***Pseudomonas aeruginosa* in cystic fibrosis: role of *mucC* in the regulation of alginate production and stress sensitivity**

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Alginate production in *Pseudomonas aeruginosa* and the associated mucoid phenotype of isolates from cystic fibrosis patients are under the control of the *algU mucABCD* cluster. This group of genes encodes AlgU, the *P. aeruginosa* equivalent of the extreme heat shock σ factor σ^E in Gram-negative bacteria, the AlgU-cognate anti- σ factor MucA, the periplasmic protein MucB and a serine protease homologue, MucD. While *mucA*, *mucB* or *mucD* act as negative regulators of AlgU, the function of *mucC* is not known. In this study the role of *mucC* in *P. aeruginosa* physiology and alginate production has been addressed. Insertional inactivation of *mucC* in the wild-type *P. aeruginosa* strain PAO1 did not cause any overt effects on alginate synthesis. However, it affected growth of *P. aeruginosa* under conditions of combined elevated temperature and increased ionic strength or osmolarity. Inactivation of *mucC* in *mucA* or *mucB* mutant backgrounds resulted in a mucoid phenotype when the cells were grown under combined stress conditions of elevated temperature and osmolarity. Each of the stress factors tested separately did not cause comparable effects. The combined stress factors were not sufficient to cause phenotypically appreciable enhancement of alginate production in *mucA* or *mucB* mutants unless *mucC* was also inactivated. These findings support a negative regulatory role of *mucC* in alginate production by *P. aeruginosa*, indicate additive effects of *muc* genes in the regulation of mucoidy in this organism and suggest that multiple stress signals and recognition systems participate in the regulation of *algU*-dependent functions.

Keywords: sigma factors, anti-sigma, mucoidy

INTRODUCTION

Conversion of *Pseudomonas aeruginosa* into the mucoid, exopolysaccharide alginate-overproducing phenotype is usually associated with chronic colonization of the respiratory tract in cystic fibrosis (Govan & Deretic, 1996). While mucoid *P. aeruginosa* can also be encountered in other chronic infections (Govan & Deretic, 1996), mucoid colony morphology is rare among environmental isolates which can produce alginate but only at very low levels (Anastassiou *et al.*, 1987; Pier *et al.*, 1986). Recent analyses have shown that the conversion to mucoidy in cystic fibrosis isolates is frequently caused by mutations in the *mucA* gene (Boucher *et al.*,

1996; Deretic *et al.*, 1994, 1995; Martin *et al.*, 1993c; Yu *et al.*, 1996). This gene is located within the *algU mucABCD* cluster at 67.5 min (Boucher *et al.*, 1996; Martin *et al.*, 1993a, b). The genes within this cluster and their organization are highly conserved in Gram-negative bacteria (Fig. 1a). In *P. aeruginosa* this locus encodes the following elements. (i) The σ factor AlgU [also known as AlgT (DeVries & Ohman, 1994) or Pa σ^E (Deretic *et al.*, 1994)] is a member of the AlgU-RpoE subfamily of alternative σ factors (Deretic *et al.*, 1995; Lonetto *et al.*, 1994; Yu *et al.*, 1996). *P. aeruginosa* AlgU and its equivalent in enteric bacteria, σ^E , control extreme stress responses in these organisms (Erickson & Gross, 1989; Martin *et al.*, 1994; Raina *et al.*, 1995; Rouvière *et al.*, 1995; Yu *et al.*, 1995). (ii) The anti- σ factor MucA which associates with AlgU and inhibits its function (Schurr *et al.*, 1996; Xie *et al.*, 1996). (iii) The periplasmic

Abbreviation: PIA, *Pseudomonas* isolation agar.

