Transfer of a chromosomal locus responsible for mucoid colony morphology in *Pseudomonas aeruginosa* isolated from cystic fibrosis patients to *P. aeruginosa* PAO

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Summary. The locus responsible for mucoid colony morphology in five independent clinical isolates of *Pseudomonas aeruginosa* from cystic fibrosis patients have been transferred by means of pM060-mediated conjugation to the genetically characterised strain *P. aeruginosa* PAO. Genetic mapping has shown that in all five strains the locus is on the chromosome between 89' and 94', although it is not possible to say that the same locus is involved in each case. The way is now open for a more detailed genetic analysis of the loci responsible for mucoid colony morphology.

Introduction

The lungs of patients suffering from cystic fibrosis (CF) invariably become infected with *Pseudomonas aeruginosa* (May *et al.*, 1972). Antibiotic treatment may not eradicate the organism but is usually clinically beneficial (Hodson *et al.*, 1981). Inevitably, however, a *P. aeruginosa* variant known as "mucoid" (from the appearance of the colonies upon culture) appears in the lung (Doggett *et al.*, 1966), and subsequently the prognosis is poor (Wood *et al.*, 1976; Henry *et al.*, 1982).

The mucoid colonial characteristics of such *P. aeruginosa* variants are due to the production of alginic acid, a 1,4-linked, selectively acetylated, copolymer of β -D-mannuronic acid and its C-5 epimer α -L-glucuronic acid (Fyfe and Govan, 1983). The enzymology of alginic acid synthesis has not yet been elucidated. Pugashetti *et al.* (1983) suggested that it is synthesised via the Entner-Doudoroff pathway—G-P-D mannose dehydrogenase, which converts G-P-D mannose to G-P-D mannuronate, being the major enzyme—whereas Banerjee *et al.* (1983) postulated that synthesis occurs via glyceraldehyde-3-phosphate.

Two different approaches have been used in attempts to elucidate the genetics of mucoid variation. Established systems of gene transfer and recombination are a basic prerequisite of any

genetic study, and *P. aeruginosa* strain PAO is the most extensively characterised pseudomonad in this respect (Holloway *et al.*, 1979; Holloway and Matsumoto, 1984). In the first approach Govan and Fyfe (1978) and Fyfe and Govan (1983) have studied the genetics of mucoid mutants derived from *P. aeruginosa* PAO. However, there is no assurance that such mutants bear any relationship to mucoid strains of *P. aeruginosa* isolated from CF patients.

The second approach was to take a clinical mucoid strain of *P. aeruginosa*, introduce into it auxotrophic and non-mucoid mutations, and by use of chromosome-mobilising plasmids previously described for PAO, attempt to map the loci involved in mucoid production in that strain (Ohman and Chakrabarty, 1981). A difficulty of this approach is that there is no easy way of relating auxotrophic and other loci mapped in this manner to the established *P. aeruginosa* PAO genetic map.

We have used a third approach which minimises objections to the above methods. We have performed experiments to demonstrate the conjugal transfer of chromosomal loci (*muc*) causing the mucoid phenotype to *P. aeruginosa* PAO, and to relate these loci to markers on the *P. aeruginosa* PAO genetic map. In these crosses we have used the enhanced chromosome mobilising (ECM) plasmid pM060 (Riess *et al.*, 1980), which is similar to R68.45 (Haas and Holloway, 1976) but showed much greater stability of chromosome-mobilising ability.

Materials and methods

Bacterial strains and plasmids

These are listed in table I.

Media and cultural conditions

Nutrient Yeast Broth, Nutrient Agar, Minimal Agar (all described by Stanisich and Holloway, 1972) and

Medium P (Leisinger *et al.*, 1972) were used for the culture of *P. aeruginosa* PAO. For mucoid clinical isolates and mucoid recombinant (PAM) strains, fructose peptone broth (Difco Bacto-peptone 1% w/v, fructose 0.3% w/v in distilled water) and peptone fructose agar (fructose peptone broth solidified with Difco Bacto-agar 1.5% w/v) were used. Stocks of amino acids and purines (50 mM) were stored over chloroform and used at a final concentration of 1 mM except for isoleucine which was used

