Microbial pathogenesis in cystic fibrosis: co-ordinate regulation of heat-shock response and conversion to mucoidy in *Pseudomonas aeruginosa*

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Summary

Conversion of *Pseudomonas aeruginosa* to the mucoid phenotype plays a major role in the pathogenesis of respiratory infections in cystic fibrosis (CF). One mechanism responsible for mucoidy is based on mutations that inactivate the anti-σ factor, MucA, which normally inhibits the alternative sigma factor, AlgU. The loss of MucA allows AlgU to freely direct transcription of the genes responsible for the production of the exopolysaccharide alginate resulting in mucoid colony morphology. In *Escherichia coli*, a close homologue of AlgU, σ^E, directs transcription of several genes under conditions of extreme heat shock. Here we examined whether AlgU, besides its role in controlling alginate production, affects the heat-shock response in *P. aeruginosa*. The *P. aeruginosa rpoH* gene encoding a homologue of the major heat-shock sigma factor, σ^{32}, was found to be transcribed by AlgU containing RNA polymerase from one of its promoters (P_3) identified in this study. Transcription of *rpoH* from P_3 was elevated upon exposure to extreme heat shock in an *algU*-dependent manner. Importantly, the AlgU-dependent promoter of *rpoH* was found to be activated in mucoid *mucA* mutants. In keeping with this observation, introduction of a wild-type *mucA* gene abrogated AlgU-dependent *rpoH* transcription in mucoid *P. aeruginosa* laboratory isolates and CF isolates. These results suggest that conversion to mucoidy and the heat-shock response are co-ordinately regulated in *P. aeruginosa*. The simultaneous activation of both systems in *mucA* mutants, selected in the lungs of CF patients, may have significance for the inflammatory processes characteristic of the establishment of chronic infection and ensuing clinical deterioration in CF.

Introduction

Chronic respiratory infections and associated inflammation are the leading cause of high mortality and morbidity in patients with cystic fibrosis (CF) (Boat, 1989; Govan and Deretic, 1996). The overproduction of the exopolysaccharide alginate, which results in mucoid colony morphology, is a characteristic pathogenic determinant expressed by *Pseudomonas aeruginosa* in CF (Govan and Deretic, 1996). The *muc* locus, a major site of genetic alterations responsible for the conversion to the mucoid phenotype, has initially been described by Fyfe and Govan (1980). The recent molecular characterization of the genes within the *muc* locus at 67.5 min of the *P. aeruginosa* map resulted in identification of the *algU mucABCD* gene cluster as the major regulatory site which encodes both positive and negative factors controlling mucoidy (Boucher *et al.*, 1996; Martin *et al.*, 1993a; Martin *et al.*, 1993b; Martin *et al.*, 1993c; Schurr *et al.*, 1994). Similar findings have been reported by others, resulting in alternative designations of *algU* as Pa σ^E or *algT* (DeVries and Ohman, 1994; Hershberger *et al.*, 1995; Wozniak and Ohman, 1994) and *mucB* as *algN* (Goldberg *et al.*, 1993). The initial analysis of *algU* showed that its predicted gene product was homologous to the *Bacillus* stationary phase and sporulation sigma factor Spo0H (Martin *et al.*, 1993a). In a subsequent study, Martin *et al.* (1994) showed that the -35 and -10 regions of promoters transcribed by AlgU shared considerable similarity with the known promoter consensus sequence for σ^E, the second heat-shock sigma factor identified at the biochemical level in *Escherichia coli* (Erickson and Gross, 1989). These and subsequent sequence analyses permitted the identification of *E. coli* and *Salmonella typhimurium rpoE* genes based on striking homologies of their gene products with AlgU (Hiratsu *et al.*, 1995; Martin *et al.*, 1994; Raina *et al.*, 1995; Rouvière *et al.*, 1995). These observations and additional analyses have uncovered the existence of a broader family of novel alternative sigma factors termed ECF (Lonetto *et al.*, 1994) or σ^E-like factors (Deretic *et al.*, 1994).

