Dual regulation of mucoidy in *Pseudomonas aeruginosa* and sigma factor antagonism

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Summary

The conversion to mucoid, exopolysaccharide alginate-overproducing phenotype in *Pseudomonas aeruginosa* during chronic respiratory infections in cystic fibrosis patients occurs via mutations that activate the alternative sigma factor AlgU (σ^E). In this study, we demonstrate that conversion to mucoidy can be caused via a second, *algU*-independent pathway, in which alginate production and transcription of the critical *algD* promoter depend on another alternative σ factor, RpoN (σ^{54}). The *algD* promoters dependent on σ^{54} and σ^E showed a complete overlap resulting in identical mRNA 5′ ends. The two pathways were not independent, as σ^{54} also repressed σ^E-dependent transcription of *algD* both *in vitro* and *in vivo*. The negative regulatory effect of σ^{54} on σ^E-dependent *algD* expression was based on σ^{54} binding to the *algD* promoter and its interference with σ^E-dependent transcription. This phenomenon, referred to here as σ factor antagonism, reflects the unique properties of σ^{54}, which lacks an intrinsic ability to form open transcription initiation complexes. We propose that this peculiar feature of σ^{54} has evolved in part to allow its recruitment as a repressor of certain promoter subsets. The repression of *algD* by σ^{54} also depends on environmental conditions, supporting the notion that σ factor antagonism plays a physiological role in controlling alginate production in *P. aeruginosa* during adaptation to different ecological sites (e.g. biofilm development, stress and other growth conditions) and unique environments in the chronically infected host.

Introduction

The conversion to exopolysaccharide alginate overproducing, mucoid phenotype provides selective advantage to *Pseudomonas aeruginosa* in the lungs of cystic fibrosis

(CF) patients (Welsh *et al.*, 1995; Govan and Deretic, 1996). This phenotypic conversion is caused by mutations that occur in two separate chromosomal loci, termed *muc* and represented by the prototypical mutations *mucA22* and *muc23* (Fyfe and Govan, 1980; Martin *et al.*, 1993a,b,c; Govan and Deretic, 1996; Boucher *et al.*, 1997). The majority (84%) of mucoid *P. aeruginosa* isolates from CF patients carry mutations in the *mucA* gene resulting in activation of the *algD* promoter, increased alginate production and reduced pulmonary clearance in animal models of respiratory infection (Boucher *et al.*, 1997; Yu *et al.*, 1998). In the absence of *muc* mutations, activation of the *algD* promoter most likely represents a major decision in the life cycle of *P. aeruginosa* possibly linked to the establishment of biofilms (Davies *et al.*, 1998) or developmental processes such as encystment in a closely related organism *Azotobacter vinelandii* (Moreno *et al.*, 1998).

In general, *algD* expression depends on regulators from the superfamily of bacterial two-component signal transduction systems (Kato and Chakrabarty, 1991; Yu *et al.*, 1997; Selvaraj *et al.*, 1998) and a specialized alternative σ factor, σ^E (also known as AlgT or AlgU) (Martin *et al.*, 1993a; DeVries and Ohman, 1994), functionally equivalent (Yu *et al.*, 1995) to the extreme heat shock sigma factor σ^E (RpoE) found in *Escherichia coli* and other Gram-negative bacteria (Rouviére *et al.*, 1995; Missiakas and Raina, 1998). Under conditions of extreme stress, σ^E is responsible for expression of the genes encoding the heat shock sigma factor σ^{32} (RpoH) and additional protective factors, by directing transcription from promoters containing the GAACTT-N$_{16/17}$-TCTGA consensus sequences (Deretic *et al.*, 1994; Missiakas and Raina, 1998). Under normal physiological conditions, σ^E is inhibited by its cognate anti-σ factor: MucA in *P. aeruginosa* (Xie *et al.*, 1996; Schurr *et al.*, 1996) or RseA in *Escherichia coli* (Missiakas and Raina, 1998). In a subset of mucoid *P. aeruginosa* isolates from CF patients, the anti-σ factor MucA is inactivated via nonsense and frameshift mutations, resulting in a constitutive production of alginate (Martin *et al.*, 1993c; Boucher *et al.*, 1997). In another set of strains, represented by the mutation *muc23*, conversion to mucoidy occurs in the absence of *mucA* alterations. The gene(s) that corresponds to the *muc23* mutation and their nature are not known at present.

The transcriptional activation of *algD* and a high-level production of alginate in *P. aeruginosa* also require the

response regulator AlgB (Selvaraj *et al.*, 1998). Para-doxically, AlgB is homologous to proteins from the NtrC (NRII) subfamily of signal transduction systems that are known to activate transcription specifically in concert with the σ^{54}-RNA polymerase holoenzyme (Ninfa *et al.*, 1995; Stock *et al.*, 1995). The NtrC-type response regulators are required for the ATP-dependent isomerization of the closed promoter complexes formed by the σ^{54}-holoenzyme into open transcriptional complexes (Lee *et al.*, 1993; Wang *et al.*, 1995; Perez-Martin and de Lorenzo, 1996; Wang and Gralla, 1996; Syed and Gralla, 1998). Because this specialized function is associated uniquely with σ^{54}, it seemed unlikely that AlgB could work in concert with σ^E (AlgU), a member of the ECF subset of the σ^{70} superfamily of σ factors (Lonetto *et al.*, 1994) possessing an intrinsic ability to form open promoter complexes (Gralla, 1993; Marr and Roberts, 1997). Interestingly, expression of the mucoid phenotype also depends on a nitrogen source in the growth medium (Deretic *et al.*, 1990). Furthermore, the promoter region of *algD* contains a potential sequence matching the consensus for σ^{54}

promoters (Kimbara and Chakrabarty, 1989; Mohr *et al.*, 1991). Taken together, these considerations suggest that σ^{54} could participate in the transcription of *algD* in *P. aeruginosa*. In this study, we show that, in a subset of mucoid *P. aeruginosa* mutants, *algD* expression and alginate production depend on σ^{54} (RpoN) and not on σ^E (AlgU), suggesting the existence of two different path-ways of conversion to mucoidy. We also demonstrate that these two pathways are not completely independent and that σ^{54} (RpoN) acts as a negative regulator when cells overexpress *algD* via the σ^E (AlgU) route.