Table I. Bacterial strains and plasmids

Strain	Genotype or phenotype	Derivation or reference
<i>Bacterium</i>		
Ps3694	Prototroph, Muc ⁺	This study
Ps3694-2	<i>met-3003, str-3001</i> , Muc ⁺	Met ⁻ Str ^r derivative of Ps3694
Ps3710	Prototroph, Muc ⁺	This study
Ps3710-17	<i>trp-3002</i> , Muc ⁺	Trp ⁻ derivative of Ps3710
Ps3712	Prototroph, Muc ⁺	This study
Ps3712-15	<i>leu-3002</i> , Muc ⁺	Leu ⁻ derivative of Ps3712
Ps3738	Prototroph, Muc ⁺	This study
Ps3738-1	<i>lys-3002</i> , Muc ⁺	Lys ⁻ derivative of Ps3738
Ps3739	Prototroph, Muc ⁺	This study
Ps3739-1	<i>leu-3004</i> , Muc ⁺	Leu ⁻ derivative of Ps3739
PAM207	<i>thr-9001, nal-26, muc-3712</i>	Pur ⁺ Cys ⁺ Pro ⁺ Muc ⁺ recombinant from the cross PS3712-15(pMO60) × PAO1052
PAM301	<i>lys-12, muc-3694</i>	Cys ⁺ His ⁺ Muc ⁺ recombinant from the cross Ps3694(pMO60) × PAO542
PAM404	<i>lys-12, muc-3739</i>	Cys ⁺ His ⁺ Muc ⁺ recombinant from the cross Ps3739-1(pMO60) × PAO542
PAM501	<i>argA171, nal-25, muc-3738</i>	Cys ⁺ His ⁺ Pro ⁺ Muc ⁺ recombinant from the cross Ps3738-1(pMO60) × PAO2022
PAM601	<i>lys-12, muc-3710</i>	Cys ⁺ His ⁺ Muc ⁺ recombinant from the cross Ps3710-17(pMO60) × PAO542
PAO542	<i>cys-5606, his-5075, lys-12</i>	Royle <i>et al.</i> (1981)*
PAO1052	<i>pur-67, thr-9001, cys-59</i> <i>proB65, nal-26</i>	Royle <i>et al.</i> (1981)*
PAO2022	<i>cys-5606, his-5075, argA171</i> <i>pro-67, nal-25</i>	Fyfe and Govan (1980)*
PAO6020	<i>pur-67, cys-59, proB65</i>	Soldati <i>et al.</i> (1984)*
PAO6021	<i>proB65, pru-365</i>	Soldati <i>et al.</i> (1984)*
<i>Plasmid</i>		
pMO60	Cb Km/Nm Tc Tra Cma	Riess <i>et al.</i> (1980)
pMO887	Cb Km/Nm Tc ^s Tra Cma	M. Nayudu†
pKT212	Sm Tc Cm	Bagdasarian <i>et al.</i> (1979)

P. aeruginosa PAO strains are derived from PAO1 (ATCC 15692), Ps strains are clinical isolates of *P. aeruginosa* and PAM strains are recombinants derived from conjugal crosses between mucoid clinical isolates and PAO which exhibit the mucoid phenotype. Genotype symbols are the same as those used by Bachmann (1983) for *Escherichia coli* except that *str* signifies streptomycin resistance, *nal* resistance to nalidixic acid, *pru* proline utilisation and *muc* involvement in mucoid colony phenotype. Phenotype symbols: Cb, carbenicillin resistance, Km/Nm, kanamycin/neomycin resistance; Tc, tetracycline resistance; Sm, streptomycin resistance; Cm, chloramphenicol resistance; Tra, conjugative ability; Cma, chromosome mobilizing ability; Muc, mucoid colony morphology (Muc⁺, mucoid; Muc⁻, nonmucoid).

*The map positions of markers relevant to this study are shown in the figure.

† pMO887 is a Tc^s derivative of R68-45 (Haas and Holloway, 1976), and carries a small deletion, generated *in vitro*, from the *SalI* site adjacent to the Tc gene (M. Nayudu, personal communication).

at 0.5 mM. When appropriate, media were supplemented with (/L) carbenicillin (Pyopen; Beecham, Australia) 500 mg, streptomycin (Sigma Chemical Co.) 500 mg, or tetracycline (oxytetracycline tetrachloride; Sigma) 200 mg. Strains with ability to use proline as sole carbon source (Pru^+) were selected on Medium P supplemented with proline 0.1% w/v. All cultures were incubated at 37°C, except PAO strains to be used as recipients in crosses with clinical strains which were grown in nutrient yeast broth at 43°C; this renders *P. aeruginosa* PAO phenotypically restrictionless for about 60 generations of growth at 37°C (Holloway, 1965).

