demple, B. and J. Halbrook.** 1983. Inducible repair of oxidative DNA damage in Escherichia coli. Nature. **304**:466-468.

45. **Demerec, M., E.A. Adelberg, A.J. Clark, and P.E. Hartmann.** 1966. A proposal for a uniform nomenclature in bacterial genetics. Genetics. **554**:61-76.

46. **Deretic, V., R. Dikshit, W.M. Konyecsni, A.M. Chakrabarty, and T.K. Misra.** 1989. The algR gene, which regulates mucoidy in Pseudomonas aeruginosa belongs to a class of environmentally responsive genes. J. Bacteriol. **171**:1278-1283.

47. **Elledge, S.J. and G.C. Walker.** 1983. The muc genes of pKM101 are induced by DNA damage. J. Bacteriol. **155**:1306-1315.

48. **Farrand, S.K., S.P. O'Morchoe, and J. McCutchan.** 1989. Construction of an Agrobacterium temefaciens C58 recA mutant. J. Bacteriol. **171**:5314-5321.

49. **Figurski, D.H. and D.R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA. **76**:1648-1652.

50. **Foster, P.L., and A.D. Sullivan.** 1988. Interactions between epsilon, the proofreading subunit of DNA polymerase III, and proteins involved in the SOS response of Escherichia coli. Mol. Gen. Genet. **214**:467-473.

51. **Foster, P.L., A.D. Sullivan, and S.B. Franklin.** 1989. Presence of the dnaQ-rnh divergent transcriptional unit on a multicopy plasmid inhibits induced mutagenesis in Escherichia coli. J. Bacteriol. **171**:144-3151

52. **Fouts, K.E., T. Wasie-Gilbert, D.K. Willis, A.J. Clark, and S.D. Barbour.** 1983. Genetic analysis of transposon-induced mutations of the Rac prophage in Escherichia coli K-12 which affect expression and function of recE. J. Bacteriol. **156**:718-726.

53. **Friedberg, E.C.** 1987. The molecular biology of nucleotide excision repair of DNA: recent progress. J. Cell. Sci. Suppl. **6**:1-23.

54. **Fruh, R., J.M. Watson, and D. Haas.** 1983. Construction of recombination-deficient strains of Pseudomonas aeruginosa. Mol. Gen. Genet. **191**:334-337.

55. **Furukawa, K., N. Hayase, and K. Taira.** 1990. Biphenyl/polychlorinated biphenyl catabolic gene (bph operon): organization, function, and molecular relationship in various Pseudomonads. pp. 111-120. In Silver, S., A.M. Chakrabarty, B. Iglewski, and S. Kaplan (eds.), Pseudomonas. Biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.

56. **Gasc, A.M., N. Sicard, J.P. Claverys, and A.M. Sicard.** 1980. Lack of SOS repair in Streptococcus pneumoniae. Mut. Res. **70**:157-165.

57. **Gillespie, K. and R.E. Yasbin.** 1987. Chromosomal locations of three Bacillus subtilis din genes. J. Bacteriol. **169**:3372-3374.

58. **Goodman, H.J.K., J.R. Parker, J.A. Southern, and D.R. Woods.** 1987. Cloning and expression in Escherichia coli of a recA-like gene from Bacteroides fragilis. Gene. **58**:265-271.

59. **Gutterson, N.I. and D.E. Koshland, Jr.** 1983. Replacement and amplification of bacterial genes with sequences altered in vivo. Proc. Natl. Acad. Sci. USA. **80:** 4894-4898.

60. **Hamood, A.N. and B.H. Iglewski.** 1990. Expression of the Pseudomonas aeruginosa toxA positive regulatory gene (regA) in Escherichia coli. J. Bacteriol. **172:**589-594.

61. **Hanahan, D.** 1983. Studies on transformation of E. coli with plasmids. J. Mol. Biol. **166:**557-580.

62. **Hanahan, D.** 1985. Techniques for transformation of E. coli. In D.M. Glover (ed.), DNA cloning. A practical approach. Volume 1. IRL Press, Oxford, England.

63. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene. **28:**351-359.

64. **Herskowitz, I.** 1987. Functional inactivation of genes by dominant negative mutations. Nature. **329:**219-222.

65. **Higgins, C.F., C.J. Dorman, D.A. Stirling, L. Waddell, I.R. Booth, G. May, and E. Bremer.** 1988. A physiological role for DNA supercoiling in the osmotic regulation of gene expression in S. typhimurium and E. coli. Cell. **52:**569-584.

66. **Holloway, B.W.** 1969. Genetics of Pseudomonas. Bacteriol. Rev. **33:**419-443.

67. **Holloway, B.W. and A.F. Morgan.** 1986. Genome organization in Pseudomonas. Ann. Rev. Microbiol. **40:**70-105.

68. **Horii, J.-I. and A.J. Clark.** 1973. Genetic analysis of the RecF pathway to genetic recombination in Escherichia coli K-12: isolation and characterization of mutants. J. Mol. Biol. **80:**327-344.