Results and discussion

Two alternative pathways lead to increased transcription of *algD* in mucoid *P. aeruginosa*

Two prototype mucoid strains of *P. aeruginosa* represen-tative of mutations in two key *muc* loci (Fyfe and Govan, 1980; Martin *et al.*, 1993c; Govan and Deretic, 1996; Boucher *et al.*, 1997) were compared: PAO578II, carrying
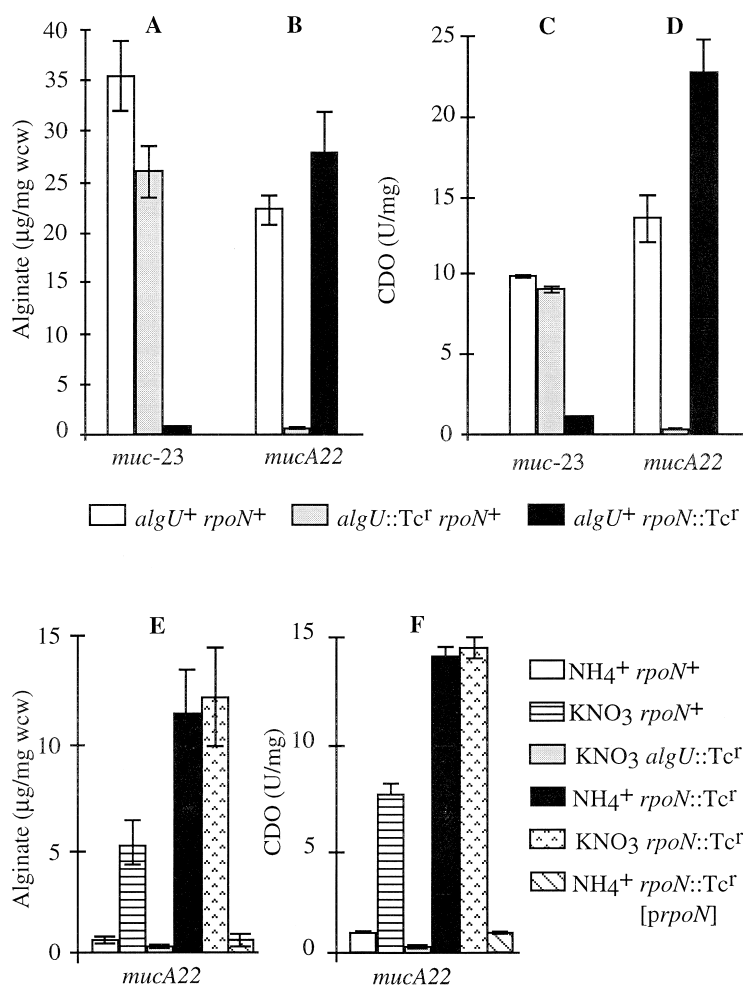

Fig. 1. Two sigma factors, σ^E and σ^{54}, can direct *algD* transcription and control mucoid phenotype.

A–D. Different sigma factors, σ^E (encoded by *algU*) and σ^{54} (encoded by *rpoN*) are required for alginate production and *algD* transcription in two prototypical *muc* mutants of *P. aeruginosa*: PAO578II (*mucA22*) and PAO579 (*muc23*).

A and B. Alginate production by *P. aeruginosa* PAO579 (*muc23*) and PAO578II (*mucA22*) and their *algU*::Tc^r and *rpoN*::Tc^r derivatives grown on *Pseudomonas* Isolation Agar.

C and D. *algD–xylE* fusion activity (chromosomally encoded) expressed as units of catechol 2,3-deoxygenase (CDO, *xylE* gene product) activity (U mg^{-1} of protein in crude extracts) as previously described (Deretic *et al.*, 1990).

E and F. Repression on nitrogen-rich media (NH$_4^+$) in *mucA22* cells is dependent on σ^{54} (encoded by *rpoN*). Strains were grown on minimal media supplemented with NH$_4^+$ (nitrogen rich) or KNO$_3$ (nitrogen poor) where indicated. (p*rpoN*), strain harbouring a functional, plasmid-borne *rpoN* gene. Strains and measurements are the same as in B and D. All values are expressed as mean ± standard error based on at least three independent experiments.

the *mucA22* mutation within the *algU mucABCD* locus (Martin *et al.*, 1993c; Boucher *et al.*, 1997), and PAO579, carrying the *muc23* mutation (Fyfe and Govan, 1980) that maps in a chromosomal locus different from *algU mucABCD* in a gene or genes that remain to be characterized. In contrast to *mucA* mutant *P. aeruginosa* strains, which depend on the alternative sigma factor σ^E (AlgU) for alginate production, inactivation of *algU* affected neither the mucoid phenotype (Fig. 1A) nor *algD* transcription (Fig. 1C) in the *muc23* mucoid strain PAO579. This suggested that *algD* expression was not dependent on σ^E (AlgU) in the *muc23* background. As expected, inactivation of *algU* (encoding *P. aeruginosa* σ^E) in the *mucA22* mutant PAO578II caused a loss of alginate production (Fig. 1B) and *algD* transcription (Fig. 1D). Alginate production in PAO579 (*muc23*) still required *algD*, as inactivation of this gene completely abrogated alginate production and mucoid phenotype in PAO579 (data not shown). Next, we tested whether an alternative σ factor different from σ^E (AlgU) was responsible for *algD* expression in PAO579. When *rpoN* was inactivated in PAO579 (*muc23*) this strain lost alginate production (Fig. 1A) and *algD* transcription (Fig. 1C). In contrast, inactivation of *rpoN* had no immediately apparent effects on the *mucA22* strain PAO578II (Fig. 1B and D). These results were consistent with the interpretation that *algD* transcription is directed by σ^E (AlgU) in the *mucA22* background (strain PAO578II) and that it is dependent on σ^{54} in the *muc23* background (strain PAO579).

