

Effects of FP2 and a Mercury Resistance Plasmid from *Pseudomonas aeruginosa* PA103 on Exoenzyme Production

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Plasmids encoding mercury resistance carried by *Pseudomonas aeruginosa* PAO1161 and PA103 were found to be involved in regulating the secretion of protease, phospholipase C, and alkaline phosphatase. Previously, mutations in *Pseudomonas* strains that caused pleiotropic effects on the production of extracellular enzymes were mapped to the bacterial chromosome. We show that pleiotropic changes in extracellular enzyme production can also be regulated by plasmids. In this study, the effects on secretion of exoenzymes by two mercury resistance plasmids, FP2 from PAO1161 and pRLW103 from PA103, were assayed in *P. aeruginosa* PAO1 and PAO18. The introduction of either plasmid into PAO1 resulted in a significant decrease in exoprotease production. Additionally, pRLW103 significantly increased the production of alkaline phosphatase by both strains. Phospholipase C was produced only in strain PAO18 containing the pRLW103 plasmid. FP2 had no effect on alkaline phosphatase or phospholipase C production in either strain and was found to decrease exoprotease secretion only in strain PAO1. The results indicate the *P. aeruginosa* mercury resistance plasmids vary in their ability to modify exoenzyme expression, and this ability is influenced by the host strain.

FP plasmids of *Pseudomonas aeruginosa* are characterized by their ability to promote chromosomal transfer (4) and error-prone DNA repair (13). These plasmids have a broad host range and are readily transferred to strains of *Escherichia coli* and *Klebsiella pneumoniae*, but they are not maintained. Finger and Krishnapillai (4) have shown the FP plasmids fall into five entry exclusion groups and four incompatibility groups. The plasmid FP2 is the sole identified representative of the incompatibility and entry exclusion group IncP-8. This plasmid also has chromosome-mobilizing ability (7) and confers resistance to mercury ions (10).

In this study, we found that *P. aeruginosa* PA103 also contains a self-transmissible mercury resistance plasmid, designated pRLW103. We measured extracellular protease, alkaline phosphatase (APh), phospholipase C (PLC), and total protein secreted by *P. aeruginosa* PAO1 and PAO18 carrying either plasmid FP2 or plasmid pRLW103. We show that the effects from FP2 and the plasmid from PA103 (pRLW103) are strain dependent. In addition, both plasmids were found to be incompatible with the chromosome-mobilizing plasmid R68.45. The data presented here suggest that the net production of *P. aeruginosa* exoenzymes is dependent upon the interaction of chromosomal and plasmid genes.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Strain construction. All mating experiments were done on agar surfaces as described by Haas and Holloway (5). The donor and recipient strains were grown overnight with aeration in Luria broth (10 g of tryptone, 5 g of NaCl, and 5 g of yeast extract per liter) or in 2× TY broth (16 g of tryptone, 5 g of NaCl, and 10 g of yeast extract per liter). The cells were harvested by centrifugation and washed with sterile saline (0.9%). Donor and recipient strains were mixed at ratios of 1:10 or 1:100 and were then spotted onto minimal M9 medium (11) supplemented with the appropriate selective and counterselective compounds. Purified single-colony isolates were replica plated onto nutrient agar containing mercury ions, antibiotics, or both or onto *Pseudomonas* isolation agar (20 g of peptone, 10 g of K₂SO₄, 1.4 g of MgCl₂, and 0.025 g of Irgasan per liter) plates. Mercury ion was added to nutrient agar as HgCl₂ at a concentration of 10⁻⁴ M. Tetracycline and chloramphenicol were used at concentrations of 250 µg/ml each.

The plasmid FP2 was transferred to *P. aeruginosa* PAO1 by mating it with PAO1161. PAO1(FP2) exconjugants were isolated by selecting against the *leu* auxotrophic marker of the donor strain and for the mercury ion resistance marker of the FP2 plasmid by plating it on minimal M9 medium containing 10⁻⁴ M HgCl₂.

