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Aeruginocin tolerant mutants of *Pseudomonas aeruginosa*

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SUMMARY

Mutants of *Pseudomonas aeruginosa* tolerant to the action of trypsin-sensitive aeruginocins can be readily isolated. They are found to be heterogeneous for a range of phenotypic characteristics (including the pattern of membrane protein components in polyacrylamide gel electrophoresis), response to bacteriophages (including both plaque formation and the ability to be lysogenized), sensitivity to various toxic agents, colonial morphology, and cellular morphology. The nature of these changes strongly supports the view that the mutants examined have undergone alteration in membrane structure. A limited genetic analysis indicates that at least two chromosomal regions are involved.

1. INTRODUCTION

An understanding of the structure, function and formation of bacterial organelles and structural components is currently being approached by means of a variety of techniques. Of particular interest is the cell membrane, both in terms of its structural relationships to other cellular components, and its importance to the bacterial genetic material (Rogers, 1970). One approach to the study of the bacterial membrane is to examine the altered genotypic and phenotypic properties of bacterial strains which have undergone mutation in genes affecting membrane structure and function. In *Escherichia coli* there is evidence that such mutations can be identified amongst mutants which have acquired tolerance to the lethal action of colicins (Bernstein, Rolfe & Onodera, 1972; Holland, 1967, 1968; Holland *et al.* 1970; Holloway, 1971; Nagel de Zwaig & Luria, 1967, 1969; Nomura & Witten, 1967; Onodera, Rolfe & Bernstein, 1970; Reeves, 1966; Rolfe & Onodera, 1971). While the modes of action of bacteriocins vary, it is clear that the bacterial cell membrane is frequently involved at one stage in a series of reactions initiated by bacteriocins which lead to the death of the cell. Presumably changes in the membrane structure may prevent the lethal action of the bacteriocins. Mutants possessing such changes acquire a variety of pleiotropic effects which are proving to be of particular value in the analysis of membrane structure and function.

One important aspect of the bacterial membrane is its relationship to the chromosome and extrachromosomal DNA structures such as plasmids. Previous studies (Holloway, Krishnapillai & Stanisich, 1971) have shown the importance of

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plasmids in the genome of *Pseudomonas aeruginosa* and we consider that a complete understanding of plasmid activity in this organism will involve a knowledge of membrane structure and function. Accordingly we have proceeded to isolate aeruginocin tolerant mutants of the genetically characterized *P. aeruginosa* strain PAO and investigate them for variation in properties associated with the membrane.

2. MATERIALS AND METHODS

(*a*) *Bacterial strains.* The *P. aeruginosa* strains used are listed in Table 1.

Table 1. *Bacterial strains used in this study*

Strain no.	Genotype	Comments	Reference
PAO 1	Prototrophic parent strain, FP⁻	—	Holloway (1969)
PAO 381	*leu-38*, *str-7*, FP2+	Male donor	Stanisich & Holloway (1969)
PAO 1403	*tol-1*	Aeruginocin-tolerant mutant derived from PAO 1 Requires tryptophan	This study
PAO 1408	*tol-2*	Requires tryptophan	This study
PAO 1414	*tol-3*	Requires tryptophan	This study
PAO 1419	*tol-4*	Aeruginocin-tolerant mutant derived from PAO 1 Requires methionine	This study
PAO 1652	*tol-5*	Aeruginocin-tolerant mutant derived from PAO 1 Prototrophic	This study
PAO 1654	*tol-6*	Prototrophic	This study
PAF 41	Prototrophic	Selected as being aeruginocinogenic for PAO	This study
PAH 108	Prototrophic	Selected as being aeruginocinogenic for PAO	This study

(*b*) *Bacteriophage strains.* F116 – a temperate, general transducing phage (Holloway, Egan & Monk, 1960); G101 – a temperate, general transducing phage (Holloway & van de Putte, 1968); B3 – a temperate bacteriophage (Holloway *et al.* 1960); D3 – a temperate bacteriophage (Holloway *et al.* 1960); E79 – a virulent bacteriophage (Holloway *et al.* 1960). The general properties of these phages were described by Holloway (1969).

(*c*) *Media.* For general cultural work, bacterial and bacteriophage assays, Oxoid Blood Agar Base plus 0·5% Oxoid Yeast extract (NA) was used as a solid medium and Oxoid Nutrient Broth No. 2 (NB) for liquid culture.

(*d*) *Culture procedures.* In general these were the same as those described previously from this laboratory (Stanisich & Holloway, 1969; Holloway, 1965). Except where stated, the incubation temperature was 37 °C.

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(*e*) *Preparations and assay of aeruginocin.* Aeruginocin production was induced by mitomycin C (Kageyama, 1964). The crude aeruginocin preparation was centrifuged to remove unlysed bacteria, DNAase being added to reduce the viscosity. Killing of bacteria was accomplished by shaking with chloroform. Assay of aeruginocin was by the technique commonly used for colicins (Mayr-Harting, Hedges & Berkeley,. 1972). NA plates were flooded with an overnight culture in NB of the indicator strain. Loopfuls of serial twofold dilutions of the aeruginocin were then spotted on to such inoculated plates, the end-point being the highest dilution to give confluent clearing; titres of 1/64 were commonly obtained.