To confirm these findings at the mRNA level and to examine the arrangement of the σ^E (AlgU) promoter and σ^{54}-dependent transcript(s) of *algD*, we next mapped the *algD* mRNA start sites in the *muc23* and *mucA22* backgrounds. Suprisingly, the 5′ ends for *algD* mRNA were identical in *muc23* (Fig. 2A, lane 2) and *mucA22* cells (Fig. 2B, lane 2). No additional mRNA 5′ ends were detected in the region spanning sequences from −1143 to +432 relative to the *algD* translational start site in either strain. In both cases, the starts matched the previously identified σ^E (AlgU) transcriptional start site for *algD* (Deretic *et al.*, 1987). Inactivation of *rpoN* in the *muc23* background resulted in the loss of transcription from this site (Fig. 2A, lane 3). Inactivation of *algU* had no effect in the *muc23* background (Fig. 2A, lane 4) but, as expected, abrogated transcription in the *mucA22* background (Fig. 2B, lane 4). This possibility was consistent with an overlap between the σ^E (AlgU) and σ^{54} promoter consensus sequences within the *algD* promoter **GGA**ACTTCCC-TC**GC**AGAGAAAACATCCTA [σ^E consensus (underlined) and σ^{54} consensus core bold]. The simplest interpretation of these observations is that σ^E (AlgU) and σ^{54} (RpoN) can both direct transcription of *algD* from overlapping promoter sequences.
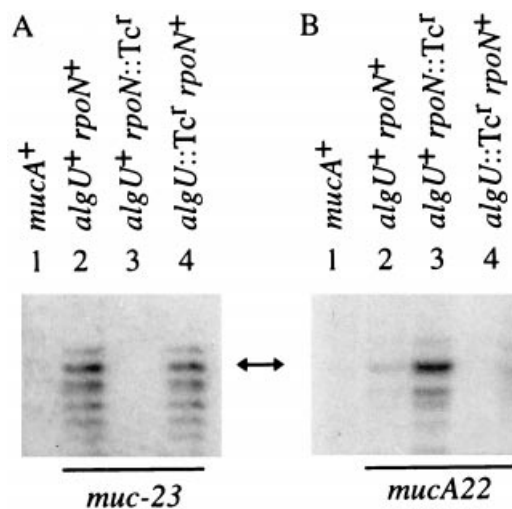
However, an alternative explanation of our data had to

Fig. 2. Coincidence of the 5′ ends of σ^E (AlgU)-and σ^{54} (RpoN)-dependent *algD* transcripts. Equal amounts of RNA from all strains were used for S1 nuclease protection analyses carried out as described (see *Experimental procedures*). Lanes: 1A and 1B, non-mucoid (*mucA+*) strain PAO381 parental to both PAO579 (*muc23*); and PAO578II (*mucA22*). 2A, PAO579; 2B, PAO578II; 3A and 3B, *rpoN*::Tc^r derivatives of PAO579 and PAO578II respectively; 4A and 4B, *algU*::Tc^r derivatives of PAO579 and PAO578II respectively. Note, increased *algD* transcription in *mucA22 algU+ rpoN*::Tc^r cells (lane B3) relative to the parental mucA22 *algU+ rpoN+* strain (lane B2).

be considered. It has been shown that, in the case of a subset of promoters in *Bacillus subtilis*, two different ECF sigma factors can substitute for each other (Huang *et al.*, 1998), while similar possibilities had been postulated in other instances (Paget *et al.*, 1998). In this alternative scenario, the following three conditions would have to be met to explain our observations: (i) another ECF sigma factor in *P. aeruginosa* had to be able to substitute for σ^E (AlgU); (ii) such ECF sigma factor had to be under control by RpoN; and (iii) its expression or activity should be abrogated or reduced in *rpoN* mutants. We addressed this issue by: (i) examining the recently completed sequence of the *P. aeruginosa* PAO1 genome (www.pseudomonas.com) for genes encoding potential ECF sigma factors; (ii) searching for the presence of a match with the known σ^{54} consensus promoter sequences (Ausubel, 1984; Hunt and Magasanik, 1985; Khan *et al.*, 1986) within the region upstream of the putative ECF-encoding open reading frames (ORFs); (iii) examining for the presence of transcripts at the predicted locations; and (iv) determining whether such transcripts were affected by inactivation of *rpoN*. In addition to the previously characterized ECF sigma factors AlgU, PvdS (Ochsner and Vasil, 1996), PigD (Ochsner and Vasil, 1996), FiuI (Ochsner and Vasil, 1996) and SigX (Duchene *et al.*, 1988), we detected within the complete nucleotide sequence of PAO1 genome a further 15 ORFs encoding potential ECF sigma factors (termed *ecfF−ecfT*; see Table 1). Of

Table 1. *P. aeruginosa* ECF σ factors and their potential for dependence on RpoN for transcription.

ECF σ[a,b]	σ^{54} promoter consensus[c]	Transcript[d]	RpoN dependence[e]
Group I			
AlgU, PvdS, PigD, FiuI, SigX	−	ND	ND
EcfF−H, K−P, R−T	−	ND	ND
Group II			
EcfI	+	−	−
EcfJ	+	+	−
EcfQ	+	−	−

a. ECF σ factors included in the analysis were: the previously characterized ECF σ factors AlgU (Martin *et al.*, 1993c); PvdS (Ochsner and Vasil, 1996); PigD (Ochsner and Vasil, 1996); FiuI (Ochsner and Vasil, 1996); and SigX (Duchene *et al.*, 1988) and EcfF−T, the putative *P. aeruginosa* ECF σ factors encoded by the PAO1 genome (www.pseudomonas.com) identified based on homologies with the known *P. aeruginosa* ECF σ factors. EcfF−T were ordered based on percent identity with AlgU as a measure of their probability of substituting for AlgU function. The locations of individual Ecf σ factors on the complete *P. aeruginosa* genomic sequence (contig 54, www.pseudomonas.com) and their percent identities with AlgU were; EcfF, 4659367−4659994, 36%; EcfG, 5086396−5086976, 32%; EcfH, 4279020−4279628, 29%; EcfI, 1903991−1903404, 28%; EcfJ, 3970477−3969959, 27%; EcfK, 2846536−2847071, 26%; EcfL, 6251742−6252284, 26%; EcfM, 1338419−1337848, 26%; EcfN, 6033389−6032880, 26%; EcfO, 663321−662786, 26%; EcfP, 5550830−5551348, 24%; EcfQ, 598403−597795, 23%; EcfR, 5696756−5697318, 22%; EcfS, 6093215−6092709, 22%; and EcfT, 4518571−4519114, 22% respectively.
b. Group I, no discernible σ^{54} promoter consensus. Group II, potential σ^{54} promoter consensus sequence present upstream of the coding region.
c. The σ^{54} promoter consensus sequences used for analysis were: CTGGYAY-n$_5$-TTGCA (Ausubel, 1984); CTGGCAC-n$_5$-TTGCA (Khan and Dixon, 1986); and CTGGYAYR-n$_4$-TTGCA (Hunt and Magasanik, 1985). A region consisting of a minimum of 250 bp upstream of the putative ATG of a given Ecf ORF was analysed in each case. −, No discernible σ^{54} consensus sequence was observed within the region investigated; +, a potential σ^{54} consensus promoter sequence was observed upstream of the putative initiation codon. Potential RpoN promoters were considered based on the following sequences (relative to the ATG codon): *ecfI*, CCGGCGC-n$_5$-CCGCA, −242/−230; *ecfJ*, CGGGCCC-n$_5$-CTGCT and CTGGCGT-n$_5$-GCGCA, −201/−189 and −186/−174 respectively; *ecfQ*, CTGGCTC-n$_5$-TTGCG, −69/−57.
d. Primer extension analyses were performed using RNA from PAO381 and PAO579 (*muc23*) as described (see *Experimental procedures*). −, No transcript of the expected size was detected; +, transcripts of the expected size were detected.
e. Dependence on RpoN was determined by primer extension analysis using RNA from PAO6867 (PAO579 *rpoN*::Tc^r) and compared with the signal obtained with RNA from PAO579 (*rpoN*). −, No change (loss) of transcript was observed in *rpoN*::Tc^r mutant relative to *rpoN* cells; ND, not determined.