69. **Horn, J.M. and D.E. Ohman.** 1988. Autogenous regulation and kinetics of induction of Pseudomonas aeruginosa recA transcription as analyzed with operon fusions. J. Bacteriol. **170:**4699-4705.

70. **Horn, J.M. and D.E. Ohman.** 1988. Transcriptional and translational analyses of recA mutant alleles in Pseudomonas aeruginosa. J. Bacteriol. **170:**1637-1650.

71. **Hromic, A. and R. Kirby.** 1989. Isolation and study of two mutants of Streptomyces cattleya affected in DNA repair and genetic instability. FEMS Microbiol. Letters. **57:**139-144.

72. **Iglewski, B.H., L. Rust, and R.A. Beaver.** 1990. Molecular analysis of Pseudomonas aeruginosa elastase. pp. 36-43. In Silver, S., A.M. Chakrabarty, B.

Iglewski, and S. Kaplan (eds.), Pseudomonas. Biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.

73. **Irbe, R.M. and M. Oishi.** 1980. Prophage induction in a permeabilized cell system: induction by deoxyribonucleases and the role of recBC- deoxyribonuclease. J. Bacteriol. **144**:1061-1067.

74. **Itoh, Y. and D. Haas.** 1985. Cloning vectors derived from the Pseudomonas plasmid pVSI. Gene. **36**:27-36.

75. **Itoh, Y., J. M. Watson, D. Haas, and T. Leisinger.** 1984. Genetic and molecular characterization of the Pseudomonas plasmid pVSI. Plasmid. **11**:206-220.

76. **Javor, G.T., C.D. Stringer, and J.I. Ryu.** 1988. Thiol-sensitive promoters of Escherichia coli. J. Bacteriol. **170**:3291-3293.

77. **Jonczyk, P. Fijalkowska, and Z. Ciesla.** 1988. Overproduction of the ε subunit of DNA polymerase III conteracts the SOS mutagenic response of Escherichia coli. Proc. Natl. Acad. Sci. USA. **85**:9124-9127.

78. **Jones, P.G., R.A. VanBogelen, and F.C. Neidhardt.** 1987. Induction of proteins in response to low temperature in Escherichia coli. J. Bacteriol. **169**:2092-2095.

79. **Kageyama, M.** 1964 Studies of a pyocin. I. Physical and chemical properties. J. Biochem. **55**:49-53.

80. **Kageyama, M.** 1975. Bacteriocins and Bacteriophages in Pseudomonas aeruginosa. p. 291-305. In S. Mitsuhashi and H. Hashimoto (ed.), Microbial Drug Resistance. University of Tokyo Press. Tokyo.

81. **Kannan, P.R. and K. Dharmalingam.** 1987. Restriction alleviation and enhancement of mutagenesis of the bacteriophage T4 chromosome in recBC sbcA strains of Escherichia coli. Mol. Gen. Genet. **209**:413-418.

82. **Karu, A.E. and E.D. Belk.** 1982. Induction of E. coli recA protein via recBC and alternate pathways: Quantitation by enzyme-linked immunosorbent assay (ELISA). Mol. Gen. Genet. **185**:275-282.

83. **Keener, S.L., K.P. McNamee, and K. McEntee.** 1984. Cloning and characterization of recA genes from Proteus vulgaris, Erwinia carotovora, Shigella flexneri and Escherichia coli B/r. J. Bacteriol. **160**:153-160.

84. **Kenyon, C.J., R. Brent, M. Ptashne, and G.C. Walker.** 1982. Regulation of damage-inducible genes in Escherichia coli. J. Mol. Biol. **160**:445-447.

85. **Kenyon, C.J. and G.C. Walker.** 1980. DNA-damaging agents stimulate gene expression at specific loci in Escherichia coli. Proc. Natl. Acad. Sci. USA. **77:** 2819-2823.

86. **Kenyon, C.J. and G.C. Walker.** 1981. Expression of the E. coli uvrA gene is inducible. Nature. **289:**808-810.

87. **Khidhir, M.A., S. Casaregola, and I.B. Holland.** 1985. Mechanism of transient inhibition of DNA synthesis in ultraviolet-irradiated E. coli: inhibition is independent of recA whilst recovery requires RecA protein itself and an additional, inducible SOS function. Mol. Gen. Genet. **199:**133-140.

88. **Kieser, H.M., D.J. Henderson, C.W. Chen, and D.A. Hopwood.** 1989. A mutation of Streptomyces lividans which prevents intraplasmid recombination has no effect on chromosomal recombination. Mol. Gen. Genet. **220:**60-64.

89. **Kokjohn, T.A. and R.V. Miller.** 1985. Molecular cloning and characterization of the recA gene of Pseudomonas aeruginosa POA. J. Bacteriol. **163:**568-572.

90. **Kokjohn, T.A. and R.V. Miller.** 1987. Characterization of the Pseudomonas aeruginosa recA analogue and its protein product: rec-102 is a mutant allele of the Pseudomonas aeruginosa PAO recA gene. J. Bacteriol. **169:**1499-1508.