The initial experimental evidence supporting the hypothesis that *algU* encodes the *P. aeruginosa* equivalent of σ^E was presented when *E. coli rpoE* was shown to complement an *algU* mutation and to induce mucoidy in *P. aeruginosa* (Yu *et al.*, 1995). Biochemical evidence that
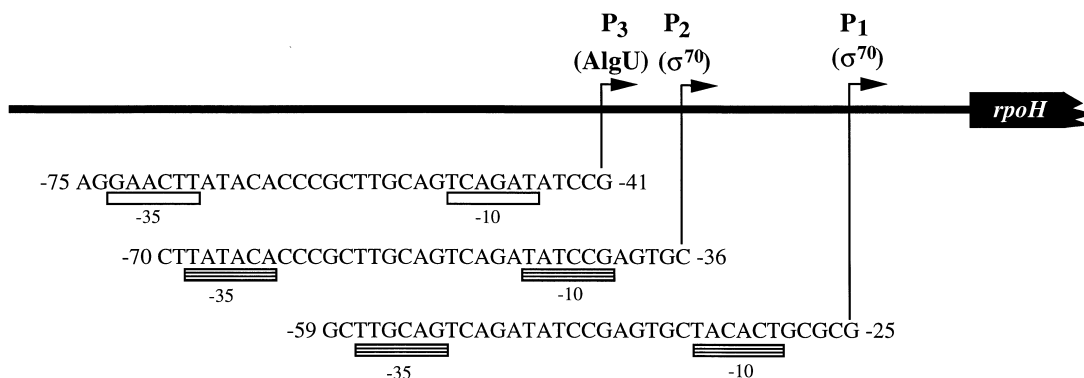
Fig. 1. The promoter region of the *rpoH* gene from *P. aeruginosa*. Numbering is given relative to the initiation codon of *rpoH*. Bent arrows, mapped mRNA 5′ ends; P_1 and P_2, σ^{70}-dependent promoters; P_3, AlgU (Pa σ^E)- dependent promoter; boxes, −35 and −10 regions of σ^{70} (horizontal stripes) and σ^E (open boxes) promoters.

algU encodes a sigma factor has been reported recently (Hershberger *et al.*, 1995; Schurr *et al.*, 1995b). AlgU activity is controlled at a post-translational level by the products of the downstream genes (*mucABCD*) that encode its negative regulators. Recently, it has been demonstrated that MucA probably functions as an anti-sigma factor (Schurr *et al.*, 1996; Xie *et al.*, 1996). Mutations in *mucA* and other *muc* genes located in the 67.5 min region are responsible for the conversion to mucoidy in CF isolates and laboratory strains (Martin *et al.*, 1993c). As a result of *mucA* inactivation, AlgU is relieved from the inhibition by its cognate anti-σ factor and is available to direct transcription of the subordinate promoters. AlgU directs transcription of its own gene (Schurr *et al.*, 1995a) and that of *algR*, a response regulator controlling critical alginate genes (Martin *et al.*, 1994; Wozniak and Ohman, 1994). AlgU and AlgR co-operate to activate transcription of the *algD* gene (Martin *et al.*, 1994; Schurr *et al.*, 1993) encoding GDP mannose dehydrogenase (Deretic *et al.*, 1987; Roychoudhury *et al.*, 1989), which catalyses the first committed step in alginate synthesis. Because of this cascade of regulatory interactions, the net result of a loss of *mucA* is alginate overproduction and mucoid colony phenotype.

The equivalence of *E. coli* σ^E and *P. aeruginosa* AlgU suggests that there may be broader physiological overlaps in the function of these sigma factors (Yu *et al.*, 1995). The sigma factor σ^{32} that controls the heat-shock response in *E. coli* is encoded by the *rpoH* gene (Grossman *et al.*, 1984). *E. coli* σ^{32} controls expression of genes encoding heat-shock proteins such as GroEL, DnaJ, DnaK, and Lon (Yura *et al.*, 1993). Interestingly, one of the *E. coli* *rpoH* promoters (P_3) is controlled by σ^E under conditions of extreme heat shock (Erickson and Gross, 1989; Wang and Kaguni, 1989). In order to address the question of whether AlgU, in addition to regulating mucoidy in *P. aeruginosa*, affects the heat-shock response in this organism, the promoter region of the recently characterized *P.*

aeruginosa rpoH gene (Naczynski *et al.*, 1995) and the effects of *algU* and *mucA* mutations on *rpoH* transcription were analysed. We report that AlgU is responsible for transcriptional initiation of one of the *rpoH* promoters (Fig. 1). We also show that *mucA* mutations in *P. aeruginosa* not only induce alginate production and mucoidy in this organism but also activate *rpoH* transcription, which may have downstream effects on the entire heat-shock response. As chronic inflammation is a well-recognized sequela of *P. aeruginosa* infections in CF, it is possible that the expected increased expression in *mucA* mutants of highly conserved antigens such as heat-shock proteins may further complicate immune processes and contribute to the pathogenesis in CF.