these ORFs, only three had a recognizable potential σ^{54} promoter sequence upstream from the coding sequence (Table 1). These genes (*ecfI, ecfJ and ecfQ*) encoded putative gene products displaying 26%−28% identity to AlgU. Using primer extension analyses and RNA isolated from a panel of *P. aeruginosa* strains, including PAO579 (*muc23*), a transcript initiating at the predicted position downstream of the putative σ^{54} promoter was observed only in the case of *ecfI*. However, when isogenic *rpoN*$^+$ and *rpoN*::Tc^r strains were compared (Table 1), the observed band was present in both strains (data not shown), suggesting that this *ecfI* transcript was not *rpoN* dependent. These experiments suggest that it is unlikely that the σ^{54} dependency of *algD* transcription can be explained by a recruitment of an RpoN-dependent ECF σ factor to substitute for AlgU in directing transcription of *algD* in the *algU* mutant derivative of PAO579. The available data strongly favour the possibility that *algD* transcription is directed by σ^E (AlgU) in *mucA* mutant mucoid strains and by σ^{54} (RpoN) in *muc23* mutant mucoid *P. aeruginosa* from promoters with overlapping recognition sequences.

Overlap between σ^E and σ^{54} pathways: nitrogen regulation of algD

One characteristic of the *P. aeruginosa* mucA22 strain PAO578II is that it does not produce significant amounts of alginate (i.e. is non-mucoid) on nitrogen-rich minimal media while it is mucoid on minimal media with poor alternative nitrogen sources, such as nitrate (Deretic *et al.*, 1990). The basis for responsiveness of *algD* expression to a nitrogen source was not previously understood. We wondered whether the involvement of σ^{54} (RpoN) uncovered in this study could be related to nitrogen regulation of *algD*. Alginate production and *algD* transcription were repressed (Fig. 1E and F; NH$_4^+$ *algU*$^+$ *rpoN*$^+$) when mucA22 cells were grown on ammonia, a nitrogen rich source for *P. aeruginosa*. Alginate production and *algD* transcription were activated when cells were grown on nitrate (Fig. 1E and F; KNO$_3^+$ *algU*$^+$ *rpoN*$^+$), a nitrogen-poor source for *P. aeruginosa* (Deretic *et al.*, 1990), consistent with *algD* repression in mucA22 cells grown under nitrogen-rich conditions. The activation of the *algD* promoter on nitrate was abolished upon inactivation of *algU*, suggesting that the transcription of *algD* under nitrogen-poor conditions was dependent on σ^E (AlgU) (Fig. 1F; KNO$_3^+$ *algU*::Tc^r). This was accompanied by a loss of alginate production (Fig. 1E; KNO$_3^+$ *algU*::Tc^r). Surprisingly, inactivation of *rpoN* in the *mucA22* background caused an increase in alginate production (Fig. 1E; NH$_4^+$ *rpoN*::Tc^r) on nitrogen-rich medium that normally represses mucoidy. This was accompanied by a derepression of *algD* transcription (Fig. 1F; NH$_4^+$

rpoN::Tc^r). Alginate production and *algD* transcription remained increased upon inactivation of *rpoN* in cells grown on nitrogen-poor medium (Fig. 1E and F; KNO$_3$ *rpoN*::Tc^r) in keeping with the repressive action of σ^{54} (RpoN) and dependence of *algD* in *mucA22* background on σ^E (AlgU) for transcription (Fig. 1E and F; KNO$_3$ *algU*::Tc^r). The RpoN-dependent repression was restored by complementation with the wild-type *rpoN* gene on a plasmid [Fig. 1E and F; NH$_4^+$ *rpoN*::Tc^r (p*rpoN*)]. The derepression of *algD* transcription in *rpoN* mutant cells was also detectable at the mRNA level as monitored by S1 nuclease protection analysis (Fig. 2). Transcription of *algD* was increased 4.8-fold in the *mucA22 rpoN*::Tc^r mutant relative to the *mucA22 rpoN*$^+$ parent (Fig. 2B, lanes 2 and 3). Collectively, the S1 nuclease protection and transcriptional fusion analyses are consistent with the interpretation that RpoN (σ^{54}) or RpoN-dependent processes repress σ^E (AlgU)-dependent transcription of *algD* in *P. aeruginosa* under nitrogen-rich conditions. This repression can be lifted by either growth under nitrogen poor conditions or when *rpoN* is inactivated. Nevertheless, σ^{54} was absolutely required as a positive factor for *algD* expression in *muc23* cells in which *algD* transcription was independent of σ (AlgU). These observations suggest a dual role for σ^{54} acting both as a positive and as a negative regulator of *algD*.

Direct repression by σ^{54} of σ (AlgU)-dependent algD transcription and σ^{54} binding to the algD promoter