Bacteriological techniques

Plate matings (Stanisich and Holloway, 1972) and carbenicillin semi-selection of auxotrophs after mutagenesis with ethyl methane sulphonate (Sigma) (Watson and Holloway, 1976) were performed as described. Pyocin typing of clinical isolates was performed by the method of Gillies and Govan (1966).

Results

During a 2-year period we have received more than 150 mucoid clinical isolates of *P. aeruginosa* from CF patients at the Royal Children's Hospital, Melbourne. These isolates showed considerable variation in growth rate and in the amount of alginate produced on peptone fructose agar; interestingly, in view of the often reported instability of the mucoid phenotype upon subculture, most of our strains were quite stable (data not shown). We chose for further study five stable mucoid isolates (listed in table I) that showed a good growth rate and which had been isolated from unrelated patients. Of these five isolates, only Ps3738 could be distinguished from the rest by pyocin typing (Gillies and Govan, 1966); it was of pyocin type 29, the other four being of type 5. Of the four type-5 isolates, Ps3694 and Ps3710 were gentamicin resistant, whereas Ps3712 and Ps3739 were gentamicin sensitive (information provided by the Royal Children's Hospital). Thus it is not possible for us to state unequivocally that our five isolates represent more than three genetically distinct strains.

Transfer of *muc* loci to *P. aeruginosa* PAO

Initial attempts to transfer the gene or genes responsible for the mucoid phenotype to *P. aeruginosa* PAO involved the use of the ECM plasmid pMO887 which does not carry genes for tetracycline resistance (Tc^R). This plasmid was transferred to a mucoid clinical isolate by selection for carbenicillin resistance. This strain was then used as a donor with various multi-marked PAO recipients carrying the IncQ plasmid pKT212, which confers Tc^R , with selection for both Tc^R and prototrophy of individual auxotrophic markers. The rationale behind this approach is that we have never observed mutation to high-level tetracycline resistance in *P. aeruginosa*. A similar procedure was used successfully to allow large numbers of clinical isolates of *P. aeruginosa* to be readily screened for chromosome-mobilising plasmids (Dean *et al.*, 1979). However, for the analysis of mucoid strains this approach proved to be unsatisfactory for two reasons. First, although pMO887 is stable in *P. aeruginosa* PAO it was highly unstable in all five clinical isolates, losing chromosome-mobilising ability at a high frequency. Second, a more serious problem was "back transfer" of pKT212 to the clinical isolate; pKT212 was mobilised by pMO887 which had transferred to the PAO recipient at high frequency without direct selection. This transfer of pKT212 resulted in the mucoid donors acquiring Tc^R and growing on the mating plates.

These problems were overcome by introducing ethyl methane sulphonate-induced auxotrophic mutations into each of the five clinical isolates to provide a contraselective marker, and by transferring pMO60 to each clinical strain by selection for carbenicillin resistance. This naturally occurring ECM plasmid proved to be quite stable in all five clinical isolates tested. Auxotrophic derivatives of the clinical isolates, carrying pMO60, were plate mated with various multi-marked *P. aeruginosa* PAO recipients, selection was applied for prototrophy of single markers, and the mating plates were screened for the appearance of mucoid recombinants. For each clinical isolate donor, prototrophic

Figure. Genetic map of the FP2 origin region of *P. aeruginosa* PAO, taken from Holloway and Matsumoto (1984); *pro-65* and *pro-67* are alleles of the *proB* locus, *pur-67* is closely linked to *pur-70*, *cys-5606* is closely linked to *cys-59*, and *his-5075* is an allele of the *hisI* locus (Royle *et al.*, 1981). Markers used for contraselection in plate matings were: *argA171* (18'), *lys-12* (20') and *thr-9001* (43') (Holloway and Matsumoto, 1984).