Since PAO1 and PA103 are both prototrophs, the mercury resistance plasmid in PA103 was transferred to the intermediate host PAO46 (*argF10 leu-10 rif28* Cm^r). A PAO46 (pRLW103) strain was isolated by selecting for chloramphenicol- and mercury-resistant exconjugates. These exconjugates were screened for the amino acid markers arginine and leucine, and a single colony [PAO46(pRLW103); *arg-10 leu-10* Cm^r Hg^r] was used as the donor in a mating with PAO1. PAO1(pRLW103) was isolated by selecting for mercury ion resistance on minimal medium.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype	Reference or source
PAO1	Prototroph FP ⁻	B. W. Holloway (6)
PAO18	<i>proB64 pur-66</i> FP ⁻	B. Holloway (9)
PAO1161	<i>leu8 res-10</i> FP2 ⁺ Hg ^r	J. Miller ^a
PA103(pRLW103)	Prototroph Hg ^r	C. Saelinger ^b
PAO25(R68.45)	<i>argF10 leu-10</i> (Tc ^r Cb ^r Km ^r Tra Cma)	B. Holloway ^c
PAO46	<i>argF10 leu-10</i> Rif28 FP ⁻ Cm ^r	B. Holloway ^c
pMD100	RP4 <i>tra</i> ColE1 <i>rep</i> Km::Tn501	R. Benzinger ^d

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^b University of Cincinnati, Cincinnati, Ohio.

^c Monash University, Clayton, Victoria, Australia.

^d University of Virginia, Charlottesville.

Production of protease, APh, and PLC. *P. aeruginosa* strains were grown overnight in peptone broth dialysate (PBD) medium (14). The bacterial cells were harvested by centrifugation and resuspended in fresh PBD medium to a cell density of 100 Klett units as measured by using a Klett-Summerson Densitometer (Klett-Summerson, New York, N.Y.) equipped with a red filter. The cultures were incubated for 3 h at 37°C with vigorous shaking. Under these conditions, the number of bacterial cells does not increase. The cells were removed by centrifugation at 12,000 × *g*, and the supernatants were filtered through a 0.45-μm-pore-size filter. These culture conditions were chosen because (i) PBD medium does not suppress the production of the iron-regulated proteases or phosphate-regulated APh and PLC, and (ii) protease degradation of the proteins in the culture supernatants is minimal.

Exoenzyme assays. Total protease activity in culture supernatants was measured by using the microazocasein assay described by Jagger et al. (8). All samples were measured in triplicate and are reported as the mean ± standard deviation of the change in the optical density at 390 nm per 60 min/ml. PLC activity was measured by using the microtiter assay described by Berka et al. (1). Samples heated at 100°C for 10 min were used as negative controls. The results are reported as the mean ± standard deviation of nanomoles of *p*-nitrophenylphosphorylcholine (Sigma, St. Louis, Mo.) hydrolyzed per minute per milliliter for four samples.

APh activity was measured by using the same microtiter assay described above for PLC, except that *p*-nitrophenyl phosphate (Sigma) was used as the substrate. The results are reported as the mean ± standard deviation of nanomoles of *p*-nitrophenyl phosphate hydrolyzed per minute per milliliter for four samples measured in triplicate.

The protein content of the culture supernatants was measured by using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.). Bovine serum albumin was used to construct a standard curve. The protein content of PBD medium was subtracted from the protein content of the experimental samples. The results are reported as the mean ± standard deviation (in micrograms per milliliter) for four samples measured in triplicate.

DNA isolation and analysis. The presence or absence of the mercury resistance plasmids FP2 and pRLW103 in the various strains constructed in this study was detected by DNA-DNA hybridizations by using the plasmid pMD100, which carries the 7.9-kb mercury resistance transposon Tn501, as a probe. The target DNA was isolated from *P.*

aeruginosa strains by using a procedure described by Snell et al. (12). The probe pMD100, a ColE1::Tn501 construct, was isolated from *E. coli* by using the cleared lysate procedure (11) and was purified by CsCl-ethidium bromide density centrifugation as described by Maniatis et al. (11).

The target DNA was digested with restriction endonucleases (Bethesda Research Laboratories, Gaithersburg, Md.) and was separated by electrophoresis through a 0.7% agarose gel. Electrophoresis was carried out for 2 h at 40 to 50 mA in TAE (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA [pH 7.6]) buffer. The DNA was transferred to nitrocellulose and was hybridized with the ColE1::Tn501 probe DNA as described by Maniatis et al. (11). The probe DNA was labeled with biotinylated dUTP by using a random hexanucleotide primer and a fast-performance liquid chromatography-purified Klenow fragment (Pharmacia, Piscataway, N.J.) (2, 3). After hybridization, the probe was detected by using streptavidin-alkaline phosphatase conjugate and the chromogenic substrates Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Bethesda Research Laboratories).