While the positive role for σ^{54} (RpoN) in *algD* transcription in *muc23* cells is consistent with its direct participation as an alternative sigma subunit of RNA polymerase, its role as a negative regulator could be indirect. To distinguish between direct and indirect effects, we employed the recently established *in vitro* system for transcription of target promoters by AlgU (σ^E)-RNA polymerase holoenzyme (Hershberger *et al.*, 1995; Schurr *et al.*, 1995). Using this assay, we tested whether σ^{54} can inhibit σ (AlgU)-directed transcription of *algD* directly. The *algD* promoter was preincubated with or without purified σ^{54}, followed by σ^E (AlgU)-RNA polymerase addition and resulting transcripts compared (Fig. 3, lanes 1 and 2). Preincubation with σ^{54} decreased the amount of the run-off product compared with the sample with no σ^{54} in the assay. The inhibitory effect of σ^{54} was absent when the experiments were performed with a mutant *algD* template (*algD*$_{G→T}$) in which the only non-overlapping residue between the GAACTT σ^E consensus sequence and the GG core of the σ^{54} promoter consensus sequence was altered (Fig. 3, lanes 3 and 4). Although the *algD*$_{G→T}$ template displayed a somewhat reduced baseline transcription by the AlgU (σ^E)-holoenzyme (Fig. 3, lane 3), the repression by σ^{54} was no longer detectable (Fig. 3, lane 4).
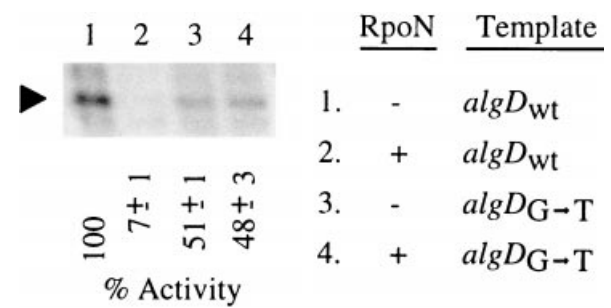
Fig. 3. σ^{54} (RpoN) inhibits *algD* transcription by σ^E (AlgU) RNA polymerase. *In vitro* transcriptional run-off assays with σ^E (AlgU)-RNA polymerase were carried out as previously described (Schurr *et al.*, 1995; Xie *et al.*, 1996). Templates, *algD*$_{wt}$, wild-type *algD* promoter; *algD*$_{G→T}$, its derivative with a destroyed σ^{54} (RpoN) consensus but retaining a preserved σ^E (AlgU) consensus sequence (see *Experimental procedures*). Triangle, σ^E (AlgU)-dependent *algD* transcripts obtained with or without preincubation with σ^{54} (RpoN; 1.1 pmol) as indicated.% Activity, phosphorimager quantitation (relative to *algD*$_{wt}$ transcription as 100%). Values represent means from three independent experiments $\pm$ SE.

The overlap of *P. aeruginosa* σ^E (AlgU) and σ^{54} promoters in *algD* suggests the possibility that σ^{54} may block access to this site by σ^E (AlgU). It has been previously shown that, in contrast to other sigma factors of the σ^{70} superfamily, σ^{54} can bind to some of its cognate promoters in the absence of core RNA polymerase (Buck and Cannon, 1992). Based on this precedent, we tested whether σ^{54} binds to the *algD* promoter. Addition of *E. coli* σ^{54} (*E. coli* and *P. aeruginosa* σ^{54} are functionally interchangeable in the latter organism; Ishimoto and Lory, 1989) to the *algD* promoter probes resulted in the formation of DNA-protein complexes (Fig. 4A). Mutation of a critical G residue (Khan *et al.*, 1986; Wang and Gralla, 1998) within the putative σ^{54} consensus sequence of the *algD* promoter (*algD*$_{G→T}$) abrogated the binding of σ^{54} (Fig. 4A, lanes 8–11). As a control, we used AlgR, another regulator previously shown to bind to *algD* and regulate its transcription in concert with σ^E (AlgU) (Mohr *et al.*, 1992). No differences in binding of AlgR to *algD*$_{G→T}$ or to the wild-type *algD* probe were observed (Fig. 4A, lanes 1 and 2). Binding of *E. coli* σ^{54} to the *algD* promoter was further confirmed by DNase I footprinting. Addition of σ^{54} to the *algD* probe resulted in protection of regions including bases corresponding to the σ^{54} promoter consensus GG and GC dinucleotide core (Fig. 4B, lane 1). The position of σ^{54} binding relative to the observed RpoN-dependent *algD* mRNA 5′ end is displaced from the conventional σ^{54} promoter −12/−24 configuration, a phenomenon that remains to be explained but could potentially be attributed to *in vivo* mRNA processing. The gel mobility shift and DNase I footprinting data described above indicate that σ^{54} binds to the σ^{54}-promoter consensus sequence within the *algD* promoter supporting
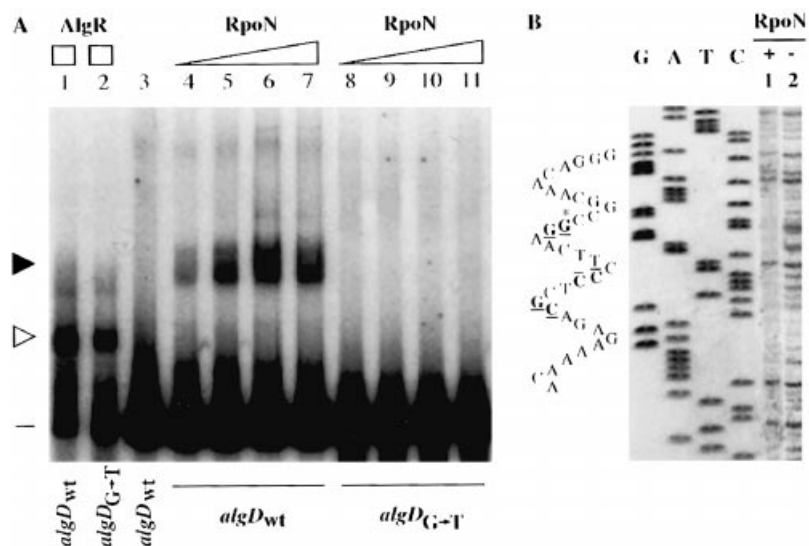
Fig. 4. σ^{54} (RpoN) binds to the *algD* promoter.

A. Gel mobility shift assays were performed as described using 1.9 nmol AlgR [a response regulator previously shown (Mohr *et al.*, 1992) to bind to *algD*] (lanes 1 and 2) or 0.14–0.84 pmol σ^{54} (RpoN) (lanes 4–7 and 8–11 containing escalating amounts of σ^{54}: 0.14 pmol, 0.28 pmol, 0.56 pmol and 0.84 pmol respectively); AlgR was used here as a control. Boxes, equal amounts of protein; wedges, increasing amounts of protein; filled triangle, σ^{54}*algD* promoter complex; open triangle, AlgR-*algD* promoter complex; bar, unbound *algD* probe; lanes or sets of lanes with *algD*wt or *algD*G→T templates are indicated below the autoradiogram.

