IncN plasmids mediate UV resistance and error-prone repair in *Pseudomonas aeruginosa* PAO

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While it seems likely that the ability to induce the expression of *recA*-controlled genes is nearly universal among the eubacteria, the expression of plasmid-borne ultraviolet (UV)-resistance and mutagenesis genes seems to be species-dependent in a complex fashion. Some plasmids encoding UV-resistance and mutagenesis functions only express these phenotypes in a select number of bacterial species. Several UV-resistance plasmids that express these functions in *Escherichia coli* are either unstable or simply do not express the UV-resistance-mutagenesis phenotype in *Pseudomonas aeruginosa*. In order to clarify the role of these plasmids in microbial ecology, we have undertaken a study of the ability of the well-characterized UV-resistance IncN plasmids pKM101 and R46 to express the UV-resistance phenotype in *P. aeruginosa*. In addition, we have examined the IncP plasmids RP4 and R68.45, observed to confer a UV-resistant phenotype upon *Myxococcus xanthus*, for the ability to express this phenotype in *P. aeruginosa*. Our experiments reveal that while pKM101 and R46 transfer to *P. aeruginosa* at a very low frequency, these plasmids, once transferred, are maintained and clearly support the expression of the UV-resistance and mutagenesis phenotype observed in *E. coli*. Studies of plasmids R68.45 and RP4 in *P. aeruginosa* revealed that they do not express UV-resistance functions in this species. UV-resistance plasmids may play an important role in the natural ecology of bacterial habitats exposed to solar radiation or to various DNA-damaging natural and man-made chemicals.

Keywords: *Pseudomonas aeruginosa*, UV-resistance plasmids, Weigle reactivation/mutagenesis, *recA*, SOS response

INTRODUCTION

A large number of bacterial plasmids have been determined to affect cell survival and mutagenic responses to ultraviolet (UV) irradiation. These plasmids frequently result in a UV-resistant (UV^r) phenotype. Plasmids producing such effects have been observed in a wide variety of bacterial genera (Pinney, 1980; Boronin, 1992).

Plasmid-encoded enhanced UV resistance may provide a selective advantage to the host in certain environments. Both enhanced mutagenesis and additional resistance to UV damage have the potential to impart additional fitness to the bacterium in selected habitats (Sedgwick & Goodwin, 1985; Sedgwick *et al.*, 1989). However, the frequency and dynamics of UV-resistance plasmids in natural ecosystems and the contribution of such plasmids to cell survival and evolution are unknown.

Molecular analyses of some of the plasmid genes responsible for the UV^r phenotype has revealed their

relationship to the *umuDC* operon of *Escherichia coli* (Walker, 1984; Strike & Lodwick, 1987). The chromosomal *umuDC* genes are required for UV-induced mutagenesis in *E. coli* and are components of the SOS network. The *mucAB* operon of pKM101 (Walker, 1984) and the *impCAB* genes of TP110 (Glazebrook *et al.*, 1986) produce analogues of the *umuDC* operon that are clearly controlled by the host *lexA-recA* regulatory system.

Many bacterial species have been shown to encode analogues of the *recA* gene of *E. coli* (Miller & Kokjohn, 1990). Some of these analogues are capable of inducing the SOS network of *E. coli* (Miller & Kokjohn, 1990). Experimental evidence makes clear that the capacity to induce expression of analogues of *mucAB* present on UV-resistance plasmids will be widely distributed among the bacteria.