Results

S1 nuclease protection mapping of the rpoH mRNA 5′ ends

In order to investigate whether *rpoH* is transcribed by AlgU (Pa-σ^E) in *P. aeruginosa* and to map the *rpoH* promoters in this organism, the region immediately upstream of the *rpoH* gene was cloned and examined by S1 nuclease protection analysis. Several bands of protection corresponding to *rpoH* mRNA 5′ ends were observed (Fig. 2). One 5′ mRNA end was located 25 bp upstream of the *rpoH* initiation codon (Figs 1 and 2). A second band of protection corresponded to the 5′ mRNA end at the position 41 bp upstream of the *rpoH* translational start (Fig. 2A, lane 1). When *P. aeruginosa* was grown on minimal media, an additional 5′ mRNA end was observed. This band of protection was located 36 bp upstream of the initiation codon (Fig. 2B), was not visible in RNA extracted from cells grown in rich medium (Fig. 2A), and was more prominent when RNA was extracted from PAO6852 (*algU*::Tc^R) (Fig. 2B, lanes 3 and 4). These results suggest
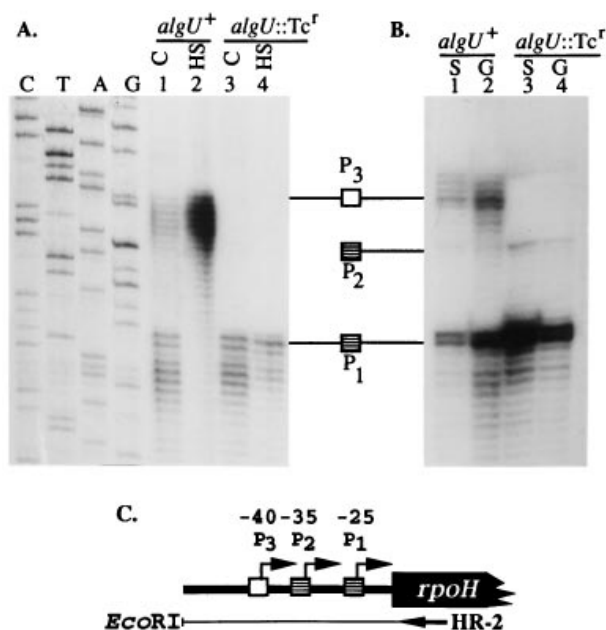
Fig. 2. Mapping of the *P. aeruginosa rpoH* promoters, analysis of transcription from P_3 in *algU*::Tc^R background and induction by heat shock of the P_3 promoter of *rpoH*.

A. *P. aeruginosa rpoH* promoters mapped using RNA from cells grown in rich medium. Lanes: 1, PAO1 grown continuously at 37°C; 2, heat-shocked PAO1; 3, PAO6852 (*algU*::Tc^R derivative of PAO1) grown at 37°C; 4, heat-shocked PAO6852.

B. S1 nuclease protection mapping of the *P. aeruginosa rpoH* P_2 promoter using RNA from cells grown on minimal media. Lanes: 1, PAO1 grown in minimal medium supplemented with succinate (S); 2, PAO1 grown in minimal medium supplemented with glucose (G); 3, PAO6852 (*algU*::Tc^R) grown in minimal medium with succinate; 4, PAO6852 (*algU*::Tc^R) grown in minimal medium with glucose.

C. Schematic representation of hybridization probe. HR-2 primer was used to generate the probe; the *Eco*RI restriction site defines the 3′ end of the radiolabelled probe for S1 nuclease protection analysis. Striped boxes, P_1 and P_2; open box, P_3.

that the *P. aeruginosa rpoH* gene is transcribed from at least three promoters (P_1, P_2 and P_3) corresponding to the mRNA start sites mapped in this study at 25 bp, 36 bp, and 41 bp upstream of the initiation codon (Fig. 1). Of the mapped mRNA 5′ ends, P_1 and P_3 were present under most conditions, while P_2 was detectable only on minimal media. Interestingly, the P_2 signal was enhanced in *algU* null mutants (Fig. 2B), suggesting a possible interference of initiation from the P_2 and P_3 promoter or masking of the signal.

Induction of rpoH P_3 by extreme heat shock and its dependence on algU

A strong consensus σ^E (AlgU) promoter sequence, previously noted upstream of the *rpoH* coding sequence (Benvenisti *et al.*, 1995; Deretic *et al.*, 1994; Naczynski *et al.*, 1995), was located at the canonical −35 and −10 positions in relation to the P_3 band. In order to determine

if *rpoH* P_3 was AlgU-dependent, RNA from *P. aeruginosa* PAO6852 (*algU*::Tc^R) was subjected to S1 nuclease protection analysis. The P_3 band was absent in these samples (Fig. 2A, lanes 3 and 4) while it was present as expected (Martin *et al.*, 1994; Schurr *et al.*, 1995a) in samples with RNA from *algU*^+ cells (Fig. 2A, lanes 1 and 2). These results indicate that the *rpoH* P_3 signal is dependent on the presence of a functional *algU* gene.