B. DNase I footprinting analysis was performed as described in *Experimental procedures* using the *algD*wt template and 0.84 pmol σ^{54}. Lanes: 1 (+) RpoN (σ^{54}) added; 2 (−) no RpoN (σ^{54}); G, A, T, C, sequencing ladder produced using the P*algD*R primer; vertical lines, protected regions. Outlined letters, conserved σ^{54} promoter core dinucleotides GG and GC; underscored letters, nucleotides *algD* matching bases demonstrated to enhance σ^{54} binding to a subset of RpoN promoters (Cannon *et al.*, 1993); asterisk, nucleotide changed in *algD*G→T (panel A).

a model of repression by blocking access to the σ^E (AlgU) holoenzyme.

Our *in vitro* experiments suggest a direct repressive effect of σ^{54} on σ-directed transcription but could not exclude the formal possibility that *in vivo* σ^{54} exerted its negative regulatory function differently. For example, σ^{54} could act not by directly binding to *algD* and repressing σ^E (AlgU)-dependent transcription but rather by increasing transcription of another negative regulator (i.e. a repressor) or by competing for the RNA polymerase core with σ^E (AlgU). Furthermore, absent in our *in vitro* experiments were putative additional factors potentially present in cells that could keep σ^{54} in its active form thus precluding its repressive function *in vivo*. To discern between the model of direct repression and alternative indirect possibilities, we reasoned that, if σ^{54} directly repressed *algD in vivo*, transcription of the *algD*G→T variant, no longer capable of binding σ^{54}, should be derepressed even under nitrogen-rich (repressing) conditions while the wild-type promoter should remain repressed. As expected, the levels of *algD* transcription in *mucA22 rpoN*+ cells harbouring the wild-type *algD*–*xylE* fusion were low under repressing conditions (Fig. 5A). By comparison, transcription of the *algD*G→T–*xylE* fusion was derepressed under identical growth conditions (Fig. 5A). The *algD*G→T promoter was still dependent on σ^E (AlgU) for activity, because

introduction of a second mutation (p*algD*G→T/T→C; destroying the σ^E consensus) or inactivation of *algU* on the chromosome abrogated transcription of the plasmid borne *algD* (Fig. 5A). A continued σ^{54} repression of the wild-type *algD* promoter on the chromosome in all experiments was evident as illustrated by the fact that cells remained non-mucoid and alginate production was low (0.51 ± 0.1–0.74 ± 0.1 μg mg^{-1} wcw). The constructs also behaved as expected in *muc23* cells, where the activity of the *algD*G→T–*xylE* fusion was much lower than that of the wild-type *algD*, because its transcription was dependent on RpoN (σ^{54}) in these cells (Fig. 5B). Consistent with these and other observations, the T to C mutation (*algD*T→C) within the −35 σ^E (AlgU) consensus sequence abrogated transcription of *algD* in *mucA22* but not in the *muc23* strain (Fig. 5A and B). These *in vivo* experiments confirmed the conclusion of our *in vitro* studies that in alginate-overproducing cells, which acquired mucoidy via the loss of the AlgU-cognate anti-σ factor MucA, σ^{54} (RpoN) played the role of a transcriptional repressor.

Sigma factor antagonism: unique design of σ^{54} and repression by a sigma factor

In this study, we have demonstrated that σ^{54} can function

A   **B**
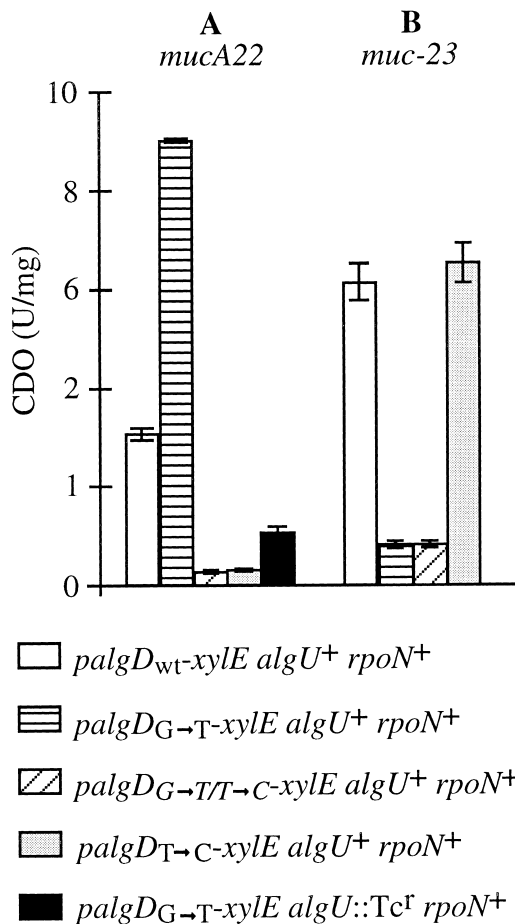*mucA22*   *muc-23*

Fig. 5. Elimination of RpoN (σ^{54}) relieves repression of *algD* promoter transcription *in vivo*. Transcriptional fusion analyses (carried out as in Fig. 1), were performed using strains harbouring the transcriptional fusion constructs: p*algD*$_{wt}$–*xylE*, p*algD*$_{G \to T}$–*xylE* (mutation destroying RpoN consensus and eliminating σ^{54} binding; see Fig. 3B), p*algD*$_{T \to C}$–*xylE* [mutation destroying σ^{E} (AlgU) consensus (see *Experimental procedures*)], or p*algD*$_{G \to T/T \to C}$–*xylE*.

Fig. 6. Model of σ^{54} as a repressor: sigma factor antagonism.
A. σ^{54} bound to the promoter blocks σ^{E}-dependent transcription under non-inducing conditions.
B. σ^{54} clears the promoter by: (B') directing transcription under inducing conditions promoting formation of OCR (open complex-competent σ^{54}-RNA polymerase) in the presence of NTA [NtrC (NRII) type activator] of the same promoter or (B'') by being engaged (recruited) in transcription elsewhere on the chromosome.
C. σ^{E} directs transcription by RNA polymerase holoenzyme at a target promoter. For simplicity, other RNA polymerase subunits are not shown.