Statistical analysis. Protease, APh, and PLC activities found in the supernatants of resting cell cultures were expressed as the mean ± standard deviation of four independent experiments measured in triplicate. Since there was variation in the amount of total protein, protease, and APh from experiment to experiment, the results were analyzed by analysis of variance with a random block design. This statistical test measures the interaction of variables within sets of experiments and between sets of experiments. Samples with significant F values were analyzed by using the Tukey or Duncan Waller tests. These statistical tests were computed by using SAS software (Statistical Analysis Systems Institute Inc., Cary, N.C.), a packaged statistical program.

RESULTS

Hybridization of Hg^r Tn501 with exconjugants. To demonstrate that the various plasmids were present in the exconjugants and that the selected colony was not due to spontaneous Hg^r, hybridizations were performed by using pMD100 as a probe for Hg^r. Both of the Hg^s parent strains, PAO18 and PAO1, showed no homology at 95% stringency washes, while the exconjugants containing FP2 and pRLW103 had the Hg^r sequences (data not shown).

Production of exoenzymes by PAO1 carrying FP2 or pRLW103. To determine whether FP2 or pRLW103 had a specific effect on the production of extracellular proteins, we independently transferred FP2 and pRLW103 into *P. aeruginosa* PAO1 as described above. We measured the production of extracellular protein production, exoprotease activity, PLC, and APh by strain PAO1 carrying either FP2 or pRLW103.

Figure 1 shows that neither FP2 nor pRLW103 affected the production of total extracellular protein by PAO1. However, the introduction of either plasmid resulted in the production of significantly ($P < 0.05$) less extracellular protease activity compared with that produced by the parent strain PAO1. When the protease activity was expressed as azounits per microgram of protein, PAO1 produced 0.62 azounits per μg of protein, while PAO1(FP2) produced 0.25 azounits per μg of protein and PAO1(pRLW103) produced 0.33 azounits per μg of protein. PAO1(pRLW103) produced significantly more APh compared with that produced by either PAO1 or PAO1(FP2). PAO1 and PAO1(FP2) produced 30 and 34 units

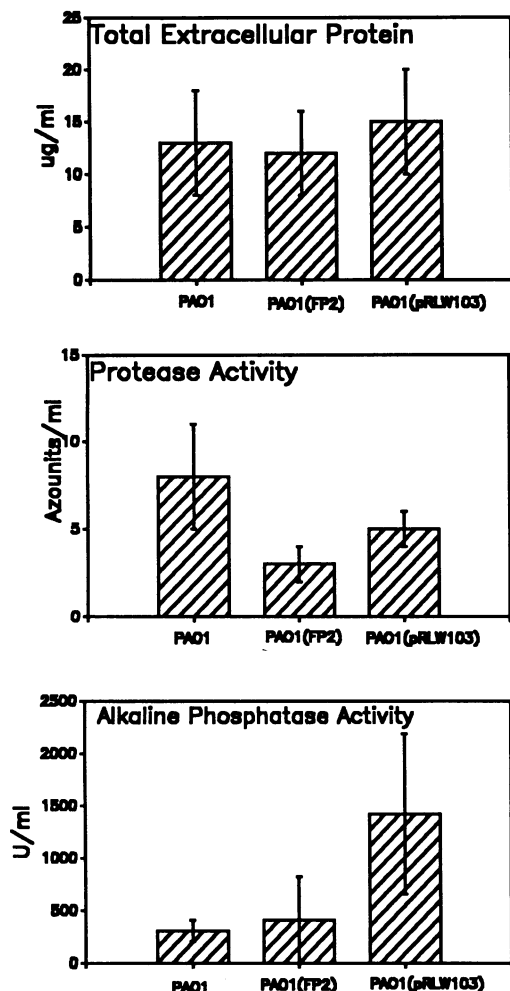


FIG. 1. Comparison of protein and exoenzyme activities from PAO1 containing either plasmid FP2 or plasmid pRLW103. The plasmids are shown in parentheses. Data are the results of triplicate samples from four independent supernatant collections and are expressed as the mean \pm standard deviation of all samples. No PLC activity was detected in any of these samples. No significant difference in the amount of protein secreted by any strain was detected, as determined by the Tukey test. Both plasmid-containing strains were found to produce significantly less ($P < 0.05$) protease activity than the parent strain did. Only PAO1(pRLW103) produced significantly more APH activity than the parent strain did.

of APH per μg of protein, respectively, while PAO1 (pRLW103) produced 95 units of APH per μg of protein. Neither of the plasmid donor strains PAO1161 or PA103 nor any of the PAO1 constructs secreted detectable levels of PLC.

The standard deviation observed within experiments for a single strain was small compared with that calculated for the strains between experiments. In all cases, significant F values ($P < 0.05$) were necessary to establish differences. Data were compared both by standardizing enzymatic activities per microgram of protein produced by each strain and by activity per milliliter. This had no effect on whether the enzymatic activities measured were significantly different or not.