recombinants were formed at a frequency of about 10^{-6} /donor cell, which is about 10–100-fold less than would be expected in PAO × PAO (pMO60) plate matings (data not shown). When selection was made for prototrophy of markers in the *pur-70-proB* region of the chromosome (see figure), 10–15% of the recombinant colonies were mucoid when Ps3694–2, Ps3710–17 or Ps3712–15 carrying pMO60 were the donors, but with Ps3738–1 and Ps3739–1 carrying pMO60 as donors, only about 1% of the recombinant colonies were mucoid (data not shown). With all five clinical isolates, however, selection for other markers failed to yield any mucoid recombinants (data not shown). This result suggested that the *muc* gene or genes being transferred had a chromosomal location; if a plasmid was being mobilised from the clinical isolates apparent linkage to all chromosomal markers used to select recombinants would be expected. It was assumed that the mucoid PAO derivatives now carried a small region (probably 5'–10', and including the *muc* gene or genes) of chromosome from the clinical isolates in place of the homologous PAO chromosomal region.

Mapping of the *muc* locus

To determine the location of the *muc* gene or genes more accurately, one recombinant mucoid clone from each cross was chosen for further study. We have designated these five clones as PAM strains, and the particular derivation of each is shown in table I. Selection for carbenicillin resis-

tance was used to transfer pMO60 into each of the PAM strains which were then used as donors in plate matings with strain PAO1052, or strain PAO6020 in the case of PAM207 (pMO60) because PAM207 required threonine. Subsequent selection was made for one or more prototrophic marker. The co-inheritance values obtained from the cross PAM404 (pMO60) × PAO1052 are shown in table II. As expected, *muc* is linked to all three markers in the *pur-70-proB* region but unlinked to *thr-48* (figure). When *cys-59⁺* was the selected marker the observation that none of the *Muc⁻* recombinants were *Pur⁺*, whereas 88% of them were *Pro⁺* suggested that *muc* is to the left of *cys-59* in the figure. This was confirmed when *cys-59⁺* and *pur-70⁺* were selected simultaneously because all of the *Cys⁺ Pur⁺* recombinants were *Muc⁺*, which indicates that *muc* is located between *pur-70* and *cys-59*. Although it would be possible to obtain such a result if *muc* were located to the left of *pur-70* providing that they were very lightly linked, it was found that the co-inheritance of *pur-70* and *muc-3739* was only 37%.

Matings between the other four PAM strains carrying pMO60 and PAO1052, or PAO6020 in the case of PAM207 (pMO60), gave similar results. Thus all five PAM strains behaved as though they differed from PAO at a single *muc* locus, located between *pur-70* and *cys-59*. However, for a given PAM strain it is not possible to say that only one *muc* locus is involved in producing the mucoid phenotype. Although the *Muc⁺* recombinant colonies from a given PAM (pMO60) × PAO cross

Table II. Conjugational mapping of the *muc-3739* mutation in *P. aeruginosa* PAO

Selected marker(s)	Recombination frequency per donor cell	Mucoid (percentage of total phenotype recombinants)	Percentage co-inheritance of unselected marker(s)			
			<i>pur-70⁺</i>	<i>cys-59⁺</i>	<i>proB65⁺</i>	<i>thr-48⁺</i>
<i>pur-70⁺</i>	9.0×10^{-7}	+ (37)	...	90	84	0
		– (63)	...	0	0	0
<i>cys-59⁺</i>	2.0×10^{-5}	+ (27)	54	...	100	0
		– (73)	0	...	88	0
<i>proB65⁺</i>	2.2×10^{-5}	+ (43)	78	100	...	0
		– (57)	0	85	...	0
<i>thr-48⁺</i>	2.4×10^{-5}	+ (0)	...	...	...	...
		– (100)	0	0	0	...
<i>cys-59⁺ pur-70⁺</i>	2.9×10^{-7}	+ (100)	...	...	70	0
		– (0)	...	...	...	...
<i>cys-59⁺ proB65⁺</i>	1.9×10^{-5}	+ (77)	81	...	...	0
		– (23)	0	...	...	0

PAM404 (pMO60) was plate mated with PAO1052 and selection made for prototrophy of individual and pairs of markers. In each case one hundred recombinants were scored for co-inheritance of unselected donor markers. The map position of markers is shown in the figure.