(*f*) *Effect of aeruginocin on incorporation of labelled substrates.* The procedure used was to take 0·9 ml of an exponential phase culture of PAO 1 in NB at 4×10^8 cells/ml to which was added 0·1 ml of partially purified aeruginocin (or 0·1 ml broth as a control). This mixture of bacterial cells and aeruginocin was incubated with shaking at 37 °C for 10 min. Then *c.* 1 μCi of a labelled substrate was added ([^{3}H]thymidine, [^{14}C]uracil or [^{14}C]isoleucine). Samples of 0·1 ml were immediately taken (0 min) and thereafter 0·1 ml samples were taken at 30 min intervals for 3 h. The samples were spotted on to filter-paper squares, dried, washed in 5% trichloracetic acid (TCA) and 1% sodium pyrophosphate for 45 min, 5% TCA for 20 min, ethyl alcohol for 20 min and ether for 5 min (using 10 ml of each solvent per filter paper). The filter papers were dried and counted for the particular radioactivity using a Packard Tricarb scintillation counter.

(*g*) *Distinguishing aeruginocin-tolerant and aeruginocin-resistant mutants.* The method of Hill & Holland (1967) was adapted for use with *P. aeruginosa* (Barbara Mills, personal communication). Only mutants shown to be tolerant by this test have been further investigated and they have been given the genotypic symbol *tol*.

(*h*) *Cell membrane preparation.* A single colony of the strain to be tested was inoculated into 60 ml NB and grown for 18 h. The culture was then all transferred to 500 ml 1% Oxoid tryptone in a 2 l baffled flask and grown for 1½ h with shaking, viable counts of $1\text{–}5 \times 10^8$/ml being obtained for most strains. The cells were harvested by centrifuging at 5800 ***g*** for 20 min, then washed twice in 0·1 M tris pH 8·1 (5800 ***g*** for 15 min). The cells were resuspended in 10 ml of the same buffer and disrupted in a French Pressure Cell at 15000 p.s.i. Whole cells were sedimented at 9100 ***g*** for 45 min. A small amount of DNAase and RNAase was added to the supernatant which was then incubated with shaking at 37 °C for 20 min. Cell debris was sedimented at 9100 ***g*** for 25 min. The supernatant from this was spun at 75000 ***g*** for 50 min. The pellet was washed twice in 0·1 M tris pH 8·1 and sedimented as above. The final pellet was suspended overnight in a solubilizing buffer (0·06 M tris, pH 6·8, 2% SDS, 10% glycerol, 0·5 M urea, 1% 2-mercaptoethanol). The samples were boiled for 3 min, and insoluble solids spun down in a bench centrifuge. For each strain tested at least three such membrane preparations were done to ensure reproducibility.

(*i*) *Procedure for running* P. aeruginosa *membranes on polyacrylamide gradient gels.* Samples were run on 2 days immediately following the completion of a membrane

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preparation, although a lapse of 3–4 days did not seem to affect the banding pattern appreciably. Preparations contained ~ 5 mg/ml of protein (Lowry, Rosebrough, Farr & Randall, 1951). The samples were diluted in the solubilizing buffer containing 1 % bromophenol blue and 40 % sucrose. Forty μl of each sample (containing 80–90 μg of protein) was placed on the gel, using a sample spacer. Each sample was run in duplicate, and two electrophoresis runs were done on each preparation.

The polyacrylamide gradient gels were made in the laboratory using the method described by Margolis & Kenrick (1968) and Margolis (1969). The gradient used was convex, and the acrylamide concentration ranged from 4 to 27 %. The running buffer used was 0·02 M tris, pH 8·1, 1·44 % glycine, 0·1 % SDS. Running time was 22 h at a constant 100 V at 4 °C, with a current of 20–40 mA. Gels were stained for 1 h in 0·25 % Coomassie brilliant blue in methanol: acetic acid: water (5:1:5, v/v/v). They were destained electrophoretically in 40 % methanol, 3·75 % acetic acid for about 5 h.

3. RESULTS

(i) *Properties of aeruginocins*

Aeruginocinogeny is extremely common in *P. aeruginosa* (Holloway, 1960; Govan & Gillies, 1969). A range of *P. aeruginosa* strains isolated from human infections was examined for aeruginocinogeny, using PAO 1 as the sensitive test strain. Two were selected, PAF 41 and PAH 108, both having the additional criterion that they were not lysogenic for any phages plating on PAO 1. Examination of lysates of both by chromatography and ultracentrifugation gave no evidence that more than one aeruginocin is produced by either strain. The aeruginocin liberated from PAF 41 will be referred to as AP 41 and that from PAH 108 as AP 108. Both were unaffected by either DNAase or RNAase but showed a marked reduction in lethal activity following treatment with pronase, protease, or trypsin. Although quantitative data on the sedimentation properties of these aeruginocins was not obtained, considerably greater centrifugal forces were required than those needed to pellet *P. aeruginosa* bacteriophages. This fact, coupled with the trypsin sensitivity, suggests that these aeruginocins were of the S type rather than the defective bacteriophage, R type, of Bradley (1967). The lethal activity of both aeruginocins was concentrated either by ultra-centrifugation or by ammonium sulphate precipitation (Higerd, Baechler & Berk, 1967). By the latter method it was possible to increase the specific activity of either aeruginocin by a factor of about 50 above that of the crude lysates and this partially purified material has been used to determine the mode of action of these aeruginocins.

