Alginate Synthesis in Mucooid *Pseudomonas aeruginosa*: a Chromosomal Locus Involved in Control

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Mucooid variants of *Pseudomonas aeruginosa* isolated *in vitro* or *in vivo* could be classified into two phenotypic groups based on whether alginate was produced on a chemically defined medium. Mucooid strains yielded lower recombination frequencies than the non-mucooid parent when used as donors in FP2-mediated plate matings. The mucooid characteristic (*muc*) was co-inherited by a proportion of recombinants selected for the inheritance of chromosomal markers *his-5075*⁺ or *cys-5605*⁺. The results of further experiments using either a mucooid recipient or a mucooid donor carrying plasmid R68.45 suggested that the control of alginate production in *P. aeruginosa* involves at least one chromosomal locus.

INTRODUCTION

Mucooid strains of *Pseudomonas aeruginosa* are frequently associated with chronic pulmonary infections in patients with cystic fibrosis (CF), but are rarely encountered in nature or in other clinical situations (Doggett, 1969). Doggett *et al.* (1966) reported that the non-mucooid form of *P. aeruginosa* precedes the mucooid form in the CF lung, but the latter eventually predominates and is associated with clinical deterioration. Mucooid and non-mucooid strains isolated from the same patient have been shown to belong to the same pyocin type (Williams & Govan, 1973) and serotype (Diaz *et al.*, 1970) suggesting that they are variants of the same strain.

Several explanations have been proposed for the emergence of mucooid strains *in vivo*. These include induction by phage (Martin, 1973) and selection of a mutant by prolonged antibiotic therapy or some other factor (Doggett & Harrison, 1969; Govan & Fyfe, 1978). Markowitz *et al.* (1978) suggested that the genetic basis of alginate production by mucooid *P. aeruginosa* may be plasmid-determined, but concluded that there was no evidence for this.

In *Escherichia coli* K12, capsular polysaccharide production is controlled by three regulator genes, *CapR*, *CapS* and *CapT*. Mutations in any of these loci result in a mucooid phenotype, and mapping studies have shown them to be situated in three distinct regions of the chromosome (Hua & Markovitz, 1972).

We have previously reported that mucooid variants of *P. aeruginosa* strain PAO and other non-mucooid strains can be isolated *in vitro* by selecting for carbenicillin resistance (Govan & Fyfe, 1978) or resistance to a virulent phage (Govan, 1975), and that the exopolysaccharide produced by such variants is an alginate-like polymer, similar to that produced by clinical isolates. The ability to produce alginate is transferable between strains of *P. aeruginosa* by conjugation (Govan, 1976; Fyfe & Govan, 1978).

This paper reports further studies on the genetic basis of alginate production in *P. aeruginosa* using mucooid derivatives of genetically marked PAO strains.

Table 1. *Strains of Pseudomonas aeruginosa used in this study*

Strain	Genotype*	Description or reference
PAO8	<i>met-28 ilv-202 str-1</i> , FP ⁻	Isaac & Holloway (1968)
PAO381	<i>leu-38 str-2</i> , FP2 ⁺	Stanisich & Holloway (1969)
PAO568	<i>leu-38 str-2 muc-2</i> , FP2 ⁺	Mucoid derivative of PAO381 isolated following incubation with phage M6 (Govan, 1975)
PAO578	<i>leu-38 str-2 muc-22</i> , FP2 ⁺	Mucoid derivative of PAO381 isolated by the method of Govan & Fyfe (1978)
PAO579	<i>leu-38 str-2 muc-23</i> , FP2 ⁺	Mucoid derivative of PAO381 isolated by the method of Govan & Fyfe (1978)
PAO581	<i>leu-38 str-2 muc-25</i> , FP2 ⁺	Mucoid derivative of PAO381 isolated following incubation with phage E79 (Govan, 1975)
PAO2018	<i>met-28 ilv-202 str-1 muc-35</i> , FP ⁻	Mucoid derivative of PAO8 isolated by the method of Govan & Fyfe (1978)
PAO2020	<i>cys-5605 argA171 pro-67 his-52 nal-25</i> , FP ⁻	His ⁻ derivative of a <i>his-5075</i> ⁺ transductant of PAO2022
PAO2021	<i>cys-5605 his-5075 argA171 pro-67 nal-25 muc-36</i> , FP ⁻	Mucoid derivative of PAO2022 isolated by the method of Govan & Fyfe (1978)
PAO2022	<i>cys-5605 his-5075 argA171 pro-67 nal-25</i> , FP ⁻	Pro ⁻ NAL ⁺ derivative of PAO540 (Haas <i>et al.</i> , 1977)
PAO2023	<i>cys-5605 his-5075 argA171 pro-67 nal-25 car-10</i> , FP ⁻	Car ⁻ derivative of PAO2022
PAO2050	<i>his-5075 argA171 pro-67 nal-25 muc-35</i> , FP ⁻ , R ⁻	Mucoid Cys ⁺ recombinant from PAO2018(R68.45) × PAO2022