Both *recA* (Miller & Kokjohn, 1990) and *lexA* (Calero *et al.*, 1991; Garriga *et al.*, 1992) structural analogues have been identified in several species of *Pseudomonas* including

Table 1. Bacterial strains used in this study

Bacterium	Relevant genotype	Reference
<i>Escherichia coli</i>		
AB1157	<i>thi-1 thr-1 leuB6 proA2 his-4 argE3</i>	Clark & Margulies (1965)
<i>Pseudomonas aeruginosa</i>		
PAO1	Prototrophic	Simonson <i>et al.</i> (1990)
PAO25	<i>argF10 leu-10</i> derivative of PAO1	
PAO303	<i>argB21</i> derivative of PAO1	

Pseudomonas aeruginosa. While *P. aeruginosa* has been shown to contain UV-inducible genes (Miller & Kokjohn, 1988; Horn & Ohman, 1988; Warner-Bartnicki & Miller, 1992), several studies have suggested that the *P. aeruginosa* chromosome does not contain functional analogues to the *umuDC* operon (Benbrook & Miller, 1986; McBeth, 1989; Simonson *et al.*, 1990). The expression of plasmid-borne UV-resistance and mutagenesis-promoting genes seems to be species-specific in a complex fashion (Jacoby, 1977; McCann & Clarke, 1981; Tardif & Grant, 1983; Hofemeister *et al.*, 1979).

Moreover, other studies have revealed that these UV-damage-resistance genes may be present on certain broad-host-range plasmids that are expressed only in certain genetic backgrounds (Hofemeister *et al.*, 1979). These studies have revealed that plasmids RP1 and R68.45 support UV-damage-induced repair and mutagenesis in *Myxococcus* sp. but not in a variety of other bacterial species. The molecular basis for the UV-resistance phenotype exhibited in *Myxococcus* sp. has not been studied.

Because of the apparent complexities of expression of plasmid-borne UV resistance genes in bacteria, we have undertaken a series of experiments to assess the potential of these plasmids to express the UV^r phenotype in *P.*

aeruginosa, a species in which repair and mutagenesis have been extensively characterized. Plasmids carrying *mucAB* analogues of the *E. coli umuDC* operons known to be regulated by the *lexA-recA* network were included. These studies allowed a determination of the ability of UV exposure to regulate *mucAB* expression in *P. aeruginosa*.

METHODS

Bacteria, plasmids, and bacteriophages. The bacterial strains and plasmids used in this study are described in Tables 1 and 2. Bacteriophage D3 is a temperate, specialized transducing phage of *P. aeruginosa* PAO (Cavenagh & Miller, 1986). A spontaneously occurring clear-plaque mutant of D3 used in our studies has been designated D3c. Phage lysates were prepared by the method of Miller & Ku (1978).

Media. Luria broth (LB) consists of 1% (w/v) tryptone, 1% (w/v) NaCl and 0.5% yeast extract. Luria agar (LA) is LB containing 1.3% (w/v) agar. Lambda top agar is 1% tryptone, 0.5% NaCl and 0.65% agar. *Pseudomonas* Isolation Agar was purchased from Difco. Saline for dilutions is 0.85% NaCl. TNM buffer is 10 mM Tris/HCl, pH 7.4, 150 mM NaCl and 10 mM MgSO₄. Antibiotics were obtained from Sigma and used at the following concentrations: rifampicin, 75 µg ml⁻¹; carbenicillin, 300 µg ml⁻¹ for *P. aeruginosa*; ampicillin, 50 µg ml⁻¹ for *E. coli*; tetracycline, 12.5 µg ml⁻¹ for *E. coli* and 250 µg ml⁻¹ for *P. aeruginosa*.

Plasmid conjugation procedure. Recipient cells (*P. aeruginosa*) were grown at 37 °C in LB to late exponential phase. Donors (*E. coli*) strains were grown at 37 °C to mid-exponential phase in LB under antibiotic selection. The cells were harvested by centrifugation, suspended in an equal volume of LB and mixed in a donor:recipient ratio of 1:1. The conjugation mixture was spread on an LA plate and incubated at room temperature for 24–48 h. The cells were removed from the plate by washing with saline and the resulting suspension was plated on *Pseudomonas* Isolation Agar containing carbenicillin.