(ii) *Mode of action of aeruginocin*

No difference was found in the mode of action of AP 108 and AP 41 on *P. aeruginosa* PAO 1 and the results given below were those obtained using AP 108. DNA, RNA and protein synthesis in *P. aeruginosa* were measured using the incorporation

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of labelled thymidine, uracil and isoleucine respectively into cold TCA-insoluble material, the uptake being measured in the presence and absence of aeruginocin.

The results (Figs. 1–3) show that aeruginocin markedly reduced the incorporation of all three substrates. The most reasonable explanation of these results is that infection of PAO 1 with aeruginocin AP 108 results in a marked decrease of DNA synthesis, RNA synthesis and protein synthesis. The effects on RNA and protein synthesis occur shortly after the addition of aeruginocin, but there is a delay of approximately 30 min before DNA synthesis is affected. These results suggest that AP 108 is acting in a manner similar to that found with colicin K on *E. coli*, particularly in view of the low titre of AP 108 used in these experiments compared to that used in the colicin K determinations, and we suggest that the observed effects result from changes in energy flux (Reeves, 1972).

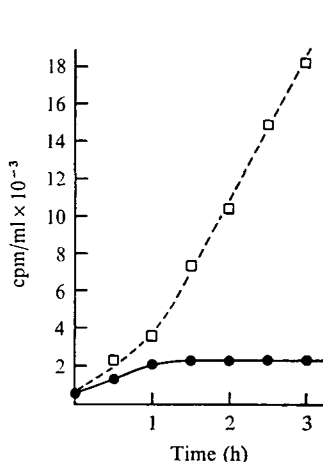

Fig. 1

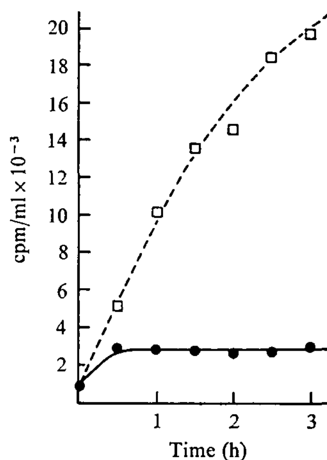

Fig. 2

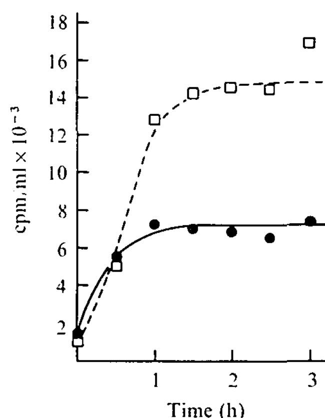

Fig. 3

Fig. 1. Effect of aeruginocin 108 on the uptake of ^{14}C-labelled isoleucine. □--□, In the absence of aeruginocin; ●—●, in the presence of aeruginocin.

Fig. 2. Effect of aeruginocin 108 on the uptake of ^{14}C-labelled uracil. □--□, In the absence of aeruginocin; ●—●, in the presence of aeruginocin.

Fig. 3. Effect of aeruginocin 108 on uptake of tritium-labelled thymidine. □--□, In the absence of aeruginocin; ●—●, in the presence of aeruginosin.

The action of both aeruginocins can be reversed by treatment with trypsin subsequent to infection. This has been previously shown for the action of colicin K on *E. coli* (Nomura & Nakamura, 1962). A culture of PAO 1 in exponential phase was treated with aeruginocin and the optical density of the culture at 37 °C followed by means of the Klett colorimeter. At various times after mixing the aeruginocin and the sensitive cells, trypsin was added to a final concentration of 250 μg/ml and changes in the optical density followed as an index of bacterial growth. From Fig. 4 it is seen that rescue of cells from the action of aeruginocin by trypsin can be achieved even after 10 min, although it is apparent from the shape

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of the curves that some action by the aeruginocin on the bacterial cells has occurred. These results are similar to those obtained with colicins and support the view that aeruginocin can exert its lethal activity without penetrating the cell.