91. **Kokjohn, T.A. and R.V. Miller.** 1988. Characterization of the Pseudomonas aeruginosa recA gene: Les⁻ phenotype. J. Bacteriol. **170:**578-582.

92. **Krasin, F. and F. Hutchinson.** 1981. Repair of DNA double-strand breaks in Escherichia coli cells requires synthesis of proteins that can be induced by UV light. Proc. Natl. Acad. Sci. **78:**3450-3453.

93. **Krueger, J.H. and G.C. Walker.** 1984. groEL and dnaK genes of Escherichia coli are induced by UV irradiation and nalidixic acid in an htpR⁺-dependent fashion. Proc. Natl. Acad. Sci. USA. **81:**1499-1503.

94. **Kung, A.H.C., and B.T.O. Lee.** 1973. The isolation and survival characterization of radiation and chemical-mutagen sensitive mutants of Pseudomonas aeruginosa. Mut. Res. **20:**175-190.

95. **Kushner, S.R., H. Nagaishi, A. Templin, and A.J. Clark.** 1971. Genetic recombination in Escherichia coli: the role of Exonuclease I. Proc. Nat. Acad. Sci. USA. **68:**824-827.

96. **Lackey, D., S.W. Krauss and S. Linn.** 1982. Isolation of an altered form of DNA polymerase I from Escherichia coli cells induced for recA/lexA functions. Proc. Natl. Acad. Sci. USA. **79:**330-334.

97. **Lehrbach, P.R., A.H.C. Kung, and B.T.O. Lee.** 1976. Mutants of <u>Pseudomonas aeruginosa</u> deficient in DNA polymerase I. Mut. Res. **41**:391-394.

98. **Lehrbach, P.R., B.T.O. Lee, and C.D. Dirckze.** 1979. Effect of repair deficiency and R plasmids on spontaneous and radiation-induced mutability in <u>Pseudomonas aeruginosa</u>. J. Bacteriol. **139**:953-960.

99. **Levine, E. and T. Thiel.** 1987. UV-inducible DNA repair in the cyanobacteria <u>Anabaena</u> spp. J. Bacteriol. **169**:3988-3993.

100. **Lindahl, T. and B. Sedgwick.** 1988. Regulation and expression of the adaptive response to alkylating agents. Ann. Rev. Biochem. **57**:133-57.

101. **Lindler, L. E. and F.L. Macrina.** 1987. Molecular cloning and characterization of a <u>Streptococcus sanguis</u> DNase necessary for repair of DNA damage induced by UV light and methyl methanesulfonate. J. Bacteriol. **169**:3199-3208.

102. **Little, J.W.** 1984. Autodigestion of <u>lexA</u> and phage lambda repressors. Proc. Natl. Acad. Sci. USA. **81**:1375-1379.

103. **Little, J.W. and D.W. Mount.** 1982. The SOS regulatory system of <u>Escherichia coli</u>. Cell. **29**:11-22.

104. **Lloyd, R.G. and C. Buckman.** 1985. Identification and genetic analysis of <u>sbcC</u> mutations in commonly used <u>recBC sbcB</u> strains of <u>Escherichia coli</u> K-12. J. Bacteriol. **164**:836-844.

105. **Love, P.E. and R.E. Yasbin.** 1986. Induction of the <u>Bacillus subtilis</u> SOS-like response by <u>Escherichia coli</u> RecA protein. Proc. Natl. Acad. **83**:5204-5208.

106. **Lovett, C.M., Jr., P.E. Love, R.E. Yasbin, and J.W. Roberts.** 1988. SOS-like induction in <u>Bacillus subtilis</u>: induction of the RecA protein analog and a damage-inducible operon by DNA damage in Rec[+] and DNA repair-deficient strains. J. Bacteriol. **170**:1467-1474.

107. **Lovett, S.T. and A.J. Clark.** 1983. Genetic analysis of regulation of the RecF pathway of recombination in <u>Escherichia coli</u> K-12. J. Bacteriol. **153**:1471-1478.

108. **Lovett, S.T. and A.J. Clark.** 1984. Genetic analysis of the <u>recJ</u> gene of <u>Escherichia coli</u> K-12. J. Bacteriol. **157**:190-196.

109. **Lovett, S.T. and A.J. Clark.** 1985. Cloning of the <u>Escherichia coli recJ</u> chromosomal region and identification of its encoded proteins. J. Bacteriol. **162**:280-285.

110. **Lu, C. and H. Echols.** 1987. RecA protein and SOS correlation of mutagenesis phenotype with binding of mutant RecA proteins to duplex DNA and LexA cleavage. J. Mol. Biol. **196**:497-504.

111. **Lu, C., R.H. Scheuermann, and H. Echols.** 1986. Capacity of RecA protein to bind preferentially to UV lesions and inhibit the editing subunit (ε) of DNA Polymerase III: a possible mechanism for SOS-induced targeted mutagenesis. Proc. Natl. Acad. Sci. USA. **83**:619-623.