UV protection and chromosomal mutagenesis procedures. *P. aeruginosa* cells were grown at 37 °C in LB. Medium for strains containing plasmids was supplemented with carbenicillin. Cells were harvested by centrifugation, and the pellet suspended in an equal volume of 0.85% saline. The saline-suspended cells were

Table 2. Plasmids used in this study

Plasmid	Incompatibility group <i>E. coli</i> / <i>P. aeruginosa</i> †	Relevant phenotype‡	Reference
RP4	IncP/IncP-1	Ap(Cb) Tc Km UV*	Jacoby (1978)
R68.45	IncP/IncP-1	Ap(Cb) Tc Km UV*	Haas & Holloway (1978)
R2	NR/IncP-9	Cb Sm Tc Su UV in <i>P. aeruginosa</i>	Simonson <i>et al.</i> (1990)
R46	IncN/?	Ap(Cb) Tc Km Su Sm UV	Mortelmans & Stocker (1976)
pKM101	IncN/?	Ap(Cb) Km UV	Walker (1977)

† NR, Does not replicate in *E. coli*; ?, IncP incompatibility group unknown, will replicate in *P. aeruginosa*.

‡ Ap(Cb), resistance to ampicillin in *E. coli* and carbenicillin in *P. aeruginosa*; UV*, resistance phenotype expressed in *Myxococcus xanthus* (McCann & Clark, 1981).

exposed to UV radiation from a GE germicidal lamp. Dose was determined with a UVX radiometer (Ultraviolet Products). Appropriate dilutions were made in 0.85 % saline with 0.1 ml samples plated on LA and incubated in the dark at 37 °C to determine percentage survival. Precautions were taken to avoid photoreactivation.

The chromosomal mutation frequency was estimated by plating 0.1 ml samples of irradiated cells on LA followed by overnight incubation at 37 °C. These plates were replica-plated onto LA containing rifampicin to determine the frequency of rifampicin-resistant cells (Simonson *et al.*, 1990).

Host cell reactivation (HCR) procedures. Lysates of phage D3c (2×10^8 p.f.u. ml⁻¹) in TNM buffer were exposed to various doses of UV radiation and used to infect host cells in the early exponential phase of growth. Lambda top agar was added, and the mixture plated on LA containing appropriate antibiotics. Phage titres were determined after incubation at 37 °C in the dark overnight.

Weigle reactivation and mutagenesis procedures. For Weigle reactivation experiments, lysates of phage D3c were irradiated with a dose of UV that resulted in approximately 0.01 % plaque survival in unirradiated host bacteria. Irradiated phage lysates were diluted in TNM buffer. Bacteria were grown in LB to early exponential phase, harvested by centrifugation, suspended in an equal volume of 0.85 % saline, and exposed to various doses of UV radiation. Samples (0.1 ml) of irradiated bacteria were mixed with dilutions of unirradiated and irradiated phage lysates. Phage adsorption was allowed to occur at 37 °C in the dark. Lambda top agar was added and the mixture poured onto LA plates containing appropriate antibiotics. The plates were incubated overnight at 37 °C in the dark. The Weigle reactivation factor (WRF) was calculated from the formula

$$\text{WRF} = \frac{[(\text{p.f.u.})_{\text{UV}} / (\text{p.f.u.})_0]_{\text{on irradiated host}}}{[(\text{p.f.u.})_{\text{UV}} / (\text{p.f.u.})_0]_{\text{on unirradiated host}}}$$

where $(\text{p.f.u.})_{\text{UV}}$ is the titre of irradiated phages and $(\text{p.f.u.})_0$ is the titre of the unirradiated lysate (Simonson *et al.*, 1990).

Weigle mutagenesis of phages was determined using cell-free lysates of wild-type D3. Irradiation of host cells and phage infections were performed as described for Weigle reactivation experiments. After overnight incubation at 37 °C in the dark, the frequency of clear-plaque mutants among the total plaques produced was determined.