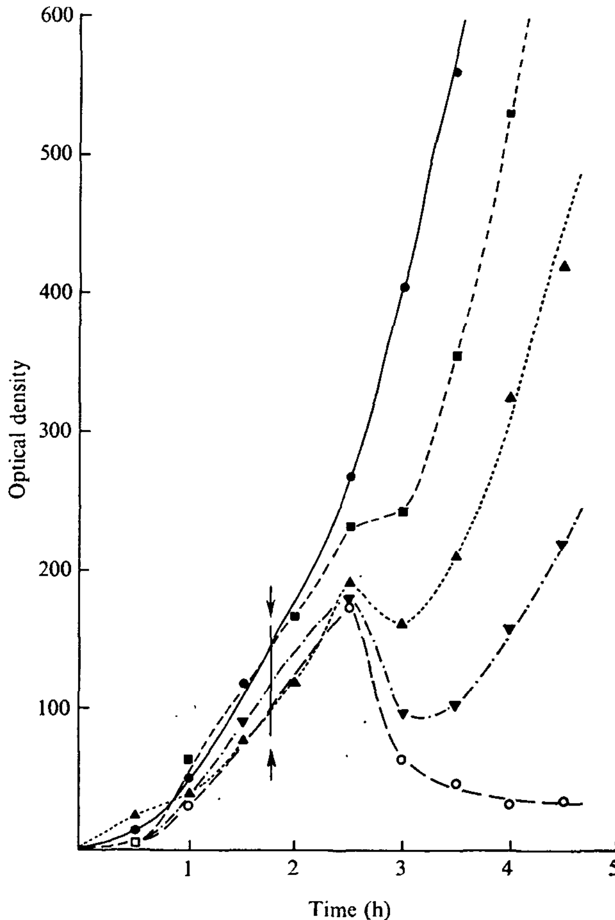

Fig. 4. Rescue of aeruginocin killing effect by addition of trypsin to an aeruginocin-bacterial culture mixture. An overnight culture of PAO 1 was diluted 1 in 9 in broth and grown for $1\frac{3}{4}$ h. One ml of AP 108 (crude preparation clearing titre 1/16) was then added. ●—●, No aeruginocin present; ○--○, aeruginocin present, no trypsin added; ■--■, aeruginocin present, trypsin added 2 min after aeruginocin; ▲--▲, aeruginocin present, trypsin added 5 min after aeruginocin; ▼-----▼, aeruginocin present, trypsin added 10 min after aeruginocin.

(iii) *Isolation of aeruginocin-tolerant mutants*

Both AP 41 and AP 108 have been used for the isolation of mutants which survive the lethal action of aeruginocin. No differences have been found in the spectrum of mutants isolated using either aeruginocin and all aeruginocin-tolerant mutants isolated using one of these aeruginocins are tolerant to the action of the other. Likewise, the types of mutants found were the same whether partially

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purified or crude preparations of aeruginocins were used and the mutants isolated were equally resistant to crude or partially purified preparations.

Potentially two types of mutants can be isolated which are capable of surviving the lethal action of aeruginocins. Resistant mutants are those which cannot adsorb aeruginocin and experience with other bacteriocins (Reeves, 1965, 1972) suggests that they are analogous to phage-resistant mutants, are unlikely to show alterations in the bacterial membrane and hence not of immediate interest to this study. Aeruginocin-tolerant mutants can adsorb aeruginocins in a manner similar to the wild-type but survive the infection. It is within this class that mutants with altered membrane properties may be found.

The procedure for isolating *tol* mutants was to spread a 1/50 dilution of an overnight NB culture of *P. aeruginosa* on NA plates on which 0·3 ml of an undiluted preparation of aeruginocin had previously been spread. After 48 h incubation, colonies appeared at a frequency of *c.* 10^{-5}. The chance of isolating sibling mutants was diminished by having a number of overnight broth cultures, each originating from a small inoculum, each broth culture being used for only one NA plate, and only colonies of different morphology being picked from each plate. The proportion of aeruginocin-tolerant mutants was much higher (about 10:1) than that of aeruginocin-resistant mutants.

The colonies on the NA + aeruginocin plates showed a wide variation in size and morphology, ranging from small translucent colonies to large, spreading, mucoid colonies, many only faintly resembling the colonial morphology of the parent strain. The viable count of all *tol* mutants tested on NA seeded with aeruginocin is not significantly different on NA.

While all the *tol* mutants isolated in this way are tolerant to the lethal action of aeruginocins AP 41 and AP 108 on solid medium, some show a degree of sensitivity to these aeruginocins in liquid medium. By appropriate modification of the isolating procedure it was possible to isolate *tol* mutants that are tolerant to the lethal action of these aeruginocins in both solid and liquid medium. Such mutants had the same range of other properties where tested as those isolated on solid medium.

If Nomura's (1967) suggestion that such tolerant mutants will have an altered membrane is correct, some predictions can be made as to pleiotropic effects which such mutants might show. We have sought to demonstrate that certain of these *tol* mutants are membrane mutants by comparing their membrane structure to that of the wild-type and comparing various functions known to be structurally associated with the membrane to the parent strain. We have then examined a wider range of cellular properties to see if they were affected in this type of mutant. A range of single step *tol* mutants of PAO 1 have been isolated and the mutants listed in Table 1 selected for further study.

(iv) *Phenotypic characteristics of* tol *mutants*

(*a*) *General cultural characteristics*

PAO 1403, PAO 1408 and PAO 1414 are similar in colonial morphology and growth rate to PAO 1. PAO 1419 and PAO 1652 are slower growing, with smaller,

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more translucent colonies and produce copious brown pigment into the medium. PAO 1654 is also slower growing than PAO 1 but does not produce any pigment.