Table 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant genotype or properties*	Source or reference
<i>P. aeruginosa</i>		
PAO1	Prototroph Alg ^{-wt}	B. Holloway (Monash University, Australia)
PAO568	PAO381 Alg ^{+m} <i>mucA2</i>	Fyfe & Govan (1980)
PAO6857	PAO1 Alg ^{+m} <i>mucB::Tc^r</i>	Schurr <i>et al.</i> (1996)
PAO6875	PAO1 Alg ^{-wt} <i>mucC::Gm^r</i>	This work
PAO6876	PAO568 Alg ^{+NaCl} <i>mucC::Gm^r</i>	This work
PAO6877	PAO6857 Alg ^{+Suc} <i>mucC::Gm^r</i>	This work
PAO6858	PAO568 Alg ^{+c} <i>mucB::Tc^r</i>	This work
Plasmids		
pKI11G	pUC18 Gm ^r cassette	S. Lory (University of Washington, USA)
pCMobA	pHC79 <i>mob</i> ⁺	Mohr & Deretic (1990)
pBSC1	pCRII <i>mob</i> ⁺ <i>mucC::Gm^r</i>	This work
<i>ptac-mucd</i> ⁺	IncQ/P4 <i>tac-mucD</i> ⁺ <i>lacI</i> ^q Cb ^r	Boucher <i>et al.</i> (1996)

* Alg^{+c}, constitutive mucoid phenotype; Alg^{+m}, mucoid phenotype dependent on medium; Alg^{+NaCl}, enhanced mucoid phenotype when grown on LB supplemented with 90 mM NaCl; Alg^{+Suc}, enhanced mucoid phenotype when grown on LB supplemented with 30 % sucrose; Alg^{-wt}, wild-type non-mucoid phenotype.

protein MucB (Martin *et al.*, 1993b; Schurr *et al.*, 1996), also known as AlgN (Goldberg *et al.*, 1993), which displays negative effects on AlgU-dependent expression. (iv) MucC (Boucher *et al.*, 1996), a protein of unknown function. (v) MucD (Boucher *et al.*, 1996), a homologue of the periplasmic serine protease HtrA (DegP) (Lipinska *et al.*, 1988; Strauch & Beckwith, 1988), which has been proposed to remove signals (e.g. denatured or otherwise damaged proteins) or other factors activating AlgU (Boucher *et al.*, 1996). The available genetic and biochemical evidence strongly supports the notion that MucA, MucB and MucD are negative regulators of AlgU activity.

AlgU (Pa σ^E) directs transcription of promoters characterized by the σ^E promoter consensus sequence -35 GAACCTT(N_{16/17}) -10 TCTGA (Deretic *et al.*, 1994; Erickson & Gross, 1989; Lam *et al.*, 1980; Martin *et al.*, 1993c; Schurr *et al.*, 1995). In addition to the P₁ and P₃ promoters of AlgU (Schurr *et al.*, 1995) at least two other genes critical for alginate production, *algD* and *algR*, have σ^E promoters and depend on AlgU for transcription (Deretic *et al.*, 1994; Martin *et al.*, 1994; Wozniak & Ohman, 1994). The *algD* gene encodes a key alginate biosynthetic enzyme, GDP-mannose dehydrogenase (May & Chakrabarty, 1994), and is located at the beginning of the gene cluster at 34 min which also encodes additional alginate biosynthetic and modification enzymes with partial overlaps with LPS biogenesis (Coyne *et al.*, 1994; May & Chakrabarty, 1994). In other organisms, elements controlled by AlgU homologues have been implicated in the heat shock response (Erickson & Gross, 1989), resistance to reactive oxygen intermediates (Johnson *et al.*, 1991), virulence in mice (Elzer *et al.*, 1996; Johnson *et al.*, 1991) and survival under conditions of high pressure and extreme cold (Chi

& Bartlett, 1995). While the role of *mucA*, *mucB* and *mucD* in the regulation of mucoidy in *P. aeruginosa* has been explored, very little is known about the function of *mucC*. Here we present data indicating a negative regulatory role for *mucC*. The effects of *mucC* are noticeable only in mucoid mutants and under conditions of environmental stress.

METHODS

Bacterial strains, plasmids and growth conditions. *P. aeruginosa* strains and plasmids used in this study are shown in Table 1. PAO6875, PAO6876 and PAO6877 were derivatives of PAO1, PAO568 (*mucA2*) and PAO6857 (*mucB::Tc^r*), respectively, with an inactivated *mucC* gene (*mucC::Gm^r*). Strain PAO6858 is a *mucB::Tc^r* derivative of PAO568. *P. aeruginosa* was grown on *Pseudomonas* isolation agar (PIA; Difco) or LB supplemented with 90 mM NaCl, 300 mM NaCl, 30 % (w/v) sucrose, tetracycline (300 or 50 μ g ml⁻¹ for PIA or LB, respectively) or gentamicin (150 or 13 μ g ml⁻¹ for PIA or LB, respectively) when required. *Escherichia coli* was grown on LB supplemented with ampicillin (100 μ g ml⁻¹), kanamycin (25 μ g ml⁻¹) or gentamicin (13 μ g ml⁻¹) when required. Bacterial growth curves were determined as described previously (Boucher *et al.*, 1996).