RESULTS

UV survival of plasmid-containing *P. aeruginosa*

Plasmids were introduced into *P. aeruginosa* and their UV survival compared to isogenic strains lacking the plasmid (Fig. 1). Plasmid R2 (IncP-9) and the IncN plasmids, R46 and pKM101, were transferrable to *P. aeruginosa* and clearly resulted in increased UV resistance (Fig. 1a). While the IncP-1 plasmids, RP4 and R68.45 (Fig. 1b), were transferrable to *P. aeruginosa*, their presence did not confer enhanced UV resistance. Thus, it appeared that the UV^r phenotype of pKM101 was stable and expressed in a *P. aeruginosa* background, while the species-specific UV-resistance character of the IncP-1 UV-resistance plasmids was not supported by the *P. aeruginosa* genetic and physiological background.

Fig. 2. Host-cell reactivation (HCR) of plasmid-containing *P. aeruginosa*. HCR was carried out as described in Methods, and strains with and without plasmids were compared. Lysates of phage D3c were irradiated with various doses of UV radiation and infected into host bacteria grown to the early exponential phase. Titres of irradiated phages were compared to unirradiated lysates in the same host. Means and standard errors are presented. *P. aeruginosa* PAO25 (○), PAO25(R2) (●), PAO25(pKM101) (□).

Fig. 1. UV survival of plasmid-containing *P. aeruginosa*. Plasmids were introduced into *P. aeruginosa* PAO strains, and their UV survival was compared with the parental strain that did not contain the plasmid. Cultures were grown to early exponential phase and exposed to various doses of UV radiation. The frequency of surviving cells was determined by plating on LB agar. Means and standard errors are presented. (a) *P. aeruginosa* PAO25 (○), PAO25(R2) (●), PAO25(R46) (□), PAO25(pKM101) (■). (b) PAO303 (○), PAO303(R2) (●), PAO303(RP4) (□), PAO303(R68.45) (■).

Fig. 3. Plasmid-stimulated Weigle reactivation of *P. aeruginosa* PAO25. Irradiated and unirradiated bacteriophages were used to infect host bacteria which had been exposed to various low doses of UV radiation. The Weigle reactivation factor (WRF) was calculated from the formula given in Methods and is a measure of the inducible DNA repair capacity of the host cell. Means and standard errors are presented. (a) PAO25 (○), PAO25(R2) (●). (b) PAO25 (○), PAO25(pKM101) (●).

Fig. 4. UV-enhanced mutagenesis of *P. aeruginosa*. Spontaneous (□) and induced (▨) levels of mutagenesis associated with DNA-damage repair were compared for strains of PAO25 with and without various plasmids. (a) Induced levels of chromosomal mutation at 50% survival are compared to levels in cell populations that were not exposed to UV radiation. (b) Weigle mutagenesis frequencies of UV-irradiated wild-type D3 phages infected into unirradiated hosts (spontaneous) are compared to rates in hosts exposed to a dose of 3 J m⁻² (induced).

Host cell reactivation (HCR)

UV resistance measured by kill curves potentially includes constitutively expressed and damage-inducible components. To assess if enhanced UV resistance encoded by plasmids in the IncP-9 and IncN incompatibility groups

includes a constitutive component, a cell-free lysate of bacteriophage D3c was irradiated with various doses of UV radiation and the ability of cells with and without the plasmids R2 and pKM101 to repair the damage done to the phage genome was evaluated (Fig. 2). Both plasmids increased the host cell's capacity to repair DNA damage in the UV-irradiated phages. These data may indicate that the introduction of damaged phage DNA into the *P. aeruginosa* host cell acts to partially induce the UV-resistance gene of these plasmids, as has been observed in *E. coli* (Sedgwick, 1986; Ossanna *et al.*, 1987). Alternatively, the increase in HCR when these plasmids are introduced into RecA⁺ *P. aeruginosa* may indicate that at least a fraction of the plasmid-encoded UV resistance potential is expressed constitutively in *P. aeruginosa*. McNally *et al.* (1990) have shown that a change of as little as one base pair in the SOS (LexA-binding) Box present in the *mucAB* genes of pKM101 allows some constitutive expression of these genes in *E. coli*. Any difference in the binding sequence required for binding of the presumptive *P. aeruginosa* LexA analogue protein could lead to low levels of expression of these plasmid-encoded UV-resistance genes even in the absence of UV exposure.