(*b*) *Membrane structure*

Membranes were isolated from PAO 1 and from each of the various *tol* mutants and, after solubilization, were examined by polyacrylamide gel electrophoresis. The banding pattern was always the same for a particular preparation run on different occasions but the bands did vary slightly between different preparations. Nevertheless over 30 bands could be positively identified. Other PAO tol^{+} strains have been examined and found to have an almost identical band pattern to that found for PAO 1.

Comparison of the various *tol* mutants to PAO 1 have been made (Figs. 5, 6). Replicate membrane preparations and replicate gel preparations have confirmed the general uniformity of the patterns shown in these photographs. A comparison of the band patterns of PAO 1 and each of the *tol* mutants shows that differences can be identified. The location of differences are indicated in Figs. 5 and 6 by lines placed against the gel of each *tol* mutant. The differences consist of missing bands, displaced bands or new bands. In general the changes in the top two-thirds of each gel are more reliable than those in the faster moving lower regions.

Clearly, the banding patterns in polyacrylamide electrophoresis of solubilized membranes from *tol* mutants are different to that of the membrane from the tol^{+} parent, and it can be concluded that the membrane structure of *tol* mutants is different to that of the wild type. Similar conclusions have been drawn from an examination of colicin tolerant mutants of *E. coli* (Rolfe & Onodera, 1971; Holland & Tuckett, 1972).

(*c*) *Sensitivity to toxic agents*

Changes in membrane structure could well result in alterations to permeability. One result of this change could be in the response to toxic agents. We have measured the relative sensitivity of the *tol* mutants to a variety of toxic substances. The results with *p*-fluorophenylalanine (FPA) are shown in Fig. 7. It is seen that whereas most of the *tol* mutants tested show a marked increase in sensitivity, one mutant (PAO 1408) showed increased resistance.

Changes in sensitivity of the *tol* mutants were also shown with sodium deoxycholate and mitomycin C. The relative sensitivities of the six strains tested were similar to that found with FPA, PAO 1408 again showing an increased resistance. However, with streptomycin and neomycin, only PAO 1419 and PAO 1652 showed any appreciable increase in sensitivity, PAO 1654 was slightly more sensitive, and the three other strains, PAO 1403, PAO 1408 and PAO 1414, showed no differences in sensitivity to that of the wild-type PAO 1.

(*d*) *Response of* tol *mutants to bacteriophage infection*

The ability of this selection of *tol* mutants to support the multiplication of a range of *P. aeruginosa* phages was examined. The results are shown in Table 2.

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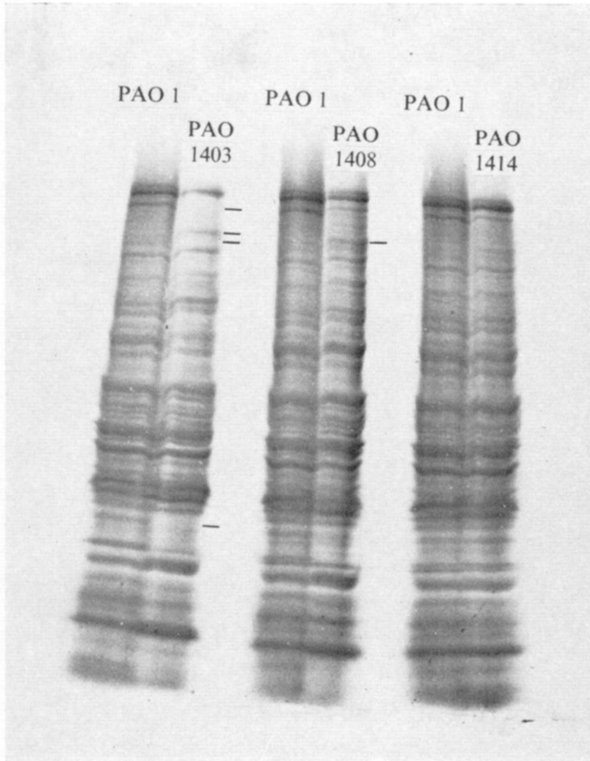

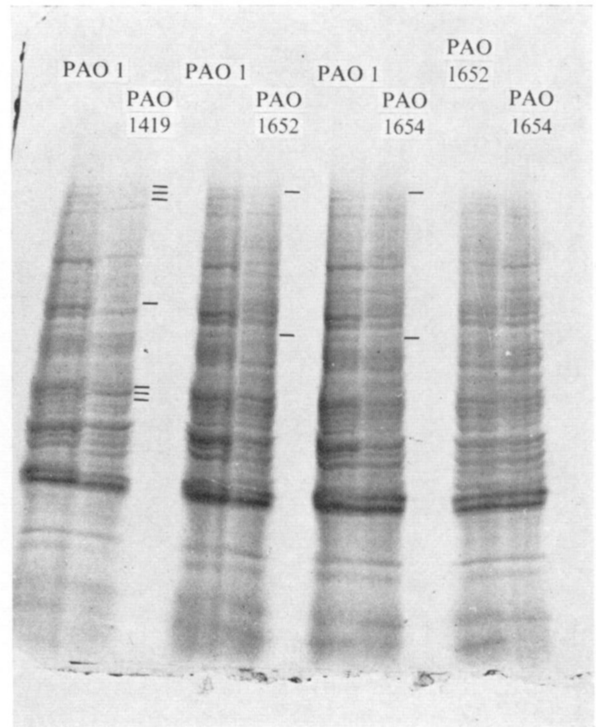

Fig. 5

Fig. 6

Fig. 5. Polacrylamide gel electrophoresis of solubilized membrane preparations from tol^{+} and *tol* strains of *P. aeruginosa*. In each case the *tol* mutant has been electrophoresed next to the tol^{+} strain from which it has been derived. From left PAO 1 (tol^{+}) and PAO 1403 (*tol*); PAO 1 and PAO 1408 (*tol*), PAO 1 and PAO 1414 (*tol*). Band differences are indicated by lines at the side of the *tol* strain.