* The genotype symbols designate the following: *arg*, arginine; *car*, carbamoylphosphate synthase; *cys*, cysteine; *his*, histidine; *ilv*, isoleucine/valine; *leu*, leucine; *met*, methionine; *muc*, mucoid (alginate-producing); *nal*, nalidixic acid resistance; *pro*, proline; *str*, streptomycin resistance. FP⁻, R⁻, recipient strain; FP2⁺, donor strain carrying the sex factor FP2.

METHODS

Bacteria and bacteriophage. The PAO strains used in this study are shown in Table 1. Donor strains carrying the plasmid R68.45 were constructed according to Haas & Holloway (1976). Nalidixic acid-resistant mutants were isolated according to Haas & Holloway (1976). Phage F116L (Krishnapillai, 1971) was used for all transductions. Phage PR4 (Stanisich, 1974) was used in the isolation of strains which had lost R68.45. Phages E79 and M6, obtained from Professor B. W. Holloway, were used in the isolation of mucoid strains using the method of Govan (1975). Clinical strains of mucoid *P. aeruginosa* were obtained from patients attending the CF clinic at the Royal Hospital for Sick Children, Edinburgh and were supplied by Dr Kenneth Watson.

Media and cultural conditions. Nutrient Broth (NB) was Oxoid no. 2 supplemented with 0.5 % (w/v) yeast extract (Oxoid), *Pseudomonas* Isolation Agar (PIA) was from Difco and Minimal Agar (MA) was as described by Vogel & Bonner (1956). Amino acid supplements were used at a concentration of 1 mM, 50 mM stock solutions being kept over chloroform. Antibiotics used were carbenicillin (Pyopen; Beecham), kanamycin (Bristol Laboratories), tetracycline (Glaxo) and nalidixic acid (NAL) (Winthrop).

Broth cultures were grown in McCartney bottles on an orbital incubator (Gallenkamp) at 140 rev. min⁻¹, and all cultures were incubated at 37 °C.

Transductions were performed according to the method of Krishnapillai (1971).

Plate matings were performed as described by Stanisich & Holloway (1972).

Interrupted matings were performed as described by Haas *et al.* (1977).

Recombinant analysis. This was performed to determine the percentage co-inheritance of unselected markers. For each selected marker, 100 recombinants were purified by streaking on to fresh selective MA. Inheritance of unselected auxotrophic markers was scored by transferring recombinants to appropriately supplemented MA. Inheritance of *muc* or *muc*⁺ was scored by transferring the recombinants to PIA, except for matings using the donors PAO579 and PAO581. In these cases, mucoid recombinants could be recognized on the mating plates, and transfer to PIA before scoring made no difference to the result obtained.

Fig. 1. Phenotypic differences between two mucooid variants of PAO381. Strains PAO578 and PAO579 after 24 h incubation on PIA and MA + leucine: PAO579 produces copious alginate under both conditions, whereas PAO578 appears rough and non-mucoid on MA + leucine.

RESULTS

Phenotypic differences between mucooid strains

The mucooid strains investigated produced copious exopolysaccharide on PIA after 24 h incubation and resembled colonial type 5 described by Phillips (1969); they could be readily distinguished from the slimy growth often observed following extended incubation of *P. aeruginosa* in media containing high concentrations of carbohydrate. However, when 100 independent, spontaneous mucooid derivatives of PAO381 were cultured on MA (+leucine), only 40 % were mucooid after 24 h, while the remaining 60 % appeared rough and non-mucoid; when transferred back to PIA, the two groups were both mucooid. Strains PAO579 and PAO581 are examples of the first category (group 1) and PAO568 and PAO578 are examples of the second group (group 2). Figure 1 shows PAO578 and PAO579 after 24 h incubation on PIA and MA + leucine.