Genetic methods. Gene replacements and insertional inactivation of chromosomal genes were carried out as described previously (Martin *et al.*, 1993b). The *mucB* and *mucC* genes were insertionally inactivated on the *P. aeruginosa* chromosome via homologous recombination using the non-replicative plasmids pDMB100 and pBSC1, respectively. For insertional inactivation of *mucC* on the chromosomes of PAO1, PAO568 and PAO6857, a 0.9 kb PCR product generated with the primers UL-9 (5' TGAAGTCCGGCAGTT 3') and UR-24 (5' CGGCACCTGCCGAAGA 3') was cloned into pCRII (Invitrogen). This construct was further modified by inserting the *Xba*I linker Gm^r cassette from pKI11G into the *Avr*II site within *mucC*, followed by cloning of *mob* from pCMobA into

the *HindIII* site to generate pBSC1. Candidate strains with gene replacements via double cross-over events between plasmid and chromosome were isolated as Gm^r Cb^s or Tc^r Cb^s exconjugants selected by replica plating. All gene replacements were confirmed by Southern blot analysis.

Alginate assay. Production of alginate was determined by assaying uronic acids as described by Knutson & Jeanes (1976).

RESULTS

The *mucC* gene is required for growth under conditions of combined elevated temperature and high salt concentration

The *algU mucABCD* cluster is conserved with minor variations in all Gram-negative bacteria investigated to date (Fig. 1a) (Schurr *et al.*, 1996; Yu *et al.*, 1996). The first four genes of the cluster, including *mucC*, are present in the majority of the organisms in which the corresponding gene cluster has been analysed (Yu *et al.*, 1996). To investigate the potential role of *mucC* in the regulation of AlgU and alginate production, we insertionally inactivated *mucC* in the standard genetic wild-type non-mucoid strain PAO1. Unlike inactivation of *mucA*, *mucB* or *mucD*, which each individually cause conversion to mucoidy in PAO1 (Boucher *et al.*, 1996; Martin *et al.*, 1993b, c), disruption of the *mucC* gene on the chromosome did not result in visible morphological changes, i.e. the resulting strain, PAO6875 (*mucC*::Gm^r), remained non-mucoid (Table 2). These results suggest that the loss of *mucC* alone, in contrast to the other members of the *mucABCD* cluster (Boucher *et al.*, 1996; Martin *et al.*, 1993b, c), is not sufficient to alter the non-mucoid phenotype.

Since the MucC homologue from *Photobacterium* sp. strain SS9 (Chi & Bartlett, 1995) appears to participate in the adaptation of this bacterium to adverse environmental conditions, we hypothesized that MucC may affect functions of AlgU other than alginate production, e.g. survival of *P. aeruginosa* at elevated temperatures and upon exposure to reactive oxygen intermediates (Martin *et al.*, 1994; Yu *et al.*, 1995). To test the possibility that *mucC* may affect *P. aeruginosa* viability, we compared growth characteristics of PAO6875 (*mucC*::Gm^r) and its parent PAO1 (*mucC*⁺) under conditions of environmental stress. No differences were observed in sensitivity to reactive oxygen intermediates (tested by exposure to paraquat or H₂O₂) or growth at 42 °C, previously shown to adversely affect *P. aeruginosa* *algU* null strains (Martin *et al.*, 1994). Since σ^E has been implicated in the response to osmolarity changes (Mecssas *et al.*, 1993) and some mucoid *P. aeruginosa* strains have been observed to alter alginate production in the presence of osmolytes (NaCl or sucrose) (Deretic *et al.*, 1990), we next tested whether *mucC* mutations can affect *P. aeruginosa* growth on high osmolarity media. No differences between PAO1 and PAO6875 (*mucC*::Gm^r) were observed on LB supplemented with 300 mM NaCl (data not shown). However, when a combination of factors was examined,