Plasmid-stimulated Weigle reactivation

The inducible nature of plasmid-augmented UV resistance was investigated by quantifying the potential of cells with and without plasmids to carry out Weigle reactivation of UV-damaged bacteriophages. If DNA-damage-inducible repair is operative, a net increase in the production of plaques resulting from infection of UV-irradiated host cells is expected. The presence of IncP-9 or IncN plasmids significantly increases the level of Weigle reactivation and is clearly DNA-damage-inducible (Fig. 3). *P. aeruginosa* PAO expresses only a marginal level of repair in the absence of plasmids.

Induction of mutagenesis by plasmid-encoded repair systems

The ability of plasmids to stimulate chromosomal mutagenesis was quantified by determining the frequency of rifampicin-resistant cells in populations of *P. aeruginosa*. The presence of IncP-9 or IncN plasmids significantly increased the ability of cells to support DNA-damage-inducible mutagenesis (Fig. 4a). Very little stimulation of mutagenesis was observed in cells that did not contain a plasmid. Spontaneous mutagenesis levels were also increased approximately tenfold in the presence of either plasmid. The spontaneous mutation frequency of *P. aeruginosa* PAO25 to Rif^r was 1.7×10^{-7} . This frequency was increased to 1.2×10^{-6} when R2 (IncP-9) was present in the cell and to 1.6×10^{-6} when pKM101 was present.

The frequency of clear-plaque mutants present among total plaques resulting from infection of cells with UV-damaged phages was determined by carrying out Weigle mutagenesis experiments (Fig. 4b). This analysis clearly demonstrates that IncP-9 and IncN plasmids resident in *P. aeruginosa* support inducible DNA repair and mutagenesis.

DISCUSSION

Taken together, our experiments clearly indicate that plasmids pKM101 and R46 can be mobilized into *P. aeruginosa* PAO, that they can replicate in this species and that these plasmids are capable of mediating DNA-damage-inducible UV resistance and mutagenesis activities in *P. aeruginosa*. However, the conjugal transfer of pKM101 from *E. coli* to *P. aeruginosa* PAO is inefficient. While some IncN plasmids or portions of IncN plasmids have been observed to integrate into the *P. aeruginosa* chromosome (Tardif & Grant, 1982), it appears that replication of pKM101 was autonomous. Agarose gel electrophoretic analysis of plasmid DNA prepared from our *P. aeruginosa* transformants revealed the presence of plasmid DNA molecules of the same molecular size as pKM101. When this DNA was used to transform *E. coli*, the UV^r phenotype was expressed and plasmid DNA could be isolated from the *E. coli* transformants (data not shown).

Our experiments suggest that control elements such as analogues of *E. coli* LexA and RecA proteins may be functioning in the induction of the UV-resistance and mutagenesis phenotype following exposure of plasmid-containing cells to UV radiation. Garriga *et al.* (1992) have recently identified a potential *lexA* analogue in *P. aeruginosa* which has significant similarity to the *lexA* gene of *E. coli* and several other Gram-negative species. In addition, the *P. aeruginosa* *recA* gene contains a LexA-binding SOS consensus sequence (Sano & Kageyama, 1987), and the *P. aeruginosa* RecA protein has been shown to regulate *E. coli* *din*-gene expression (Kokjohn & Miller, 1987). Expression of the R46 and pKM101 *mucAB* genes is regulated by LexA in *E. coli* and the regulatory sequence of these genes contains an SOS Box to which LexA protein has been shown to bind (McNally *et al.*, 1990). Thus, regulation of *mucAB* in *P. aeruginosa* is likely to be via RecA–LexA antagonism.