Mucooid strains isolated from the sputa of patients with cystic fibrosis could also be classified into groups 1 and 2, and in several cases appeared concurrently in the same specimen.

Mucooid strains as donors and recipients in FP2-mediated plate matings

Figure 2 is a chromosome map of *P. aeruginosa* PAO showing the locations of markers relevant to this study. To examine the effect of alginate production on chromosome transfer, a series of plate matings was performed using mucooid strains as donors or recipients.

Table 2 shows the recombination frequencies obtained using the non-mucooid recipient strain PAO2022 and the mucooid donors PAO568, PAO578, PAO579 and PAO581. Strain PAO381 was included as a control donor. The mucooid donors yielded lower recombination frequencies than PAO381 for all selected markers. Non-mucooid revertants of these strains had recovered donor ability, giving recombination frequencies equivalent to those obtained using PAO381.

When mucooid derivatives of PAO2022 were used as recipients in plate matings with PAO381, recombination frequencies were similar to those obtained in the PAO381 × PAO2022 mating. Hence alginate production does not impair recipient ability.

Fig. 2. Chromosome map of *P. aeruginosa* strain PAO showing the markers relevant to this study (based on Holloway *et al.*, 1979). See legend to Table 1 for genotype symbols. Very close linkage exists between the following pairs of markers: *pro-67* and *proB*, *car-10* and *car-9*, *his-5075* and *hisI*, *his-52* and *hisII*.

Table 2. Frequency of recombinants obtained in plate matings between PAO2022 and mucoid derivatives of PAO381

Selected marker	No. of recombinants per 10 ⁸ donors				
	× PAO381	× PAO568	× PAO578	× PAO579	× PAO581
<i>pro-67</i> ⁺	1186	25	81	13	47
<i>his-5075</i> ⁺	98	9	14	3	17
<i>cys-5605</i> ⁺	81	7	8	2	9
<i>argA171</i> ⁺	958	100	108	36	119

Co-inheritance of *muc* with *his-5075* and *cys-5605*

Examination of the recombinants from the PAO579 × PAO2022 and PAO581 × PAO2022 matings revealed mixtures of mucoid and non-mucoid clones. All the recombinants from the matings using PAO568 and PAO578 appeared non-mucoid on the selection plates. However, when purified and replica-plated to PIA, a proportion was mucoid.

Table 3 shows the percentage co-inheritance of alginate production with each of the selected markers in these plate matings. All mucoid clones from the mating plates were checked for NAL^r to ensure that they were recombinants and not *leu*⁺ revertants of the donors.

In a reciprocal plate mating between a non-mucoid donor strain (PAO381) and a group 2 mucoid recipient (PAO2021), co-inheritance of *muc-36*⁺ (non-mucoid) with *his-5075*⁺ and *cys-5605*⁺ was observed. Analysis of recombinants (outlined in Table 4) indicated a location for *muc-36* distal to *cys-5605* rather than between *his-5075* and *pro-67*, since the co-inheritance of *muc*⁺ by *cys*⁺*pro*⁺ and *his*⁺*pro*⁺ double recombinants was low. However, low co-inheritance of *muc*, *his* and *cys* was obtained when the selected marker was *argA171*.

Fig. 3. Interrupted mating between PAO381 and the mucoid recipient PAO2021. (a) Time of entry kinetics of *pro-67* (○), *cys-5605* (●), *his-5075* (□) and *argA171* (■). (b) Percentage co-inheritance with *cys-5605*⁺ of the unselected markers *muc-36*⁺ (○), *pro-67*⁺ (●) and *argA171*⁺ (□).