Production of exoenzymes by PAO18 carrying FP2 or pRLW103. The plasmids FP2 and pRLW103 were trans-

ferred into an auxotrophic mutant of PAO1, PAO18. The introduction of FP2 into PAO18 significantly increased ($P < 0.05$) the overall protein production, from 17 $\mu\text{g}/\text{ml}$ in PAO18 to 24 $\mu\text{g}/\text{ml}$ in the plasmid-containing strain (Fig. 2). However, pRLW103 had the opposite effect by significantly decreasing overall protein production from 17 to 14 $\mu\text{g}/\text{ml}$. In contrast to the results with PAO1, the introduction of FP2 into PAO18 resulted in no significant change in the production of extracellular protease (Fig. 2). When the protease activity was expressed as azounits per microgram of protein, PAO18 produced 0.17 azounits per μg of protein and PAO18(FP2) produced 0.14 azounits per μg of protein. However, the introduction of pRLW103 into PAO18 resulted in a marked decrease in protease activity (0.01 azounits per μg of protein). In addition, only PAO18 carrying the pRLW103 plasmid produced extracellular APH and PLC (Fig. 2). Protease activity was also determined per microgram of protein produced by each strain, which resulted in the same correlations.

The chromosome-mobilizing plasmid R68.45 was also introduced into strain PAO18 by conjugation. This exconjugant was assayed for total protein secreted, along with protease, PLC, and APH activities. There was no statistical difference in the levels of detectable enzyme activities between the parent PAO18 and the conjugant PAO18(R68.45). This suggests that the results observed by the introduction of the plasmids FP2 and pRLW103 into strain PAO18 were not artifacts due to exogenous plasmid maintenance. There was no correlation between the parent strains PAO1161 (containing plasmid FP2) and PA103 (containing plasmid pRLW103) and the amount of exoenzymes secreted by the exconjugants. PAO1161 produced 26 units of PLC per μg of protein, 53 units of APH per μg of protein, and 0.56 azounits per μg of protein. PA103 secreted no detectable amounts of PLC or APH and 0.02 azounits per μg of protein.

The introduction of plasmid R68.45 into strain PAO18 demonstrated that this plasmid, which was found to be incompatible with FP2 and pRLW103, did not alter the production of exoenzymes. Conjugation of exconjugants PAO18(FP2) and PAO18(pRLW103) with PAO25(R68.45) and selection for Tc^r PAO18 resulted in the loss of the Hg^r -containing plasmid. This demonstrates that the effects observed in the exconjugants PAO18(FP2) and PAO18 (pRLW103) were not due to exogenous plasmid maintenance or to chromosome alteration, since PAO18(R68.45) was indistinguishable from the parent strain PAO18 with respect to exoenzyme production.

DISCUSSION

In *P. aeruginosa*, mutations with pleiotropic effects on the production of extracellular enzymes have been mapped to the bacterial chromosome (15). We reported here that plasmids FP2 and pRLW103 also can exert pleiotropic effects on *P. aeruginosa* protease, PLC, and APH secretion. The effects of the plasmids on the production of these enzymes were strain dependent and varied for each plasmid. There was no correlation between the amount of exoenzymes secreted by the plasmid-containing parent strains PAO1161 and PA103 and the amount of exoenzymes secreted by the PAO1 and PAO18 exconjugants.

The pRLW103 plasmid decreased protease activity in PAO18 by 87%, while the FP2 plasmid had no significant effect on the secretion of protease. Neither APH nor PLC activity could be detected in the parent strain PAO18 nor in the exconjugant PAO18(FP2). However, culture superna-