112. **Madiraju, M.V.V.S., A. Templin, and A.J. Clark.** 1988. Properties of a mutant recA-encoded protein reveal a possible role for Escherichia coli recF- encoded protein in genetic recombination. Proc. Natl. Acad. Sci. USA. **85**:6592-6596.

113. **Mahajan, S.K. and A.R. Watta.** 1977. Nature of recombinants produced by the RecBC and the RecF pathways in Escherichia coli. Nature. **266**:652-653.

114. **Maniatis, T., E.F. Fritsch, J.M. Sambrook.** 1982. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

115. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**:208-218.

116. **Marrerro, R. and R.E. Yasbin.** 1988. Cloning of the Bacillus subtilis recE⁺ gene and functional expression of recE⁺ in B. subtilis. J. Bacteriol. **170**:335-344.

117. **Marsh, L. and G.C. Walker.** 1987. New phenotypes associated with mucAB: alteration of a MucA sequence homologous to the LexA cleavage site. J. Bacteriol. **169**:1818-1823.

118. **Matsushima, P. and R.H. Baltz.** 1987. recA gene of Escherichia coli complements defects in DNA repair and mutagenesis in Streptomyces fradiae JS6 (mcr-6). J. Bacteriol. 1987. **169**:4834-4836.

119. **McEachern, F. and L. M. Fisher.** 1989. Regulation of DNA supercoiling in Escherichia coli: genetic basis of a compensatory mutation in DNA gyrase. FEB. **253**:67-70.

120. **McPartland, A., L. Green and H. Echols.** 1980. Control of recA gene RNA in E. coli: regulatory and signal genes. Cell. **20**:731-737.

121. **McEvoy, J.L., H. Murata, and A.K. Chatterjee.** 1990. Molecular cloning and characterization of an Erwinia carotovora subsp. carotovora pectin lyase gene that responds to DNA-damaging agents. J. Bacteriol. **172**:3284-3289.

122. **Mercer, A.A. and J.S. Loutit.** 1978. Transformation and transfection of Pseudomonas aeruginosa with linear DNA. Proc. Unov. Otago Med. Sch. **56**: 56-57.

123. **Miller, J.H.** 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

124. **Miller, R.V. and T.A. Kokjohn.** 1988. Expression of the recA gene of Pseudomonas aeruginosa PAO is inducible by DNA damaging agents. J. Bacteriol. **170**:2385-2387.

125. **Miller, R.V. and T.A. Kokjohn.** 1990. General microbiology of recA: environmental and evolutionary significance. Ann. Rev. Micro. **44**:365-394.

126. **Miller, R.V., T.A. Kokjohn, and G.S. Sayler.** 1990. Environmental and molecular characterization of systems which affect genome alteration in Pseudomonas aeruginosa. pp. 252-268. In Silver, S., A.M. Chakrabarty, B. Iglewski, and S. Kaplan (eds.), Pseudomonas. Biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.

127. **Miller, R.V. and C.-M.C. Ku.** 1978. Characterization of Pseudomonas aeruginosa mutants deficient in the establishment of lysogeny. J. Bacteriol. **134**:875-883.

128. **Moreau, P.L. and M.-F. Carlier.** 1989. RecA protein-promoted cleavage of LexA repressor in the presence of ADP and structural analogues of inorganic phosphate, the fluoride complexes of aluminum and beryllium. J. Biolog. Chem. **264**:2302-2306.

129. **Morelle, G.** 1989. A plasmid extraction procedure on a miniprep scale. Focus **11**:7-8.

130. **Mount, D.W.** 1977. A mutant of Escherichia coli showing constituitive expression of the lysogenic induction and error-prone DNA repair pathways. Proc. Natl. Acad. Sci. **74**:300-304.

131. **Murphy, R.C., G.E. Gasparich, D.A. Bryant, and R.D. Porter.** 1990. Nucleotide sequence and further characterization of the Synechococcus sp. strain PCC 7002 recA gene: complementation of a cyanobacterial recA mutation by the Escherichia coli recA gene. J. Bacteriol. **172**:967-976.

132. **Nakayama, K., N. Irino, H. Nakayama.** 1985. The recQ gene of Escherichia coli K-12: molecular cloning and isolation of insertion mutants. Mol. Gen. Genet. **200**: 266-271.

133. **Neidhardt, F.C., R.A. VanBogelen, and V. Vaughn.** 1984. The genetics and regulation of heat-shock proteins. Ann. Rev. Genet. **18**:295-329.

134. **Nohmi, T., J.R. Battista, L.A. Dodson, and G.C. Walker.** 1988. RecA-mediated cleavage activates UmuD for mutagenesis: mechanistic relationship between transcriptional derepression and posttranslational activation. Proc. Natl. Acad. Sci. USA. **85**:1816-1820.

135. **Notani, N.K. and J.K. Setlow.** 1980. Inducible repair system in <u>Haemophilus influenzae</u> unaccompanied by mutation. J. Bacteriol. **143:**516-519.