directly as a negative regulator (repressor) of transcription. The finding that σ^{54} (or, *in vivo*, σ^{54} RNA polymerase holoenzyme) can bind to a promoter and block transcription initiation by an RNA polymerase holoenzyme containing a different alternative σ factor, suggests a novel physiological function for this σ subunit of RNA polymerase. RNA polymerase holoenzymes, with the notable exception of σ^{54}-containing polymerase, form transcription-ready open-promoter complexes (Gralla, 1993; Marr and Roberts, 1997). σ^{54} is unique in its absolute requirement for additional transcriptional activators that are needed to isomerize σ^{54} polymerase-promoter complexes from a closed to a transcription-competent open state (Lee *et al.*, 1993; Ninfa *et al.*, 1995; Stock *et al.*, 1995; Wang *et al.*, 1995; Perez-Martin and de Lorenzo, 1996; Wang and Gralla, 1996; Syed and Gralla, 1998). It is possible that the unique features of σ^{54}, which
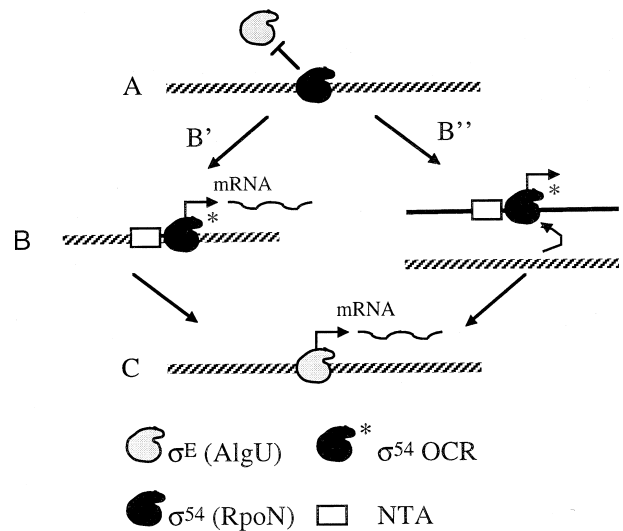
cannot spontaneously support the isomerization of target promoters from closed to open complexes, increase its repertoire as a regulator of transcription to include a role functionally equivalent to that of a repressor.

Repression by σ^{54} offers versatile combinatorial effects not possible with conventional repressor molecules as depicted in the model shown in Fig. 6. In the case of an overlapping promoter configuration, such as in *algD*, σ^{54} leads to repression when there are no external activating inputs (Fig. 6A). When environmental conditions dictate, such inputs can be supplied by a cognate response regulator of the NtrC (NRII) type (Ninfa *et al.*, 1995; Stock *et al.*, 1995). A role for at least one regulator of this type, AlgB, has been demonstrated in alginate production and *algD* transcription (Selvaraj *et al.*, 1998). Thus, AlgB is a likely factor involved in activation of *algD* via σ^{54} (RpoN) and it or another NtrC-type factor could play a role in transducing nitrogen availability signals in *algD* regulation. When activation of σ^{54} takes place (e.g. nitrogen-poor conditions), it can initiate transcription of the subordinate promoter (Fig. 6B') or be engaged elsewhere (Fig. 6B''). Under such conditions, σ^{54} clears the site which now can be transcribed by an RNA polymerase holoenzyme containing a different σ factor (Fig. 6C). In the case of mucoid *mucA* mutants, this sigma factor is σ^{E} (AlgU) which is no longer held inactive by the antisigma factor MucA due to mutations such as *mucA22*.

In wild-type cells, the relative contributions of either σ to the overall transcription levels most likely depend on environmental inputs. While the mutants tested in the present study helped reveal mostly antagonistic relationships between σ^{54} and σ^E, this does not preclude their synergy under certain conditions, with such effects probably masked by the dominant route of transcription in two types of *muc* mutants investigated. In the absence of inhibitory activity by σ^{54} (RpoN), the unbridled σ^E (AlgU)-dependent transcription of *algD* in *mucA22* cells is sufficient to render cells mucoid. This does not preclude the possibility of additional transcription by σ^{54} holo-enzyme under inducing conditions, but this transcription alone is apparently not sufficient to render cells mucoid as inactivation of *algU* in *mucA22* cells results in a severe reduction of *algD* transcription. Conversely, in *muc-23* cells, σ^{54} (RpoN) dominates *algD* transcription and σ^E (AlgU) appears to play a negligible role as it is inhibited by the antisigma factor MucA. The nature of the genes and mutations in *muc-23* cells is not known at present, but it is evident that such mutations activate σ^{54} (RpoN)-dependent transcription of *algD* to high levels. The *muc-23* mutation could be either in a response regulator interacting with RpoN at *algD* or might activate the system by a novel mechanism.

While the dual (positive and negative) regulatory activity of σ^{54} revealed in this study appears to be reserved for overlapping promoters such as in the case of *algD*, additional binding site configurations may be possible. It has also been suggested that the *pilE* promoter of *Neisseria gonorrhoeae* (Fyfe *et al.*, 1995), which contains overlapping σ^{54} consensus and σ^{70} consensus sequences, may be under negative regulation by σ^{54} albeit this phenomenon was observed only in a heterologous host. A potentially similar effect has been noted during mutational analysis of the σ^{70}-dependent promoter of the *E. coli glnA* gene (Reitzer *et al.*, 1987). Additional variations on this theme involving σ^{54} and its interacting factors may exist in other systems (Bertoni *et al.*, 1998; Farewell *et al.*, 1998; Marques *et al.*, 1998). However, the exact design involving superimposed σ^{54} and σ^E promoters uncovered here may be reserved only for a narrow subset of critical promoters directing major physiological decisions in Gram-negative organisms. In the case of the system described here, phenomena such as biofilm formation (Davies *et al.*, 1998), developmental processes potentially similar to encystment (Moreno *et al.*, 1998) and prolonged survival under harsh environmental conditions and persistence during chronic infection (Govan and Deretic, 1996; Welsh *et al.*, 1995) may be subject to σ factor antagonism as a versatile mechanism providing instant coordination of major physiological systems in the cell. This system also has a built-in circuit breaker that can abort commitment for a major change in the morphological and physiological state of the bacterium.