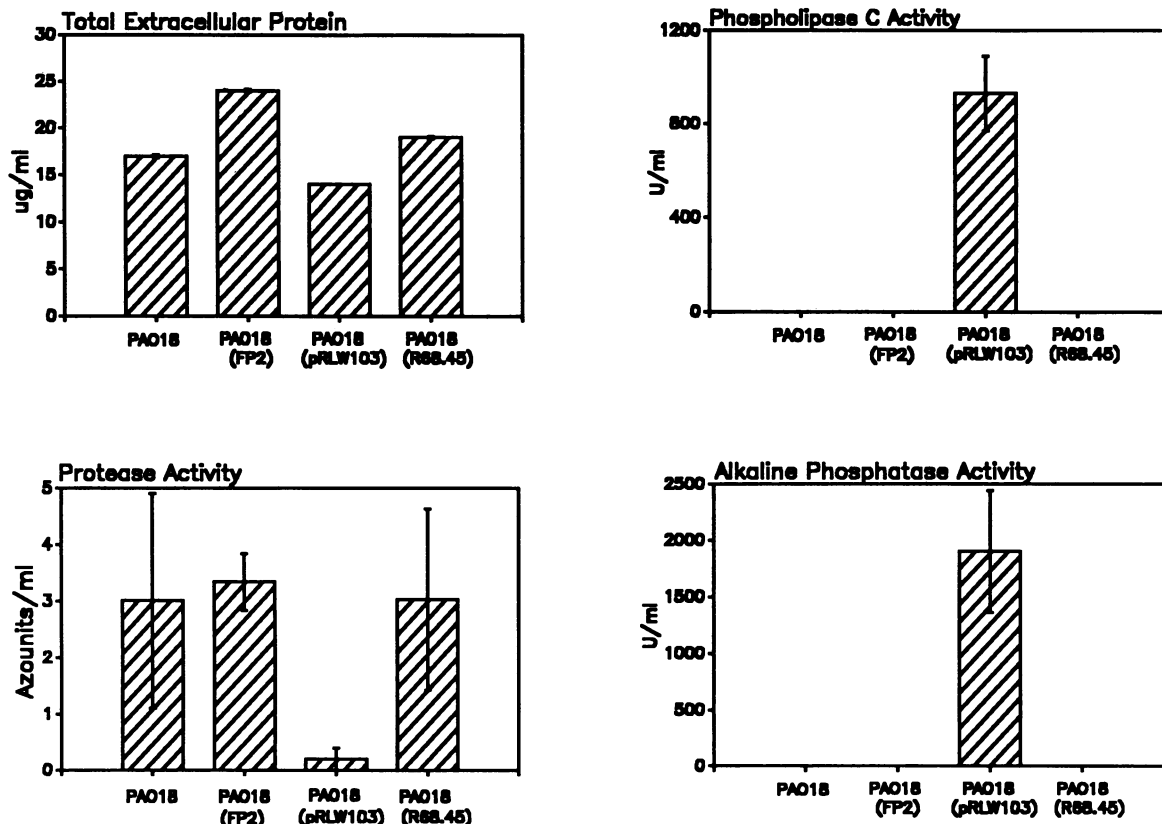


FIG. 2. Comparison of protein and exoenzyme activities from PAO18 containing plasmid FP2 or plasmid pRLW103. The plasmids are shown in parentheses. Data are the results of triplicate samples from four independent supernatant collections and are expressed as the mean \pm standard deviation of all samples. PAO18 secreted significantly less total protein than PAO18-containing FP2 did, as determined by the Tukey test for significant F values ($P < 0.05$). By the same method, PAO18 secreted significantly more total protein and protease activity per microgram of protein than PAO18(pRLW103) did. Plasmid R68.45 was introduced into PAO18 as a negative control. Only PAO18(pRLW103) produced detectable levels of PLC and Aph.

tants of PAO18(pRLW103) had significant amounts of both enzymes.

In contrast, the amounts of protease activity detected in culture supernatants of PAO1(FP2) and PAO1(pRLW103) were reduced by 62 and 38%, respectively, compared with that detected in culture supernatants of the parent strain. PAO1(pRLW103) produced significantly more Aph than did either PAO1 or PAO1(FP2). In our assays, none of these strains produced detectable levels of PLC, even though PAO1 has been shown by Berka et al. (1) to produce PLC.

We could not differentiate between plasmid maintenance and integration into the chromosome, but the strains could be cured of either plasmid FP2 or plasmid pRLW103 by an incompatibility with plasmid R68.45. This is unusual since FP2 has been shown to be the only member of its incompatibility group (4). The functional similarity between the plasmids may be a factor. However, no chromosomal linkage was found with the conjugal transfer of the marker for Hg^r between strains.

In summary, these results show that the transferable Hg^r elements FP2 and pRLW103 have pronounced effects on the host strain's capacity to secrete protease, Aph, and PLC. The genetic background of the host strain altered the effect that these plasmids had on secretion. The most striking observation is the production of Aph and PLC by PAO18(pRLW103), while neither the donor strain PA103 nor

the recipient strain PAO18 produced detectable levels of either of these enzymes. The Hg^r plasmids FP2 and pRLW103 appear to be functionally different with respect to their abilities to affect extracellular enzyme production, and there appears to be a complex interaction between plasmid genes and chromosomal genes that affects the production of exoenzymes.

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