Fig. 6. As for Fig. 5. From left PAO 1 (tol^{+}) and PAO 1419 (*tol*); PAO 1 and PAO 1652 (*tol*); PAO 1 and PAO 1654 (*tol*). The right-hand pair of gels show a comparison of PAO 1652 and PAO 1654. Band differences are indicated by lines at the side of the *tol* strain.

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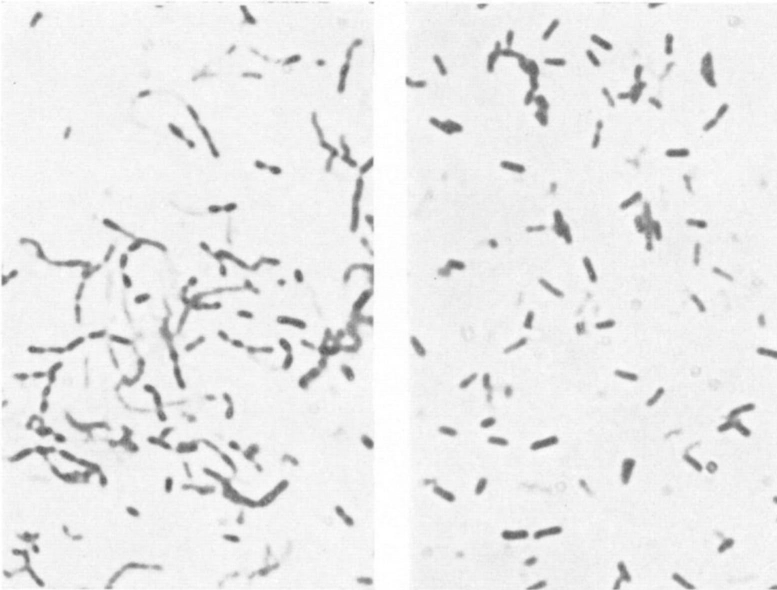

Fig. 8. Appearance of PAO 1654 grown in NB at 37 °C (left) compared to the parent *tol*+ strain PAO 1 (right).

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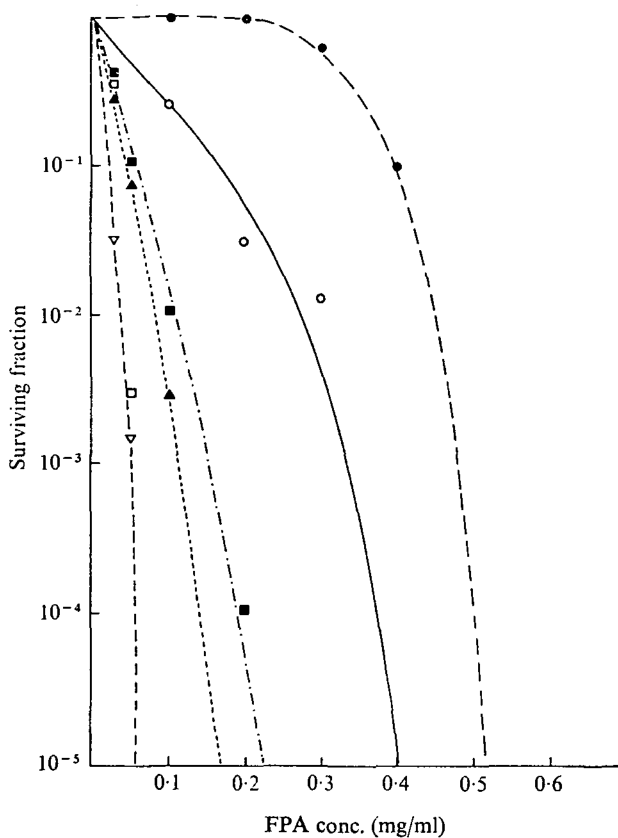

Fig. 7. Sensitivity levels of *tol* mutants to *p*-fluorophenylalanine (FPA) measured as survival at various levels. An overnight NB culture of each bacterial strain was washed, then assayed for viable count on MM (plus tryptophan or methionine as required) containing various concentrations of FPA, with a 24 h incubation period. □--□, PAO 1419; ▲······▲, PAO 1654; ■--■, PAO 1414; ▽--▽, PAO 1652; ○—○, PAO 1 (*tol*⁺); ●--●, PAO 1408.