136. **Ohman, D.E., J.B. Goldberg, and J.L. Flynn.** 1990. Molecular analysis of the genetic switch activating alginate production. pp. 28-35. <u>In</u> Silver, S., A.M. Chakrabarty, B. Iglewski, and S. Kaplan (eds.), <u>Pseudomonas</u>. Biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.

137. **Ohman, D.E., M.A. West, J.L. Flynn, and J.B. Goldberg.** 1985. Method for gene replacement in <u>Pseudomonas aeruginosa</u> used in construction of <u>recA</u> mutants: <u>recA</u>-independent instability of alginate production. J. Bacteriol. **162:**1068-1074.

138. **Oishi, M. and C.L. Smith.** 1978. Inactivation of phage repressor in a permeable cell system: Role of <u>recBC</u> DNAase in induction. Proc. Natl. Acad. Sci. **75:** 3569-3573.

139. **Orrego, C. and E. Eisenstadt.** 1987. An inducible pathway is required for mutagenesis in <u>Salmonella typhimurium</u> LT2. J. Bacteriol. **169:**2885-2888.

140. **Ossanna, N., K.R. Peterson, and D.W. Mount.** 1986. Genetics of DNA repair in bacteria. Trends Genet. **2:**55-8.

141. **Ossanna, N., K.R. Peterson, and D.W. Mount.** 1987. UV-inducible SOS response in <u>Escherichia coli</u>. Photochem. Photobiol. **45:**905-908.

142. **Owttrim, G.W. and J.R. Coleman.** 1987. Molecular cloning of a <u>recA</u>-like gene from the cyanobacterium <u>Anabaena variabilis</u>. J. Bacteriol. **169:**1824-1829.

143. **Peak, M.J. and J. Peak.** 1989. Solar-ultraviolet- induced damage to DNA. Photodermatol. **6:**1-15.

144. **Perry, K.L., S.J. Elledge, B.B. Mitchell, L. Marsh, and G.C. Walker.** 1985. <u>umuDC</u> and <u>mucAB</u> operons whose products are required for UV light- and chemical- induced mutagenesis: UmuD, MucA, and LexA proteins share homology. Proc. Natl. Acad. Sci. USA. **82:**4331-4335.

145. **Peterson, K.R., N. Ossanna, A.T. Thliveris, D.G. Ennis, and D.W. Mount.** 1988. Derepression of specific genes promotes DNA repair and mutagenesis in <u>Escherichia coli</u>. **170:**1-4.

146. **Phillips, I., E. Culebras, F. Moreno, and F. Baquero.** 1987. Induction of the SOS response by new 4-quinolones. J. Antimicrob. Chemother. **20:**631-638.

147. **Piddock, L.J.V. and R. Wise.** 1987. Induction of the SOS response in <u>Escherichia coli</u> by 4-quinolone antimicrobial agents. FEMS Microbiol. Letters. **41:**289-294.

148. **Pollard, E.C., and P.M. Achey.** 1975. Induction of radioresistance in Escherichia coli. Biophys. J. **15**:1141-1154.

149. **Potter, A.A. and J. S. Loutit.** 1982. Exonuclease activity from Pseudomonas aeruginosa which is missing in phenotypically restrictionless mutants. J. Bacteriol. **151**:1204-1209.

150. **Quinones, A., R. Piechocki, and W. Messer.** 1988. Expression of the Escherichia coli dnaQ (mutD) gene is inducible. Mol. Gen. Genet. **211**:106-112.

151. **Ramesar, R.S., V.Abratt, D.R. Woods and D.E. Rawlings.** 1989. Nucleotide sequence and expression of a cloned Thiobacillus ferrooxidans recA gene in Escherichia coli. Gene. **78**:1-8.

152. **Rella, M. and D. Haas.** 1982. Resistance of Pseudomonas aeruginosa PAO to nalidixic acid and low levels of β-lactam antibiotics: mapping of chromosomal genes. Antimicrob. Agents Chemother. **22**:242-249.

153. **Rhee, D.-K. and D.A. Morrison.** 1988. Genetic transformation in Streptococcus pneumoniae: molecular cloning and characterization of recP, a gene required for genetic recombination. J. Bacteriol. **170**:630-637.

154. **Rolfe, B. and B.W. Holloway.** 1968. Genetic control of DNA specificity in Pseudomonas aeruginosa. Genet. Res. Camb. **12**:99-102.

155. **Ronson, C.W., B.T. Nixon, and F.M. Ausubel.** 1987. Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. Cell. **49**: 579-581.

156. **Ruvkun, G.B. and F.M. Ausubel.** 1981. A general method for site-directed mutagenesis in prokaryotes. Nature. **289**:85-88.

157. **Salles, B., J.M. Weisemann, and G.M. Weinstock.** 1987. Temporal control of colicin E1 induction. J. Bacteriol. **169**:5028-5034.