+ = Mucoid; – = non-mucoid; ... = not isolated.

were morphologically indistinguishable, and segregation of *muc* loci in these crosses might have been expected to yield mucoid colonies of distinguishable phenotype, it is possible that segregation of *muc* loci was occurring undetected. However, if more than one locus is involved, such loci must be tightly linked.

It is also impossible to say that the five PAM strains differ from strain PAO at the same locus. Although they were indistinguishable from each other in colony morphology, further genetic analysis is required to answer this point.

The *P. aeruginosa* PAO genetic map carries a proline utilisation marker (*pru-365*) between *pru-70* and *cys-59* (figure). To determine the relative positions of *muc-3739* and *pru-365* on the genetic map, PAM739 (pMO60) was plate mated with PAO6021. When *proB65*⁺ was the selected marker, 99% of Muc⁺ recombinants were Pru⁺. Because Muc⁻ Pru⁺ recombinants were not recovered, the most probable order of the markers is *muc-pru-pro*. For reasons that we are unable to explain, Pru⁺ recombinants were not recovered by direct selection, even though such recombinants can be recovered at the expected frequencies in PAO × PAO crosses.

Discussion

The transfer of a locus causing the production of alginate in *P. aeruginosa* isolates from CF patients to *P. aeruginosa* PAO, and its subsequent expression in that strain, opens the way to a genetic analysis of the nature of this locus, and will allow a comparison with those *P. aeruginosa* PAO loci that have been identified by Fyfe and Govan (1983) as being responsible for alginate production. These workers have mapped two such loci, closely linked to but on either side of *cys-59*, and phenotypically distinguishable on the basis of colony morphology. Group-1 mutations, represented by *muc-23* which maps to the right of *cys-59*, yield mucoid colonies on minimal agar, whereas group-2 mutations, represented by *muc-22* which maps to the left of *cys-59*, gives rise to non-mucoid colonies on minimal agar. All five PAM strains generated in this study form mucoid colonies on minimal agar and hence carry group-1 mutations but our linkage data clearly indicate a position for *muc* to the left of *cys-59*. Furthermore, although *muc-22* maps to the right of *pru* (Fyfe and Govan, 1983), our data suggest that *muc-3739* (carried by PAM404) maps

to the left of *pru*. Clearly, more than one locus is involved, and more precise mapping of further mutations either generated in *P. aeruginosa* PAO or transferred to PAO from mucoid clinical isolates is required before any conclusions can be drawn.

It is more difficult to correlate our findings with those of Ohman and Chakrabarty (1981) who investigated three closely linked loci, denoted *alg*, in a mucoid clinical isolate that could mutate to suppress the mucoid phenotype. Moreover, the location of these *alg* loci was determined by Ohman and Chakrabarty in relation to a range of auxotrophic markers generated in their clinical isolate. Thus it is not possible to relate the location of any of these loci to the *P. aeruginosa* PAO chromosome which serves as a reference point for genetic studies in this organism. In a later study, and using another mucoid clinical isolate, Darzins and Chakrabarty (1984) mapped a mutation that suppressed the mucoid phenotype to 19' on the PAO chromosome map. They were able to do this because a clone from a cosmid gene bank, identified by its ability to complement the *alg*⁻ suppressor mutation (i.e., restore the mucoid phenotype), also carried the *argH*⁺ wild-type gene which is located at 19' (Holloway and Matsumoto, 1984). These workers also identified three other *alg*⁻ loci on the basis of complementation by different clones from their cosmid bank, but were not able to determine the chromosomal locations of these loci. Isolation and mapping of the mucoid suppressor mutations in the PAM strains described in this paper will determine the map positions of suppressor loci.

A reported feature of mucoid strains is their instability during culture (Zierdt and Schmidt, 1964; Govan, 1975; Fyfe and Govan, 1983). Govan (1975) reported that the use of desoxycholate citrate agar allows maintenance of the mucoid colony morphology. We noted early in this study that our strains appeared quite stable for the mucoid phenotype. Boyce and Miller (1981) reported that supplementation of the growth medium with 0.03M FeCl₃ led to rapid overgrowth of the culture by non-mucoid revertants, and preliminary studies with our PAM strains support this (data not shown). This leads to interesting speculations as to the role of Fe⁺⁺⁺ in the emergence and maintenance of the mucoid phenotype in the lungs of CF patients.

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