The AlgU promoters of *algU* (P_1 and P_3) and *algR* (R_P) are induced during exposure to extreme heat shock (Martin *et al.*, 1994; Schurr *et al.*, 1995a). In order to examine whether the P_3 promoter of *rpoH* can be induced by extreme heat shock, *P. aeruginosa* PAO1 RNA was isolated from cells that were exposed to a temperature shift from 37°C to 50°C. This treatment resulted in a dramatic increase in *rpoH* P_3 transcription (Fig. 2A, lane 2) as compared to cells grown at 37°C (Fig. 2A, lane 1). The observed activation of *rpoH* transcription from P_3 under conditions of extreme heat shock (Fig. 2A, lane 2) and the absence of the corresponding transcripts in *algU* mutants (Fig. 2A, lanes 3 and 4) are consistent with the interpretation that *rpoH* P_3 is an AlgU-dependent promoter.

In vitro *transcriptional analysis of AlgU and* σ^{70} *promoters of* P. aeruginosa rpoH

Purified AlgU has been used *in vitro* to demonstrate its activity as a sigma factor on one of the promoters of *algU* (P_1) (Schurr *et al.*, 1995b). In order to examine whether AlgU directs transcription of the *rpoH* P_3 promoter, purified AlgU was preincubated with core RNA polymerase and added to the standard *in vitro* transcriptional run-off assay (Schurr *et al.*, 1995b) with *rpoH* as a template. The predicted distance from P_3 initiation to the end of the truncated *rpoH* is 77 nucleotides and a transcript of this size was observed upon addition of AlgU to the mixture (Fig. 3A, lanes 2 and 3). This transcript had the expected size and was absent when the same template was incubated with RNA polymerase core alone (Fig. 3A, lane 1). These findings support the conclusion that the *rpoH* P_3 promoter is transcribed by the AlgU (Pa σ^E) RNA polymerase holoenzyme.

In a set of complementary experiments, *in vitro* transcription of P_1 and P_2 was investigated. Examination of the −10 (TAcAcT) and −35 (TTGcag) regions of the strong *rpoH* P_1 promoter suggested the possibility that this promoter may be transcribed by the σ^{70} holoenzyme. In *E. coli*, it is known that σ^{70} directs transcription of several *rpoH* promoters (Erickson *et al.*, 1987; Fujita *et al.*, 1987; Nagai *et al.*, 1990). In order to examine the possibility that σ^{70} directs transcription of the P_1 promoter of *P. aeruginosa rpoH*, *in vitro* transcriptional run-off analyses were performed either with core RNA polymerase reconstituted with exogenously added σ^{70} or with σ^{70} saturated
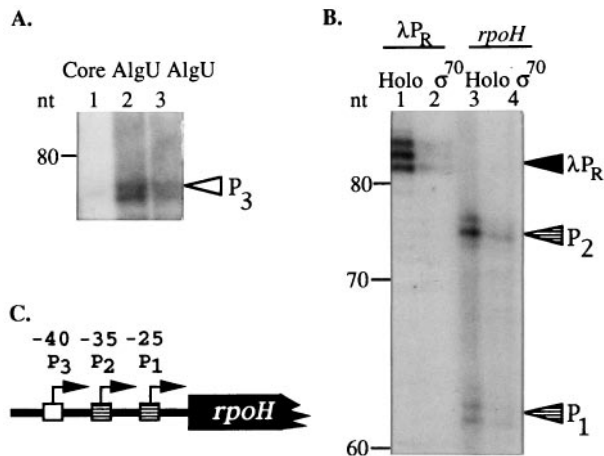
Fig. 3. AlgU directs transcription of the *rpoH* P_3 promoter and σ^{70} directs transcription from *rpoH* P_1 and P_2 promoters.
A. *In vitro* transcription from the *rpoH* P_3 promoter. Lanes: 1, core RNA polymerase; 2 and 3, core RNA polymerase with AlgU. Open triangle, *rpoH* P_3 transcript.
B. σ^{70}-directed *in vitro* transcription from the λP_R promoter and the r*poH* P_1 and P_2 promoters. Lanes: 1, RNA polymerase holoenzyme and the λP_R promoter; 2, core RNA polymerase with σ^{70} and the λP_R promoter; 3, RNA polymerase holoenzyme and the *rpoH* promoter; 4, core RNA polymerase with σ^{70} and the *rpoH* promoter.
C. Schematic representation of *rpoH* promoter positions. Open box, *rpoH* P3 promoter; striped box, *rpoH* P_1 and P_2 promoters; filled triangle, λP_R transcript; striped triangles, *rpoH* P_1 and P_2 transcripts.