Table 2. *Response of* tol *mutants to bacteriophages. Figures given in the body of the table are plating efficiencies relative to PAO 1*

Bacterial strain	Bacteriophage strain				
	B3	D3	E79	F116	G101
PAO 1	1	1	1	1	1
PAO 1403	1	1	1	1	1
PAO 1408	1	1	1	1	1
PAO 1414	1	1	1	1	1
PAO 1419	1	$< 10^{-6}$	$< 10^{-6}$	1	10^{-3}
PAO 1652	1	$< 10^{-6}$	$< 10^{-6}$	1	$< 10^{-6}$
PAO 1654	1	1	1	1	1

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In those cases where plating efficiency was reduced, it was shown that adsorption of the phage was at the same rate as with PAO 1, indicating that the reduced plating was due to lack of multiplication or reduction in burst size of the phage.

(e) *Properties of PAO 1654*

One mutant, PAO 1654, was selected for further study in view of a number of differences from the other mutants characterized. The additional features of this mutant are:

(*a*) Growth in NB at 37 °C produces elongated, non-motile, multiseptate cells with a morphology suggestive of defective septum formation at cell division (Fig. 8).

(*b*) PAO 1654 is killed in media in which the ionic content is low, such as 1% tryptone. However, when 2% sodium chloride is added to the 1% tryptone, viability and growth is very similar to that found with the parent strain PAO 1.

(*c*) PAO 1654 is markedly deficient in its ability to be lysogenized by temperate phages, thus having the Les phenotype previously described for other mutants (Holloway & van de Putte, 1968; van de Putte & Holloway, 1968; Holloway, 1969).

Plating efficiency of temperate phages such as D3, F116 and G101 is normal (see Table 2), but the plaques are clear, and without solid centres of lysogenized growth. Quantitative measurements of lysogenization frequency confirmed the plaque appearance, with the frequency of lysogenization of PAO 1654 being markedly lower than that found with PAO 1. Other *tol* mutants (including PAO 1419) with the Les phenotype have been isolated and they will be the subject of a separate communication (Dodge & Holloway, in preparation). It appears that procedures used previously for the isolation of *les* mutants may have been selective for the isolation of mutants with properties similar to those of some *tol* mutants.

Together these properties are similar to those of strains of *E. coli* carrying the *lon* mutation, which is considered to be a defect in cell-envelope synthesis (Takano, 1971). It has not yet been possible to determine if the pleiotropy of PAO 1654 is due to a single mutation in a gene affecting membrane structure or function. Like other mutants showing the Les phenotype, PAO 1654 accepts the sex factor FP2 at a frequency some 1000-fold less than that of the other *tol* mutants of this paper and *tol*$^+$ strains.

(*f*) *Genetic analysis of* tol *mutants*

The phenotypic variability of the various *tol* mutants suggests that more than one locus may be involved. We have attempted to map the *tol* locus of the various mutants using the transduction and conjugation systems available in *P. aeruginosa* (Holloway, 1969; Holloway, Krishnapillai & Stanisich, 1971). When the *tol* mutants were examined it was found that some had acquired an auxotrophic requirement for tryptophan (not satisfied by indole) or methionine (not satisfied by cysteine) (Table 1).

This auxotrophic requirement may be due to loss of function in one or more

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membrane associated enzymes. Alternatively, the genetic change resulting in aeruginocin tolerance could involve a chromosomal deletion, in the case of say, PAO 1403, involving one of the genes of the tryptophan pathway. It is not as yet possible to distinguish with certainty between the two alternatives, but in either case, the auxotrophic phenotype provides a useful way of selecting for the *tol*$^+$ allele, because selection for prototrophy can be made. Using one *tol* mutant as a donor, with selection for either Trp$^+$ or Met$^+$, and an auxotrophic *tol* mutant as the recipient, the occurrence of *tol*$^+$ transductants amongst the Trp$^+$ progeny indicated that the *tol* loci of donor and recipient are different (no Met$^+$ progeny were found) (Table 3).

Table 3. *Transductions with various* tol *mutants using phage F116*

		Recipient (selection for Trp$^+$ or Met$^+$) figures given represent % of transductants which were Tol$^+$			
		PAO 1403	PAO 1408	PAO 1414	PAO 1419
Donor	PAO 1	NTF	100	NTF	NTF
	PAO 1403	—	NTF	NTF	NTF
	PAO 1408	NTF	—	NTF	NTF
	PAO 1414	NTF	NTF	—	NTF
	PAO 1419	NTF	100	NTF	—
	PAO 1652	NTF	100	NTF	NTF
	PAO 1654	NTF	100	NTF	NTF

NTF, No transductants formed.

A number of conclusions can be drawn from these results.

(1) PAO 1403 and PAO 1414 did not show transduction for the auxotrophic requirement associated with the *tol* mutation. This was not a reflexion of a recombination deficient phenotype, since when these strains were made auxotrophic for markers at other loci they could form recombinants in transduction at normal frequency. PAO 1403, PAO 1408 and PAO 1414 acted as normal recipients in conjugation with an FP2$^+$ male, again with selection for prototrophy. Twenty-five Trp$^+$ recombinants from such crosses were found to be *tol*$^+$. This supports the view that a chromosomal deletion has resulted in the concomitant Tol and auxotrophic phenotypes, the deletion being larger than the chromosomal fragment carried by the transducing phage thus preventing synapsis of the transduced fragment and therefore transduction of this region. The chromosome segment transferred in conjugation does not have this size limitation and hence conjugation frequencies are normal. Additional support for the deletion hypothesis comes from the observation that revertants to prototrophy did not occur spontaneously or following mutagen treatment of PAO 1403, PAO 1408 and PAO 1419.