Table 3. Co-inheritance of *muc* mutations with selected auxotrophic markers following plate matings between mucoid donors and the non-mucoid recipient PAO2022

Donor	Percentage of recombinants co-inheriting <i>muc</i>			
	<i>pro-67</i> ⁺	<i>his-5075</i> ⁺	<i>cys-5605</i> ⁺	<i>argA171</i> ⁺
PAO568 (<i>muc-2</i>)	1	21	15	<1
PAO578 (<i>muc-22</i>)	1	11	11	<1
PAO579 (<i>muc-23</i>)	2	21	32	<1
PAO581 (<i>muc-25</i>)	<1	7	14	<1

Table 4. Recombinant analysis following a plate mating between PAO381 and the mucoid recipient PAO2021

Selected marker	No. of recombinants per 10 ⁸ donors	Percentage co-inheritance of unselected marker				
		<i>muc-36</i> ⁺	<i>pro-67</i> ⁺	<i>his-5075</i> ⁺	<i>cys-5605</i> ⁺	<i>argA171</i> ⁺
<i>pro-67</i> ⁺	2182	1	—	2	2	1
<i>his-5075</i> ⁺	164	15	34	—	90	2
<i>cys-5605</i> ⁺	155	18	30	93	—	1
<i>argA171</i> ⁺	1273	<1	<1	<1	<1	—

So confirmation of the proposed location of *muc-36* could not be obtained by the analysis of *arg*⁺*cys*⁺ and *arg*⁺*his*⁺ double recombinants.

Co-inheritance of unselected auxotrophic markers was not affected by alginate production by either the donor or recipient in these plate matings.

Transfer of *muc-36*⁺ during an interrupted mating

To obtain further evidence for the location of *muc-36* distal to *cys-5605* an interrupted mating was performed between PAO381 and PAO2021 (Fig. 3). Time of entry kinetics displayed by the selected markers *pro-67*⁺, *his-5075*⁺, *cys-5605*⁺ and *argA171*⁺ (Fig. 3a) were consistent with the published order and times of entry for these loci (Holloway *et al.*, 1979). The transfer of *muc-36*⁺ could not be measured directly as a selection procedure is not available. However, the *cys*⁺ recombinants obtained at each interruption time were scored for co-inheritance of *muc*⁺ as well as the proximal marker *pro*⁺ and the distal marker *arg*⁺ (Fig. 3b). The percentage co-inheritance of *pro*⁺ by *cys*⁺ recombinants was approximately 25 %, irrespective of the interruption time. Conversely, the percentage co-inheritance of *muc*⁺ increased significantly with mating time, indicating a time of entry later than for *cys*.

Table 5. *Recombinant analysis following a plate mating between PAO2018(R68.45) and PAO2022*

Selected marker	No. of recombinants per 10 ⁸ donors	Percentage co-inheritance of unselected marker				
		<i>muc-35</i>	<i>pro-67</i> ⁺	<i>his-5075</i> ⁺	<i>cys-5605</i> ⁺	<i>argA171</i> ⁺
<i>pro-67</i> ⁺	3000	6	—	43	42	3
<i>his-5075</i> ⁺	4100	16	49	—	92	2
<i>cys-5605</i> ⁺	5600	22	46	93	—	4
<i>argA171</i> ⁺	19000	2	13	10	8	—

Table 6. *Recombinant analysis following a plate mating between PAO8(R68.45) and PAO2050*

Selected marker	No. of recombinants per 10 ⁸ donors	Percentage co-inheritance of unselected marker			
		<i>muc-35</i> ⁺	<i>pro-67</i> ⁺	<i>his-5075</i> ⁺	<i>argA171</i> ⁺
<i>pro-67</i> ⁺	1500	5	—	31	2
<i>his-5075</i> ⁺	3100	26	49	—	7
<i>argA171</i> ⁺	17000	3	7	4	—

However, low co-inheritance of *arg*⁺ was again obtained, and when the *arg*⁺ recombinants were scored for *cys*⁺ and *muc*⁺, <1 % co-inheritance was observed.

Mapping muc-35 using R68.45

In a further attempt to map a *muc* locus unambiguously, a series of plate matings was performed using donor strains carrying R68.45. The group 2 mucoid strain PAO2018 (R68.45) was crossed with PAO2022, and the recombination frequencies and co-inheritance of unselected markers are shown in Table 5. One of the *cys*⁺ recombinants from this mating had inherited *muc-35* and R68.45, but was still *pro his arg*. Strain PAO2050 was derived from this recombinant by isolating a clone resistant to the phage PR4. This clone had acquired sensitivity to carbenicillin, tetracycline and kanamycin and could act as a recipient for R68.45. Hence, it was concluded that PAO2050 was an R⁻ derivative of the original *cys*⁺ recombinant. A plate mating was performed between PAO8(R68.45) and PAO2050 in which *muc-35* was a recipient marker rather than a donor marker, as in the previous mating. The results are shown in Table 6.