Fig. 1. Genetic organization of the *algU mucABCD* gene cluster which controls expression of mucoidy in *P. aeruginosa*, organization of its equivalents in other Gram-negative organisms and conservation of MucC homologues. (a) Genes and their functions. *algU* encodes the alternative σ factor AlgU (Pa σ^E) (Martin *et al.*, 1993a; Yu *et al.*, 1995). *mucA* is a common site of spontaneous mutations causing conversion to mucoidy in cystic fibrosis (Martin *et al.*, 1993c); it encodes an anti- σ factor which binds to AlgU and inhibits its activity (Schurr *et al.*, 1996; Xie *et al.*, 1996). *mucB* encodes a periplasmic protein which also acts as a negative regulator of mucoidy (Martin *et al.*, 1993b; Schurr *et al.*, 1996). *mucC* (Boucher *et al.*, 1996) was shown in this study to play a negative regulatory role. *mucD* encodes a homologue of the serine protease HtrA (DegP) and has been proposed to remove denatured or damaged proteins, thus eliminating the signals activating the extreme stress σ factor AlgU (Boucher *et al.*, 1996). Pa, *P. aeruginosa*; Av, *Azotobacter vinelandii*; Ph, *Photobacterium* spp.; Ec, *E. coli*; Hi, *Haemophilus influenzae*. The asterisk indicates that *mucC* homologues in *Haemophilus influenzae* are unlinked (see b). (b) Conservation of the putative transmembrane domains (I and II) and a motif present in MucC homologues resembling the sequence of the active centre of SoxR (Wu & Weiss, 1991). Hatched boxes, predicted transmembrane domains; open boxes, the CX₅CX₂CX₅C motif; filled portions of open boxes, T and A instead of the first C within the Pa and Av motifs, respectively. Numbers represent the position of charged amino acid residues demarcating each putative transmembrane domain.

Table 2. Inactivation of *mucC* enhances alginate production in *mucA* and *mucB* mutants of *P. aeruginosa*

Strain†	Genotype	Phenotype:‡ Alginate production [$\mu\text{g (mg wet wt of cells)}^{-1} \pm \text{SE}$]\\$			
		PIA	LB		
			42 °C	42 °C 90 mM NaCl	42 °C 30 % sucrose
PAO1	<i>algU</i> ⁺ <i>mucABCD</i> ⁺	NM (1.14 ± 0.05)	NM (1.93 ± 0.14)	NM (ND)	NM (1.26 ± 0.09)
PAO6875	<i>mucC</i> ::Gm ^r	NM (2.04 ± 0.01)	NM (2.39 ± 0.33)	NM (ND)	NM (1.20 ± 0.08)
PAO6857	<i>mucB</i> ::Tc ^r	M (23.81 ± 1.79)	NM (2.72 ± 0.33)	NM (ND)	NM (1.47 ± 0.16)
PAO6877	<i>mucB</i> ::Tc ^r	M (24.56 ± 2.25)	NM (2.82 ± 0.26)	NM (ND)	M (7.06 ± 0.11)
PAO6877	<i>mucC</i> ::Gm ^r				
	<i>mucB</i> ::Tc ^r	M (ND)	NM (ND)	NM (ND)	M (10.82 ± 0.97)
	<i>mucC</i> ::Gm ^r [<i>ptac-mucD</i> ⁺]				
PAO568	<i>mucA2</i>	M (86.59 ± 5.35)	NM (1.64 ± 0.08)	M* (4.15 ± 0.32)	M (ND)
PAO6876	<i>mucA2</i>	M (84.37 ± 9.53)	NM (2.87 ± 0.08)	M (14.19 ± 1.77)	M (ND)
	<i>mucC</i> ::Gm ^r				

† The strains PAO6875, PAO6857 and PAO6877 are derivatives of *P. aeruginosa* PAO1. Strain PAO6876 is a derivative of *P. aeruginosa* PAO568.

‡ Phenotypes: NM, non-mucoid; M, mucoid; M*, mucoid after 48 h. Strains plated on PIA medium were grown at 37 °C. Strains plated on LB were grown at 42 °C with indicated supplements.