158. **Sano, Y. and M. Kageyama.** 1987. The sequence and function of the recA gene and its protein in Pseudomonas aeruginosa PAO. Mol. Gen. Genet. **208**:412-419.

159. **Sargentini, N.J. and K.C. Smith.** 1986. Quantitation of the involvement of the recA, recB, recC, recF, recJ, recN, lexA, radA, radB, uvrD, and umuC genes in the repair of X-ray-induced DNA double-strand breaks in Escherichia coli. Radiation Research. **107**:58-72.

160. **Schendel, P.F., M. Fogliano, and L.D. Strausbaugh.** 1982. Regulation of the Escherichia coli K-12 uvrB operon. J. Bacteriol. **150**:676-685.

161. **Schoemaker, J.M., R.C. Gayda, and A. Markovitz.** 1984. Regulation of cell division in Escherichia coli: SOS induction and cellular location of the SulA protein, a key to lon-associated filamentation and death. J. Bacteriol. **158**:551-561.

162. **Schultz, J.E., G.I. Latter, and A. Matin.** 1988. Differential regulation by cyclic AMP of starvation protein synthesis in Escherichia coli. J. Bacteriol. **170:** 3903-3909.

163. **Sedgwick, S.G.** 1986. Inducible DNA repair in microbes. Micro. Sci. **3:**76-83.

164. **Sedgwick, S.G.** 1986. Stability and change through DNA repair. pp. 233-289. In D.J. Galas, T.B.L. Kirkwood and R.F. Rosenberger (eds.), Accuracy in Molecular Processes. Chapman and Hall, London.

165. **Sedgwick, S.G. and P.A. Goodwin.** 1985. Differences in mutagenic and recombinational repair in enterobacteria. Proc. Natl. Acad. Sci. USA. **82:** 4172-4176.

166. **Setlow, J.K., D. Spikes, and K. Griffin.** 1988. Characterization of the rec-1 gene of Haemophilus influenzae and behavior of the gene in Escherichia coli. J. Bacteriol. **170**:3876-3881.

167. **Shen, L.L., W.E. Kohlbrenner, D. Weigl, and J. Baranowski.** 1989. Mechanism of quinolone inhibition of DNA gyrase. J. Biol. Chem. **264**:2973-2978.

168. **Shinagawa, J., H. Iwasaki, T. Shiba, and A. Nakata.** 1988. RecA dependent proteolytic cleavage of UmuD and MucA proteins in SOS mutagenesis. Abstr. 17th Ann. Meet. J. Cell. Biochem., Taos, New Mexico, 17-30 January 1988, p.249, Abst. No. E032.

169. **Shinagawa, J., H. Iwasaki, J. Kato, and A. Nakata.** 1988. RecA protein-dependent cleavage of UmuD protein and SOS mutagenesis. Proc. Natl. Acad. Sci. USA. **85**:1806-1810.

170. **Shinomiya, T.** 1984. Phenotypic mixing of pyocin R2 and bacteriophage PS17 in Pseudomonas aeruginosa PAO. J. Virol. **49**:310-314.

171. **Shinomiya, T.** 1974. Studies on the biosynthesis and morphogenesis of R-type pyocins of Pseudomonas aeruginosa J. Biochem. **76**:1830-1840.

172. **Shinomiya, T. and S. Ina.** 1989. Genetic comparison of bacteriophage PS17 and Pseudomonas aeruginosa R-type pyocin. J. Bacteriol. **171**:2287-2292.

173. **Shinomiya, T., S. Shiga, and M. Kageyama.** 1983. Genetic determinant of pyocin R2 in Pseudomonas aeruginosa PAO. I. Localization of the pyocin R2 gene cluster between the trpCD and trpE genes. Mol. Gen. Genet. **189**:375-381.

174. **Shinomiya, T., S. Shiga, A. Kiguchi, and M. Kageyama.** 1983. Genetic determinant of pyocin R2 in Pseudomonas aeruginosa PAO. II. Physical characterization of pyocin R2 genes using R-prime plasmids constructed from R68.45. Mol. Gen. Genet. **189**:382-389.

175. **Simonson, C.S., T.A. Kokjohn, and R.V. Miller.** 1990. Augmentation of the UV repair potential of Pseudomonas aeruginosa PAO by plasmid R2. J. Gen. Micro. **136**:1241-1249.

176. **Singer, J.T.** 1989. Molecular cloning of the recA analog from the marine fish pathogen Vibrio anguillarum 775. J. Bacteriol. **171**:6367-6371.

177. **Skavronskaia, A.G., G.I. Aleshkin, I.G. Tiganova, O.I. Rusina, I.V. Andreeva.** 1988. SOS-induction of the RP4 plasmid tet-determinant. Mol. Gen. Mikrobiol. Virusol. **8**:17-23.

178. **Smith, G.R.** 1989. Homologous recombination in E. coli: multiple pathways for multiple reasons. Cell. **58**:807-809.

179. **Smith, C.M. and E. Eisenstadt.** 1989. Identification of a umuDC locus in Salmonella typhimurium LT2. J. Bacteriol. **171**:3860-3865.

