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

J. C. Boucher, M. J. Schurr, H. Yu, D. W. Rowen and V. Deretic

Alginic acid 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 σE 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 mucC 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, indicating 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 σE (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, σE, 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...
Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Relevant genotype or properties*</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>Prototroph Alg&lt;sup&gt;−&lt;/sup&gt;</td>
<td>B. Holloway (Monash University, Australia)</td>
</tr>
<tr>
<td>PAO568</td>
<td>PAO381 Alg&lt;sup&gt;+&lt;/sup&gt; mucA2</td>
<td>Fyfe &amp; Govan (1980)</td>
</tr>
<tr>
<td>PAO6857</td>
<td>PAO1 Alg&lt;sup&gt;−&lt;/sup&gt; mucB::Tc&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Schurr et al. (1996)</td>
</tr>
<tr>
<td>PAO6875</td>
<td>PAO1 Alg&lt;sup&gt;−&lt;/sup&gt; mucC::Gm&lt;sup&gt;−&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>PAO6876</td>
<td>PAO6856 Alg&lt;sup&gt;−&lt;/sup&gt; mucC::Gm&lt;sup&gt;−&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>PAO6877</td>
<td>PAO6857 Alg&lt;sup&gt;−&lt;/sup&gt; mucC::Gm&lt;sup&gt;−&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>PAO6858</td>
<td>PAO6856 Alg&lt;sup&gt;−&lt;/sup&gt; mucB::Tc&lt;sup&gt;−&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>plII1G</td>
<td>pUC18 Gm&lt;sup&gt;−&lt;/sup&gt; cassette</td>
<td>S. Lory (University of Washington, USA)</td>
</tr>
<tr>
<td>pCMobA</td>
<td>pHC79 mob&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Mohr &amp; Deretic (1990)</td>
</tr>
<tr>
<td>pBSC1</td>
<td>pCRIL mob&lt;sup&gt;+&lt;/sup&gt; mucC::Gm&lt;sup&gt;−&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>ptae-mucd&lt;sup&gt;+&lt;/sup&gt;</td>
<td>IncQ/P4 tac-mucD&lt;sup&gt;−&lt;/sup&gt; lacI&lt;sup&gt;−&lt;/sup&gt; Cb&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Boucher et al. (1996)</td>
</tr>
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</table>

* Alg<sup>−</sup>, constitutive mucoid phenotype; Alg<sup>+</sup>, mucoid phenotype dependent on medium; Alg<sup>−</sup> muc<sup>+</sup>, enhanced mucoid phenotype when grown on LB supplemented with 90 mM NaCl; Alg<sup>+</sup> muc<sup>+</sup>, enhanced mucoid phenotype when grown on LB supplemented with 30% sucrose; Alg<sup>−</sup>, 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<sup>E</sup>) directs transcription of promoters characterized by the σ<sup>E</sup> promoter consensus sequence —35 GAACCTT<sub>N16/17</sub> —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 P<sub>1</sub> and P<sub>3</sub> promoters of AlgU (Schurr et al., 1995) at least two other genes critical for alginate production, algD and algR, have σ<sup>E</sup> 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 biosynthesis (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 (mucA<sup>2</sup>) and PAO6857 (mucB<sup>−</sup>: Tc<sup>−</sup>), respectively, with an inactivated mucC gene (mucC::Gm<sup>−</sup>). Strain PAO6858 is a mucB::Tc<sup>−</sup> 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 μg ml<sup>−1</sup>) for PIA or LB, respectively) or gentamicin (150 or 13 μg ml<sup>−1</sup>) for PIA or LB, respectively) when required. Escherichia coli was grown on LB supplemented with ampicillin (100 μg ml<sup>−1</sup>), kanamycin 25 μg ml<sup>−1</sup>) or gentamicin (13 μg ml<sup>−1</sup>) 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 insertional 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' CGGCACCTTGCCGAAGA 3') was cloned into pCRII (Invitrogen). This construct was further modified by inserting the XbaI linker Gm<sup>−</sup> cassette from pKII1G into the AvrII site within mucC, followed by cloning of mob from pCMobA into...

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

**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 PA01. Unlike inactivation of mucA, mucB or mucD, which each individually cause conversion to mucoidy in PA01 (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'), 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') and its parent PA01 (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 σK has been implicated in the response to osmolarity changes (Mecsas 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 PA01 and PAO6875 (mucC::Gm') were observed on LB supplemented with 300 mM NaCl (data not shown). However, when a combination of factors was examined,
Table 2. Inactivation of mucC enhances alginate production in mucA and mucB mutants of P. aeruginosa

<table>
<thead>
<tr>
<th>Strain†</th>
<th>Genotype</th>
<th>Phenotype‡</th>
<th>Alginate production [µg (mg wet wt of cells)⁻¹ ± SE]§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PIA</td>
<td>42 °C</td>
</tr>
<tr>
<td>PAO1</td>
<td>algU⁺ mucABCD⁺</td>
<td>NM (1±14 ±0.05)</td>
<td>NM (1±93 ±0.14)</td>
</tr>
<tr>
<td>PAO6875</td>
<td>mucC::Gm'</td>
<td>NM (2±04 ±0.01)</td>
<td>NM (2±39 ±0.33)</td>
</tr>
<tr>
<td>PAO6857</td>
<td>mucB::Tc'</td>
<td>M (2±81 ±1.79)</td>
<td>NM (2±72 ±0.33)</td>
</tr>
<tr>
<td>PAO6877</td>
<td>mucB::Tc' mucC::Gm'</td>
<td>M (2±56 ±2.25)</td>
<td>NM (2±82 ±0.26)</td>
</tr>
<tr>
<td>PAO6877</td>
<td>mucB::Tc' mucC::Gm' [ptac-mucD⁺]</td>
<td>M (ND)</td>
<td>NM (ND)</td>
</tr>
<tr>
<td>PAO568</td>
<td>mucA2</td>
<td>M (8±59 ±5.35)</td>
<td>M (1±64 ±0.08)</td>
</tr>
<tr>
<td>PAO6876</td>
<td>mucC::Gm'</td>
<td>M (8±37 ±9.53)</td>
<td>NM (2±87 ±0.08)</td>
</tr>
</tbody>
</table>

† 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 × 10⁻⁴ 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' 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') and PAO6877 (mucB::Tc' mucC::Gm'), were compared to their parental strains PAO568 (mucA2) and PAO6857 (mucB::Tc'), 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.
Induction of mucoidy in P. aeruginosa mucA2 mutant PA0568 would have an effect on alginate production under such conditions. It appeared reasonable to test whether 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 PA06877 (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 mucA2 background. The mucB double mutant assumed a mucoid phenotype under conditions which rendered single mutant strains (mucA2 or mucB::Tc) 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 PA06875 (the mucC::Gm derivative of PA01) al-
Although the growth was retarded in this strain at levels similar to those in PAO6876 (mucA2 mucC::Gm\(^{+}\)) (Fig. 2a and b). Furthermore, mucoidy was induced in PAO6877 (mucB::Tc\(^{+}\) mucC::Gm\(^{+}\)) 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 Missiañas et al. (1997), have indicated a possible positive effect of RseC, the E. coli homologue of MucC, on \(\sigma^{2}\)-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 \(\sigma\) factor AlgU (Martin et al., 1993c; Schurr et al., 1996; Xie et al., 1996), the anti-\(\sigma\) 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 \(\sigma\) 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.

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