§ The *P* values (*t* test) were 8.67×10^{-6} for PAO6857 and PAO6877 grown at 42 °C on LB in the presence of 30 % sucrose and 5.10×10^{-3} for PAO568 and PAO6876 grown at 42 °C on LB in the presence of 90 mM NaCl.

i.e. growth at elevated temperature (42 °C) in the presence of 300 mM NaCl, a dramatic effect of the *mucC* mutation on *P. aeruginosa* growth was observed (Fig. 2a). The *mucC*::Gm^r strain PAO6875 displayed severe inhibition of growth, similar to that seen in the *algU* mutant strain PAO6852 (Fig. 2a). The effect of *mucC* inactivation was not due to polar effects on *mucD*, since the *mucC* mutant PAO6875 did not show increased sensitivity to H₂O₂ or retarded growth at 42 °C characteristic of *mucD* mutants. Furthermore, introduction of the plasmid *ptac-mucD* in PAO6875 did not complement the growth inhibition phenotype, although this plasmid complements the *mucD* mutation in strain PAO6860 (Boucher *et al.*, 1996). However, several attempts to obtain a direct complementation of the chromosomal *mucC* mutation using the *mucC* gene cloned behind the *tac* promoter were inconclusive. The negative results of these complementation studies are complicated by the fact that *mucC* does not have a separate ribosome-binding site and is likely translationally coupled with *mucB*, as reflected in the partial overlap of the last codon of *mucB* and the initiation codon of *mucC* in *P. aeruginosa* (Boucher *et al.*, 1996). However, the specificity of effects of *mucC* inactivation is illustrated by the similar phenotypes of several independently obtained *mucC* mutants. In further support of the specificity of *mucC* inactivation, growth inhibition under the same conditions (Fig. 2b) was also observed in PAO6876, a

mucC strain made in a different genetic background. However, *mucC* inactivation did not significantly decelerate *P. aeruginosa* growth when sucrose was used as an osmolyte as exemplified in Fig. 2(c). These results suggest that *mucC* is required for uninhibited growth of *P. aeruginosa* under conditions of combined high salt concentration and elevated temperature.

The *mucC* gene is a negative regulator of alginate production in mucoid *mucA* and *mucB* mutants

Although inactivation of *mucC* in the non-mucoid wild-type strain PAO1 had an effect on its survival under conditions of combined elevated temperature and increased salt concentration in the medium, it did not affect the non-mucoid phenotype in the PAO1 background under such conditions (Table 2). In a converse set of experiments, we investigated whether the *mucC* gene may have an effect on alginate production in mucoid mutants. To test this, *mucC* was inactivated in *mucA* and *mucB* backgrounds and two double mutant strains, PAO6876 (*mucA2 mucC*::Gm^r) and PAO6877 (*mucB*::Tc^r *mucC*::Gm^r), were compared to their parental strains PAO568 (*mucA2*) and PAO6857 (*mucB*::Tc^r), respectively. No effect on mucoidy (e.g. reduction or increase in alginate production) was seen in such strains grown on PIA, a medium that supports the mucoid phenotype in all *muc* mutants tested thus far (Table 2). Although *mucA* and *mucB* mutants of *P.*

Fig. 2. Effects of *mucC* inactivation on growth of *P. aeruginosa* under stress conditions. (a) Growth curves of PAO1 (*algU*⁺ *mucC*⁺) (●), its *mucC*::Gm^r and *algU*::Tc^r isogenic derivatives, PAO6875 (○) and PAO6852 (◆), respectively, and PAO6875 harbouring *ptac-mucD*⁺ (◇). (b) Growth curves of the *P. aeruginosa* *mucA*2 mutant PAO568 (●), its *mucC*::Gm^r derivative, PAO6876 (○), and PAO6876 harbouring *ptac-mucD*⁺ (◇). (c) Growth curves of PAO6857 (*mucB*::Tc^r) (●) and PAO6877 (*mucB*::Tc^r *mucC*::Gm^r) (○). All strains were grown at 42 °C in LB supplemented with either 300 mM NaCl or 30% sucrose as indicated.

aeruginosa (e.g. PAO568 and PAO6857) are mucoid on PIA, they are non-mucoid on LB unless this medium is supplemented with salt, with maximum alginate production in PAO568 at 300 mM NaCl (Deretic *et al.*, 1990). Since salt concentration seemed to play a role in induction of mucoidy in *mucA* and *mucB* mutants while *mucC* had an effect (albeit at an elevated temperature) on *P. aeruginosa* growth in high ionic strength media, it appeared reasonable to test whether *mucC* inactivation would have an effect on alginate production under such conditions. In experiments carried out to explore these possibilities, induction of alginate production and significant morphological differences (i.e. mucoid phenotype in *mucC* mutants vs. slightly mucoid or non-mucoid

phenotype in parental *mucC*⁺ strains) were observed on media supplemented with two different osmolytes (NaCl and sucrose) (Table 2).