180. **Smith, K.C. and R.C. Sharma.** 1987. A model for the recA-dependent repair of excision gaps in UV-irradiated Escherichia coli. Mut. Res. **183**:1-9.

181. **Southern, E.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503-517.

182. **Spangler, R., R. Zhang, J. Krueger, and G. Zubay.** 1985. Colicin synthesis and cell death. J. Bacteriol. **163**:167-173.

183. **Stachel, S.E., G. An, C. Flores, and E.W. Nester.** 1985. A Tn3 lacZ transposon for the random generation of β-galactosidase gene fusions: application to the analysis of gene expression in Agrobacterium. EMBO J. **4**:891-898.

184. **Talmadge, K., and W. Gilbert.** 1980. Construction of plasmid vectors with unique PstI cloning sites in a signal sequence coding region. Gene. **12**:235-241.

185. **Tessman, I.** 1985. UV-induced mutagenesis of phage S13 can occur in the absence of the RecA and UmuC proteins of Escherichia coli. Proc. Natl. Acad. Sci. USA. **82**:6614-6618.

186. **Tessman, E.S. and P.K. Peterson.** 1985. Isolation of protease-proficient, recombinase-deficient recA mutants of Escherichia coli K-12. J. Bacteriol. **163**: 688-695.

187. **Thoms, B. and W. Wackernagel.** 1984. Genetic control of damage-inducible restriction alleviation in Escherichia coli K12: an SOS function not repressed by LexA. J. Bacteriol. **169**:4816-4821.

188. **Thoms, B. and W. Wackernagel.** 1987. Regulatory role of recF in the SOS response of Escherichia coli: impaired induction of SOS genes by UV irradiation and nalidixic acid in a recF mutant. J. Bacteriol. **169**:1731-1736.

189. **Thoms, B. and W. Wackernagel.** 1988. Suppression of the UV-sensitive phenotype of Escherichia coli recF mutants by recA(srf) and recA (Tif) mutations requires recJ⁺. J. Bacteriol. **170**:3675-3681.

190. **Tonetti, D.A.** 1990. Molecular characterization of qin: a novel gene of Pseudomonas aeruginosa involved in quinolone sensitivity. Ph.D. Dissertation, Loyola University of Chicago.

191. **Totten, P.A., J. Cano Lara, and S. Lory.** 1990. The rpoN gene product of Pseudomonas aeruginosa is required for expression of diverse genes, including the flagellin gene. J. Bacteriol. **172**:389-396.

192. **Torrey, T.A. and T. Kogoma.** 1987. Genetic analysis of constitutive stable DNA replication in rnh mutants of Escherichia coli K12. Mol. Gen. Genet. **208**:420-427.

193. **Trgovcevic, Z., D. Petranovic, E. Salaj-Smic and M. Petranovic.** 1987. Kinetics of recB-dependent repair: relationship to post-UV inactivation of the prophage. Mut. Res. **184**:1-6.

194. **Van Bogelen, R.A., P.M. Kelley, and F.C. Neidhardt.** 1987. Differential induction of heat shock, SOS, and oxidation stress regulons and accumulation of nucleotides in Escherichia coli. J. Bacteriol. **169**:26-32.

195. **Van Houton, B. and A. Sancar.** 1987. Repair of N-methyl-N'-nitro-N-nitrosoguanidine induced DNA damage by ABC excinuclease. J. Bacteriol. **169**:540-545.

196. **Vericat, J.-A., R. Guerrero, and J. Barbe.** 1988. Inhibition of the SOS response of Escherichia coli by the Ada protein. J. Bacteriol. **170**:1354-1359.

197. **Volkert, M.R. and M.A. Hartke.** 1984. Suppression of Escherichia coli recF mutations by recA-linked srfA mutations. J. Bacteriol. **157**:498-506.

198. **Volkert, M.R. and M.A. Hartke.** 1987. Effects of the Escherichia coli recF suppressor mutation, recA801, on recF-dependent DNA-repair associated phenomena. Mutat. Res. **184**:181-186.

199. Volkert, M.R., L.J. Margossian, and A.J. Clark. 1984. Two-component suppression of recF143 by recA441 in Escherichia coli K-12. J. Bacteriol. **160:** 702-705.

200. Walker, G.C. 1984. Mutagenesis and inducible responses to deoxyribonucleic acid damage in Escherichia coli. Micro. Rev. **48:**60-93.

201. Walker, G.C. 1985. Inducible DNA repair systems. Ann. Rev. Biochem. **54:** 425-57.

202. Walker, G.C. 1989. General principles of DNA repair in microorganisms and implications for future research. Birth Defects. **25:**45-49.

203. Walker, G.C., C.J. Kenyon, A. Bagg, S.J. Elledge, K.S. Perry, and W.G. Shanabruch. 1982. Regulation and functions of Escherichia coli genes induced by DNA damage. pp. 42-63. In J.F. Lemontt and W.M. Generoso (ed.), Molecular and Cellular Mechanisms of Mutagenesis. Plenum Publishing Corp., New York.