Linkage of muc-35 to other markers

Since *muc-35* showed linkage to *his-5075* and *cys-5605* in R68.45-mediated plate matings, it was expected that co-inheritance of *muc-35* would also be observed with other markers in the 8 to 15 min region of the PAO chromosome. Hence, plate matings were performed using PAO2018(R68.45) as donor and the non-mucoid recipient strains PAO2020 (*pro-67 cys-5605 his-52 argA171*) and PAO2023 (*pro-67 car-10 his-5075 cys-5605 argA171*). Recombinant analysis following these matings indicated that co-inheritance of *muc-35* with *his-52* and *car-10* was low (3 % in each case).

Attempts to isolate new auxotrophic markers in the 8 to 15 min region were unsuccessful.

DISCUSSION

The mucoid PAO strains and mucoid strains of *P. aeruginosa* isolated from CF patients could be divided into two major groups based on alginate production or non-production on MA after 24 h at 37 °C. Further characterization of these strains should reveal the extent of phenotypic variation and lead to an understanding of the nature of the control system.

Production or non-production of alginate cannot be selected for in plate or interrupted

mating experiments. As in the case of *E. coli*, the mucooid determinants can only be mapped indirectly by measuring co-inheritance with markers which can be selected for. Markovitz *et al.* (1967) used this method to show that *CapS*, a mutation resulting in mucoidness of *E. coli* K12, was located on the chromosome near a *trp* gene. They performed a series of interrupted and uninterrupted matings between a non-mucoid donor (*trp*⁺ *CapS*⁺ Hfr) and a mucooid recipient (*trp* *CapS*) screening the *trp*⁺ recombinants for co-inheritance of *CapS*⁺.

Our initial experiments with *P. aeruginosa* using mucooid donors (FP2⁺) showed that four, independently isolated *muc* mutations could be transferred to a non-mucoid recipient by selecting for the inheritance of chromosomal markers *cys-5605* and *his-5075*. Similar results were obtained when PAO381 was crossed with a mucooid recipient, PAO2021. However, more precise mapping of the mucooid determinant (*muc-36*) proved difficult because of linkage anomalies between the selected markers. The segregation data in Table 4 and the results of the interrupted mating (Fig. 3) serve to illustrate this problem. Although *argA171* enters approximately 7 min after *his-5075* and *cys-5605*, *arg*⁺ recombinants show no co-inheritance of the earlier markers.

Low linkage between markers with similar times of entry has been previously described in PAO, and several hypotheses have been put forward to explain this observation. These included the 'two linkage group' theory (Stanisich & Holloway, 1969; Loutit, 1969) and the 'two origin' hypothesis (Holloway *et al.*, 1975); however, insufficient evidence has been obtained to support either of these alternatives. The development of an improved technique for FP2-mediated interrupted matings (Haas *et al.*, 1977) and the introduction of R68.45 into PAO genetics have provided alternative approaches to chromosome mapping in *P. aeruginosa*.

We attempted to map *muc-35* by means of R68.45-mediated plate matings; however, the results were essentially the same as those obtained using FP2.

A possible explanation for the low linkage between *argA171* and the proximal markers in our experiments could be that the recipient strain PAO2022 was derived from PAO540, which had been mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine on two occasions. However, this is unlikely because we have obtained similar results using PAT strains with comparable markers.

Despite these anomalies, which are not in themselves associated with alginate production in *P. aeruginosa* strain PAO, this investigation has shown that all the *muc* mutations examined are linked to the chromosomal markers *pro-67*, *his-5075* and *cys-5605*, and that at least one, *muc-36*, is distal to *cys-5605* and probably at about 14 min. A catabolic marker, *puuB*, is also reported to be in the 14 min region (Matsumoto *et al.*, 1978) but we have not yet determined whether *muc-36* is closely linked to this locus.

Before the number of genes involved in the control of alginate production can be determined, a closely linked marker will need to be found which can be selected for in matings and transductions.

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