Interestingly, *mucC* inactivation in the *mucA* parent expressed its effect specifically when NaCl was used, while its inactivation in the *mucB* parent caused an effect when sucrose was used as an osmolyte (Table 2). This observation suggested a degree of specificity and differences in effects of double *mucA mucC* and *mucB mucC* mutations. In combination with the *mucA*2 allele, inactivation of *mucC* (strain PAO6876) caused a significant increase in alginate production [from 4.15 to 14.19 µg (mg wet cell wt)⁻¹] on LB supplemented with 90 mM NaCl at 42 °C relative to the parental strain PAO568 (*mucA*2). The use of 90 mM NaCl (instead of 300 mM NaCl) was optimal for the detection of the effects of *mucC* inactivation on alginate production in the *mucA*2 background. Strain PAO6877 (*mucB*::Tc^r *mucC*::Gm^r) displayed increased alginate production [a change from 1.47 in the *mucC*⁺ parent to 7.06 µg (mg wet cell wt)⁻¹ in *mucC*::Gm^r cells] and mucoid phenotype when grown in the presence of 30% sucrose at 42 °C. The parental strain PAO6857 (*mucB*::Tc^r *mucC*⁺) remained non-mucoid under identical conditions (Table 2). These differences were not observed when the incubation temperature was 37 °C (data not shown). The effect of *mucC* mutation could not be attributed to a polar effect on *mucD* since expression of plasmid-borne *mucD* (known to complement mucoidy in *mucD* mutants; Boucher *et al.*, 1996) did not suppress mucoidy in PAO6877 (Table 2). These results suggest that *mucC* has a negative regulatory role in alginate production, which becomes appreciable only in the presence of *mucA* or *mucB* mutations and under a combination of adverse growth conditions.

Additive effects of *mucA* and *mucB* mutations

The results with *mucC* inactivation suggest that the *muc* genes have additive effects on alginate production and that *mucC* may act synergistically with *mucA* or *mucB*. A further example of cumulative effects of the inactivation of negative regulators in the *algU mucABCD* gene cluster was observed when *mucB* was insertionally inactivated in the *mucA*2 background. The *mucA*2 *mucB* double mutant assumed a mucoid phenotype under conditions which rendered single mutant strains (*mucA*2 or *mucB*::Tc^r) non-mucoid (Table 3).

DISCUSSION

In this study we have examined the role of *mucC* in alginate production and in general resistance to stress in *P. aeruginosa*. Interestingly, *mucC* expresses its negative effect on alginate production under similar environmental conditions which impose a requirement for *mucC* to maintain full stress tolerance. However, the inhibition of growth in *mucC* mutants was neither the cause nor a prerequisite for increased alginate production. For example, mucoidy was not induced in PAO6875 (the *mucC*::Gm^r derivative of PAO1) al-

Table 3. Additive effects of *mucA* and *mucB* mutations on alginate production in *P. aeruginosa*

Strain†	Genotype	Phenotype‡	Alginate production [µg (mg wet wt of cells) ⁻¹ ± SE]§
PAO568	<i>mucA2</i>	NM	1.87 ± 0.04
PAO6857	<i>mucB::Tc^r</i>	NM	1.58 ± 0.11
PAO6858	<i>mucA2 mucB::Tc^r</i>	M*	3.83 ± 0.09

† PAO6858 is a *mucB::Tc^r* derivative of PAO568.

‡ Phenotypes of PAO568, PAO6857 and PAO6858 on LB medium: NM, non-mucoid; M*, mucoid after 48 h. PAO568 and PAO6857 are mucoid on PIA (Table 2). All incubations were at 37 °C.