However, alternative explanations for the regulation of IncN plasmid-encoded enhancement of UV resistance in *P. aeruginosa* should be considered. For instance, it is possible that the *mucAB* genes are expressed at low, but constitutive levels and that *P. aeruginosa*-specific, UV-inducible gene products act to enhance their action following UV exposure. This explanation would be consistent with the HCR results we obtained in this study. Additionally, Sano & Kageyama (1993) recently sequenced the *P. aeruginosa* UV-inducible pyocin AP41 gene and could not identify an SOS Box in the sequence. They also found that, when the gene was introduced into *E. coli*, production of pyocin AP41 was not inducible by exposure to UV radiation. Our research group has made similar observations following introduction of *P. aeruginosa* *din*-gene promoters (Warner-Bartnicki & Miller, 1992) into *E. coli* (A. L. Warner-Bartnicki & R. V. Miller, unpublished). Thus, proteins other than LexA may be required for full induction of SOS gene expression in *P. aeruginosa*.

Final resolution of the question of regulation of the *mucAB* operon in *P. aeruginosa* must await the isolation of

a *P. aeruginosa* *lexA*(def) mutation which does not produce functional LexA repressor. Use of a mutant containing such a mutation will allow measurement and comparison to wild-type cells of *mucAB*-specific mRNA and protein synthesis in an environment in which their expression should be unequivocally constitutive.

The data obtained in this study demonstrate that the IncP-1 plasmids RP4 and R68.45 fail to express UV^r phenotypes in *P. aeruginosa*. Experiments by other investigators have revealed the ability of these plasmids to express UV-inducible repair and mutagenesis phenotypes in some species of bacteria (McCann & Clarke, 1981; Hofemeister *et al.*, 1979; Tardif & Grant, 1983). The reason for species-specific expression of UV repair phenotypes is not clear and is under investigation.

The dynamics and ecology of UV-resistance plasmids in natural populations of bacteria are virtually unknown at present. The exact contribution to cell survival conferred by either plasmid-borne or chromosomal mutagenesis genes is unclear. However, it is now certain that the presence of analogues of *umuDC* is not universally found in the eubacteria. Experiments with *Enterobacteriaceae* have revealed that *umuDC* is not conserved even within a single genus (Sedgwick & Goodwin, 1985). Other investigations with *Pseudomonas* spp. likewise reveal variability in the presence of chromosomally encoded, functional analogues of the *E. coli* *umuDC* genes in this genus (McBeth, 1989; Simonson *et al.*, 1990).

Comprehension of the ecology of UV-resistance plasmids is further complicated by consideration of the effects of solar UV radiation on bacteria. Solar UV radiation consists of the UV portion of the spectrum not filtered out by the ozone layer of Earth, 290 nm and longer wavelengths (Jagger, 1985), but little is known concerning the ability of these wavelengths to induce SOS functions in microbes native to natural environments and their UV-resistance plasmids. Surprisingly, expression of the UV-inducible genes of pKM101 is effectively blocked by solar UV irradiation (Eisenstark, 1989). However, UV-resistance plasmids are readily obtainable in natural aquatic ecosystems (Bale *et al.*, 1988), thus surviving the test of selection.

Our current knowledge is insufficient to allow an estimation of the contribution of UV-resistance genes to plasmid and cell survival in natural ecosystems. We have observed that UV^r markers are often found associated with plasmids which also confer resistance to one or more heavy metals or other toxic compounds. The environmentally significant functions of the systems we equate with UV resistance may be in the protection of the host from the toxic and mutagenic effects of the exposure to, or detoxification of, these elements and compounds. A fuller understanding of the molecular basis for UV resistance associated with these plasmids will provide insight into the ways in which bacteria have dealt with various kinds of DNA-damaging stress through their evolutionary and recent history.

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