204. Walker, G.C., L. Marsh, and L.A. Dodson. 1985. Genetic analysis of DNA repair: inference and extrapolation. Ann. Rev. Genet. **19:**103-26.

205. Walkup, L.K. and T. Kogoma. 1989. Escherichia coli proteins inducible by oxidative stress mediated by the superoxide radical. J. Bacteriol. **171:**1476-1484.

206. Wang, T-C.V., and K.C. Smith. 1983. Mechanisms for recF-dependent and recB-dependent pathways of postreplication repair in UV-irradiated Escherichia coli uvrB. J. Bacteriol. **156:**1093-1098.

207. Wang, T-C.V., and K.C. Smith. 1985. Mechanism of sbcB-suppression of the recBC-deficiency in postreplication repair in UV-irradiated Escherichia coli K-12. Mol. Gen. Genet. **201:**186-191.

208. Wang, T.-C.V., and K.C. Smith. 1986. recA (Srf) suppression of recF deficiency in the postreplication repair of UV-irradiated Escherichia coli K-12. J. Bacteriol. **168:**940-946.

209. Wanner, B.L., and R. McSharry. 1982. Phosphate-controlled gene expression in Escherichia coli K12 using Mud1-directed lacZ fusions. J. Mol. Biol. **158:** 347-363.

210. Weigle, J.J. 1953. Induction of mutation in a bacterial virus. Proc. Natl. Acad. Sci. USA. **39:**628-636.

211. Weiss, B. and L. Grossman. 1987. Phosphodiesterases involved in DNA repair. Adv. Enzymol. **60:**1-34.

212. **Willett, H.P.** 1980. Action of chemotherapeutic agents. pp.234-277. In Joklik, W.K., H.P. Willett, and D.B. Amos, Zinsser Microbiology, 17th Edition. Appleton-Century-Crofts, NY.

213. **Witkin, E.M.** 1976. Ultraviolet mutagenesis and inducible DNA repair in Escherichia coli. Bacteriol. Rev. **40**:869-907.

214. **Witkin, E.M., V. Roegner-Maniscalco, J.B. Sweasy, and J.O. McCall.** 1987. Recovery from ultraviolet light-induced inhibition of DNA synthesis requires umuDC gene products in recA718 mutant strains but not in recA⁺ strains of Escherichia coli. Proc. Natl. Acad. Sci. USA. **84**:6805-6809.

215. **Wong, S.C. and A.T. Abdelal.** 1990. Unorthodox expression of an enzyme: evidence for an untranslated region within carA from Pseudomonas aeruginosa. J. Bacteriol. **172**:630-642.

216. **Woodgate, R., B.A. Bridges, G. Herrara, and M. Blanco.** 1987. Mutagenic DNA repair in Escherichia coli XIII. Proofreading exonuclease of DNA Polymerase III holoenzyme is not operational during UV mutagenesis. Mut. Res. **183**:31-37.

217. **Youngman, P., P. Zuber, J.B. Perkins, K. Sandman, M. Igo, R. Losick.** 1985. New ways to study developmental genes in spore-forming bacteria. Science. **228**:285-91.

218. **Zink, R.T., Engwall, J.K., J.L. McEvoy, A.K. Chatterjee.** 1985. recA required in the induction of pectin lyase and carotovoricin in Erwinia carotovora subsp. carotovora. J. Bacteriol. **164**:390-396.

VITA

Audrey L. Warner was born in Chicago, Illinois on August 23, 1962, to Kristy M. Hope Warner and Clifford A. Warner.

In 1981, she was fortunate to receive a Scholarship from St. Xavier College in Chicago, IL. During her first two years of college, Audrey majored in psychology. However, her interest in chemistry and genetics persuaded her to change her major to biology in her third year. This change was fortuitous, as the following year she was nominated by St. Xavier College to The National Dean's List and Who's Who Among Students in American Universities and Colleges.

Audrey received a Bachelor of Science degree in January of 1985, and that same year entered the doctoral program of the Department of Biochemistry and Biophysics at Loyola. Soon after, she initiated her research on Pseudomonas aeruginosa stress responsive genes in the laboratory of Dr. Robert V. Miller. While enrolled at Loyola, Audrey was honored to receive a Loyola University Basic Science Fellowship, a National Science Foundation Honorable Mention, and an Arthur J. Schmitt Foundation Dissertation Fellowship. However, she considers her greatest accomplishment during that period to be her marriage to Gregory J. Bartnicki on August 29, 1987.

The author recently completed her doctoral research. Currently she is conducting postdoctoral studies on human cell differentiation in the laboratory of Dr. Eliezer Huberman at Argonne National Laboratory in Argonne, Illinois.

DISSERTATION APPROVAL SHEET

The dissertation submitted by Audrey L. Warner-Bartnicki has been read and approved by the following committee:

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

8 November, 1990 _Robert V. Miller_
Date   Director's Signature