§ The *P* value (*t* test) was 3.23×10^{-5} for PAO568 and PAO6858.

though the growth was retarded in this strain at levels similar to those in PAO6876 (*mucA2 mucC::Gm^r*) (Fig. 2a and b). Furthermore, mucoidy was induced in PAO6877 (*mucB::Tc^r mucC::Gm^r*) in the presence of 30 % sucrose at 42 °C while growth was not significantly reduced relative to the *mucC⁺* parent (Fig. 2c). It is not possible to exclude the possibility that the new negative regulator of mucoidy, *mucC*, reported in this study also plays a positive regulatory role under certain environmental conditions or in genetic backgrounds different from those tested in this study. For example, recent studies by Missiakas *et al.* (1997), have indicated a possible positive effect of RseC, the *E. coli* homologue of MucC, on σ^E -dependent transcription, albeit such effects were not corroborated by De Las Penas *et al.* (1997).

In one model that is currently being considered, it is possible that the effects of MucC are exerted via its putative role in the control of AlgU activity along with the other Muc factors. In this scenario, MucC may help direct AlgU to enhance the expression of factors necessary for survival under conditions of high ionic strength/osmotic shock at high temperature which could compete with the *alg* system for the available AlgU. Alternatively, MucC could be an effector which, in a fashion similar to that proposed for MucD, may remove or neutralize signals that induce AlgU activity (e.g. denatured proteins or other products of thermal and osmotic shock). While elimination of such putative inducing molecules may improve bacterial viability, at the same time such events may reduce AlgU activity, thus negatively affecting alginate production. However, unlike MucD which shows significant similarity with the serine protease HtrA (DegP) (Lipinska *et al.*, 1988; Strauch & Beckwith, 1988), MucC has no obvious motifs that would suggest such a function.

A modulatory role for MucC affecting interactions between the σ factor AlgU (Martin *et al.*, 1993c; Schurr *et al.*, 1996; Xie *et al.*, 1996), the anti- σ factor MucA (Schurr *et al.*, 1996; Xie *et al.*, 1996) and the periplasmic protein MucB (Schurr *et al.*, 1996) must also be considered. The transduction of signals between the

periplasmic compartment [where MucB is located (Schurr *et al.*, 1996)] and the cytoplasmic compartment where the σ factor activity of AlgU is expressed may require complicated multi-component machinery. In that regard we have noticed two potential transmembrane domains in MucC (aa 76–99 and 104–123) which are also predicted for all of its homologues (Fig. 1b). In a model that takes into account a possible membrane association of MucC, this factor could transduce stress signals to the other components inhibiting AlgU activity and modulate their action. Further experiments are needed to determine the precise role of MucC and to discern between direct and indirect effects that its inactivation may have on the physiology of the cell which could have broader implications for those Gram-negative organisms that have MucC equivalents (Fig. 1a).

If AlgU is unchecked and overexpressed in the absence of at least one of its negative regulators, it becomes toxic to the cell (Schurr *et al.*, 1994). In this context, the apparent redundancies in the system may have evolved as a mechanism for reducing the likelihood of events leading to runaway AlgU. Furthermore, overexpression of the alginate system in wild-type cells may not be desirable unless precise environmental conditions are met, e.g. biofilm formation (Lam *et al.*, 1980), or defence against extreme stress conditions is needed (Boucher *et al.*, 1996; Martin *et al.*, 1994; Yu *et al.*, 1995). In support of these considerations is the lack or only rare occurrence of mucoid variants among environmental isolates (Anastassiou *et al.*, 1987; Pier *et al.*, 1986). In clinical situations, such as in cystic fibrosis, under strong selective pressures during prolonged chronic infections, mucoid mutants may be selected as the result of a compromise between the survival in the lung infiltrated with phagocytic cells (Speert, 1994) and the loss of adaptation flexibility or some toxicity of AlgU.

ACKNOWLEDGEMENTS

This work was supported by grants AI31139 from the National Institute for Allergy and Infectious Diseases and DERET-196PO from the Cystic Fibrosis Foundation. M. Schurr was

supported by grant SCHURR95IO from the Cystic Fibrosis foundation. D. W. Rowen and H. Yu were Cystic Fibrosis Foundation postdoctoral fellows.

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Received 17 March 1997; revised 4 July 1997; accepted 21 July 1997.