Experimental procedures

Strains and constructs

Pseudomonas aeruginosa PAO381 and the its mucoid derivatives PAO578II and PAO579 have been previously described (Fyfe and Govan, 1980). The plasmids pDMU100 (Martin *et al.*, 1993c) and pKI11 (Ishimoto and Lory, 1989) were used to inactivate *algU* and *rpoN* via triparental matings (Martin *et al.*, 1993c). The plasmid p*rpoN* (pPT212) (Totten *et al.*, 1990) was used for complementation of *rpoN*::Tc^r mutants. The plasmid pHYDX was used to place the *algD*::*xylE* fusion on the *P. aeruginosa* chromosome (Yu *et al.*, 1995). The plasmids pVDX18 and p*algD*$_{wt}$ (pVD533) have been previously described (Deretic *et al.*, 1990). A 918 bp PCR product from *P. aeruginosa* PAO1 was the source of the wild-type *algD* promoter. The *algD* promoter containing fragment was cloned into the invitrogen pCR2.1 vector generating pCBD-3 and *algD* sequence verified. The plasmids p*algD*$_{G \to T}$–*xylE*, p*algD*$_{T \to C}$–*xylE* and p*algD*$_{G \to T/C \to T}$–*xylE* contained the *algD* promoter sequence (APS; **GG**AA**CTT**CCCTCGCAGAGAAAACATCCTA) with the following changes generated by cross-over PCR using the appropriate oligonucleotides: G→T (APS, bold **G**), TT→CC (APS, bold **TT**), and a combination of both G→T and T→C changes. For transcriptional fusion analyses, the *algD* promoter variants were fused with the *xylE* reporter gene as *Hin*dIII–*Eco*RI fragments cloned into pVDX18. To generate the single stranded *algD* probe for S1 nuclease assays, the *algD* promoter region was subcloned as a *Hin*dIII–*Xba*I fragment from pCBD-3 into M13 mp19 and the uniformly labelled single-stranded probe generated using the oligonucleotide P*algD*$_R$ (5′-CCGATTATTCGAGACGGTTTC-3′) as described by Deretic and Konyecsni (1989).

S1 nuclease protection and primer extension analyses

For S1 nuclease protection assays, RNA was isolated, hybridized to single-stranded *algD* probes, treated with S1 nuclease and digestion products analysed as previously described by Deretic and Konyecsni (1989). Primer extension analysis was carried out by end-labelling the primers ECF11 (for *ecfI*) 5′-CGGCGCCATGGGAATG-3′, ECF13 (for *ecfJ*) 5′-GTGGCGCCATGGGCTT-3′ and ECF18 (for *ecfQ*) 5′-ATGGGGGTCGAGTGTA-3′ with 15U T4 polynucleotide kinase (GibcoBRL) in 10 mM DTT, 30 μCi [γ-^{32}P]-ATP, 1∞ polynucleotide kinase buffer supplied with enzyme for 1 h at 37°C. Reations were stopped by adding 100 mM EDTA, 50 μl TE and heating to 65°C for 10 min. Unincorporated nucleotides were removed by Boehringer Mannheim G-25 Sephadex spin columns. Thirty micrograms of RNA per sample was annealed to primers in hybridization buffer (500 mM KCl, 250 mM Tris-HCl pH 8.3) for 1 min at 95°C followed by 2 min at 55°C and 15 min on ice. Extensions were carried out in 10 mM DTT, 250 μM dNTP′s, GibcoBRL Superscript II RT (GibcoBRL) and reverse transcriptase buffer supplied with enzyme at 44°C for 45 min. Reactions

were stopped, boiled and analysed on a polyacrylamide sequencing gel along the side of the sequencing ladder generated using the same primer and corresponding PCR fragment as the template.

In vitro *transcriptional run-off analysis*

For *in vitro* transcriptional run-off analyses, a 142 bp fragment containing wild-type *algD* and *algD*$_{G \to T}$ were generated by PCR using the oligonucleotides 5′-TCCAAA-TATTTCGCGAGCGGGACGAACGGTCG-3′ 5′-GTTTGTC-CCTCGGAGCGGAA-3′ and pCBD-3 and p*algD*$_{G \to T}$ as templates respectively. For inhibition studies, *E. coli* σ^{54} (1.1 pmol) was preincubated at room temperature with the *algD* promoter template for 10 min in 10 mM Tris-HCl pH 7.5, 30 mM KCl, 10 mM MgCl$_2$ and 1 mM DTT. AlgU and RNA polymerase were incubated together on ice for 10 min in 10 mM Tris-HCl pH 8.0, 10 mM KCl, 10 mM β-mercapto-ethanol, 1 mM EDTA, 0.4 mg ml^{-1} BSA and 0.1% Triton X-100 to allow association. AlgU and RNA polymerase was added to each reaction, incubated for 10 min at room temperature, 0.2 mM ATP, GTP, CTP and 3 μCi [α-^{32}P]-UTP was then added and further incubated for 15 min and reaction stopped by adding 150 mM sodium acetate and ethanol. Precipitated DNA was dissolved and analysed on a polyacrylamide sequencing gel.

Mobility shift DNA-binding assay

Probes were generated by PCR using the oligonucleotides 5′-GGCCATCAAGTTGGTATCAA-3′ and P*algD*$_R$ as primers and pCBD-3 and p*algD*$_{G \to T}$, as templates. End-labelled probes (^{32}P) were incubated with AlgR (1.9 nmol) or *E. coli* RpoN (0.14–0.83 pmol) in a total volume of 10 μl containing 25 mM Tris-HCl pH 8.0, 6 mM MgCl$_2$, 0.5 mM EDTA, 0.5 mM DTT, 20 mM KCl, 5% glycerol, 100 μg ml^{-1} poly(dI-dC), and 100 μg ml^{-1} ssDNA. DNA-protein complexes were separated from the unbound probe on a 5% native polyacrylamide gel (Mohr *et al.*, 1992) and visualized by autoradiography.

DNase I footprinting analysis

The primers P*algD*$_R$ and ADL2 (5′-GGCCATCAAGTTCC-TATCAA-3′) were used to generate a 160 bp probe. The probe was end-labelled using 3 μl of PCR product, 2 μl of polynucleotide kinase buffer (Boehringer Mannheim), 3 μl of [^{32}P]-ATP, and 15 U polynucleotide kinase (Boehringer Mannheim), digested with *Eco*RV and purified. Gel mobility shift σ^{54}-DNA binding reaction mixtures were incubated for 10 min at room temperature, MgCl$_2$ added (final concentration of 5 mM) and DNase I digestions carried out on ice for 2 min. Reactions were stopped by addition of 40 mM EDTA and separated on a non-denaturing polyacrylamide gel as described for gel mobility shifts. Bands corresponding to protein bound and unbound DNA were excised from the gel and eluted in 500 mM NH$_4$OAc, 1% SDS and 1 mM EDTA. Eluates were phenol extracted and DNA precipitated in 550 μl of EtOH, 1 μg of tRNA and 10 μg of glycogen. DNA was resuspended in 10 μl of formamide buffer and run

alongside a sequencing ladder generated using the P*algD*$_R$ primer on an 8% polyacrylamide sequencing gel.

Alginate assay

Alginate was determined by the method of Knutson and Jeanes (1976). The amount of alginate was expressed in μg of uronic acid per mg of wet-cell weight.

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