No Met$^+$ transductants were obtained using PAO 1419 as a recipient. This strain showed much reduced transduction frequencies for other auxotrophic markers. Likewise recovery of recombinants in conjugation between PAO 1419 and FP2$^+$

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donors was much reduced both for the Met region and for other auxotrophic markers. The nature of this recombination deficient phenotype is being investigated.

(2) The fact that PAO 1403 and PAO 1414 cannot act as donors in transduction for PAO 1408 with selection for prototrophy suggests that the same *trp* locus is involved in all three mutants, and hence that the same *tol* region is deficient in all three mutants.

(3) That PAO 1419, PAO 1652 and PAO 1654 can act as donors of both the *trp* and *tol* loci in PAO 1408, indicates that the *tol* loci of these three mutants must be different to that of PAO 1408. It is not possible by this test to determine if PAO 1419, PAO 1652 and PAO 1654 have the same or different *tol* loci.

Attempts to accurately map the *tol* loci and the associated *trp* and *met* loci by transduction or conjugation have been unsuccessful. For the present we can merely conclude that at least two chromosomal regions are responsible for the Tol phenotype in *P. aeruginosa.*

4. DISCUSSION

It is clear that aeruginocin tolerant mutants of *P. aeruginosa* show pleiotropic effects which are consistent with a mutational alteration of membrane structure. The changes in membrane structure as identified by polyacrylamide gel electrophoresis comparisons with the parent strain from which they are derived provide strong support of this view. As yet we do not know what the observed differences betweeen the *tol* and tol^{+} forms mean. The simplest explanation is that what we can refer to as a *tol* gene is coding for some structural component of the membrane. We do not know if the variety of band changes observed in any one mutant represent only one mutational change or whether a more extensive genetic change has occurred. Certainly more than one gene is involved, as shown by the transduction results. The fact that PAO 1403, PAO 1408 and PAO 1414 appear to involve the same chromosomal region as shown by transduction results, yet the membrane analysis by polyacrylamide gel electrophoresis for PAO 1408 and PAO 1414 is clearly different to that for PAO 1403 indicates an additional genetic complexity of the loci producing these phenotypic effects.

The other phenotypic changes support the view that there have been structural changes in the membrane of *tol* mutants. The changes in sensitivity to various toxic agents are in agreement with changes in permeability, but this will need to be confirmed by actual measurements of uptake of various molecules.

The phenotype of PAO 1654 is of particular interest. It is to be expected that osmotic fragility would be associated with membrane mutations (Nagel de Zwaig & Luria, 1967) and the morphological effects as seen in this mutant, similar to the *lon* mutation (Takano, 1971) and to some *tol* mutants of *E. coli* (Holland *et al.* 1970). The reduced ability of PAO 1654 to be lysogenized by temperate phages is unexpected. It could be argued that this may reflect a membrane attachment site of the prophages concerned. However, it has been found that various phages known to have a chromosomal attachment site in *P. aeruginosa* (Krishnapillai & Carey,

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1972; K. Carey, personal communication), show a *les* phenotype with PAO 1654 so that this argument cannot be supported.

It is of interest that a mutant such as PAO 1654 should show a much reduced ability to acquire the sex factor FP2. Pemberton & Clark (1973) have shown that the sex factors FP2 and FP39 are extrachromosomal entities and it is reasonable to conclude from their results that both plasmids could be associated with the membrane. Perhaps changes in the membrane such as occur in PAO 1654 result in a reduced ability of the plasmid to form a stable association with the mutant membrane.

The phenotypic description of these *tol* mutants in fact raises more problems than solutions. It has not yet been possible to devise a satisfactory reversion test so that it is not known whether mutation in a single gene can produce the multiple phenotypic effects observed. As the mutations examined were all spontaneous, it is unlikely that independent unlinked mutations have occurred. It is likely that the *tol* strains are either mutations at a single locus with pleiotropic effects, or deletions of a group of genes affecting the various properties involved. While it is clear that membrane structure and function is involved, we do not know the actual functions of the various tol^{+} genes. Clearly we must be able to distinguish between primary and secondary effects of gene mutations on membrane structure and function. Perhaps the best way of doing this would be by means of some conditional mutation affecting membrane structure. To date, attempts to find a temperature-sensitive or cold-sensitive *tol* mutant have been unsuccessful. The range of phenotypic and genotypic variation found in these mutants demonstrates the interrelationships of the membrane with many cellular functions and it can be expected that a range of genes will be involved in the formation of such a structure. *tol* mutants are easy to isolate in *P. aeruginosa* and preliminary examination of other mutants shows an even greater degree of pleiotropy. The phenotypic and genetic characterization of additional mutants is proceeding.

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