holoenzyme preparations. In these experiments, where the σ^{70}-dependent promoter of λP_R was used as a positive control (Fig. 3B, lanes 1 and 2), the expected size transcript corresponding to *rpoH* P_1 was observed with the σ^{70}-containing polymerase (Fig. 3B, lanes 3 and 4). As expected, no transcript corresponding to P_3 was observed with σ^{70}. Interestingly, a stronger second band was observed with the size corresponding to the transcript from P_2, suggesting that σ^{70} polymerase may transcribe both the P_1 and P_2 promoters of *rpoH*.

Inactivation of mucA *induces AlgU-dependent* rpoH *transcription*

As *mucA* has been shown to act as a negative regulator of AlgU activity (Martin *et al.*, 1993c; Schurr *et al.*, 1994; 1996), we tested the hypothesis that the AlgU-dependent promoter P_3 of *rpoH* may also be affected by *mucA* mutations. RNA from the strain PAO578I (Schurr *et al.*, 1994) carrying the characterized *mucA22* mutation (Martin *et al.*, 1993c) was examined by S1 nuclease protection analysis. Increased transcription was observed from *rpoH* P_3 in *mucA22* cells when compared to *mucA*$^+$ cells (Fig. 4, lane 8 versus lane 7). This effect was similar to the increased transcription of *algU* P_1 and P_3 in the same genetic background (Fig. 4, lanes 4 versus 5 for P_1; lanes 1 versus 2 for P_3). These observations indicate

that expression from the *rpoH* P_3 promoter is negatively regulated by MucA and that the transcription from this promoter is elevated in *mucA* mutants. As another level of control, we also tested transcription of *rpoH* P_3 in a *mucB*::Tc^R background. MucB encodes a periplasmic protein which also negatively regulates alginate production (Martin *et al.*, 1993b; Schurr *et al.*, 1996). Although *mucB* mutations cause conversion to mucoidy, this phenotype is medium dependent and under the conditions used in the present study, the strains are normally not induced for alginate production. In keeping with the absence of increased alginate synthesis, the loss of *mucB* did not alter expression of *rpoH* P_3, *algU* P_1 and *algU* P_3 under the conditions used (Fig. 4, lanes 3, 6, and 9). Collectively, these findings support the model in which mucoidy and increased *rpoH* expression are co-ordinated in *P. aeruginosa*.

Plasmid-borne wild-type mucA *suppresses AlgU-dependent transcription from* rpoH P_3

In order to confirm that the observed increase in *rpoH* P_3 transcription in PAO578I (*mucA22*) was due to the lack of MucA, a wild-type *mucA* was introduced into this strain. In addition to the laboratory strain PAO578I, a previously characterized CF isolate carrying a sequenced *mucA22* (CF23) (Martin *et al.*, 1993c) was also tested. As previously described, introduction of the p*tac-mucA*$^+$ plasmid into these mucoid strains resulted in a non-mucoid phenotype (Martin *et al.*, 1993c). RNA was isolated from PAO578I harbouring p*tac-mucA*$^+$ and CF23 containing p*tac-mucA*$^+$ and subjected to S1 nuclease protection analysis. Examination of the P_3 promoter of *rpoH* in the complemented strains showed that its AlgU-dependent transcription was suppressed when a functional *mucA* was present *in trans* (Fig. 5, lanes 2 and 4). Interestingly, the presence of extra copies of *mucA* suppressed *rpoH* transcription below the levels seen in wild-type *P. aeruginosa* (Fig. 5, lane 2 compared to Fig. 2A, lane 1). These results are consistent with the model in which MucA inhibits AlgU and prohibits initiation of transcription from the P_3 promoter of *rpoH*. Collectively, these findings support the notion that *mucA* mutations, in addition to being an important mechanism responsible for conversion to mucoidy in *P. aeruginosa*, also increase expression of the gene encoding the major heat-shock sigma factor in this organism.

Discussion

In this study, we have mapped the 5′ mRNA ends of the *P. aeruginosa rpoH* gene and found that there are three promoters located at positions −25 (P_1), −36 (P_2) and −41 (P_3) relative to the start codon. The conclusion that *P. aeruginosa rpoH* contains an AlgU-dependent promoter (P_3) is supported by the following experimental
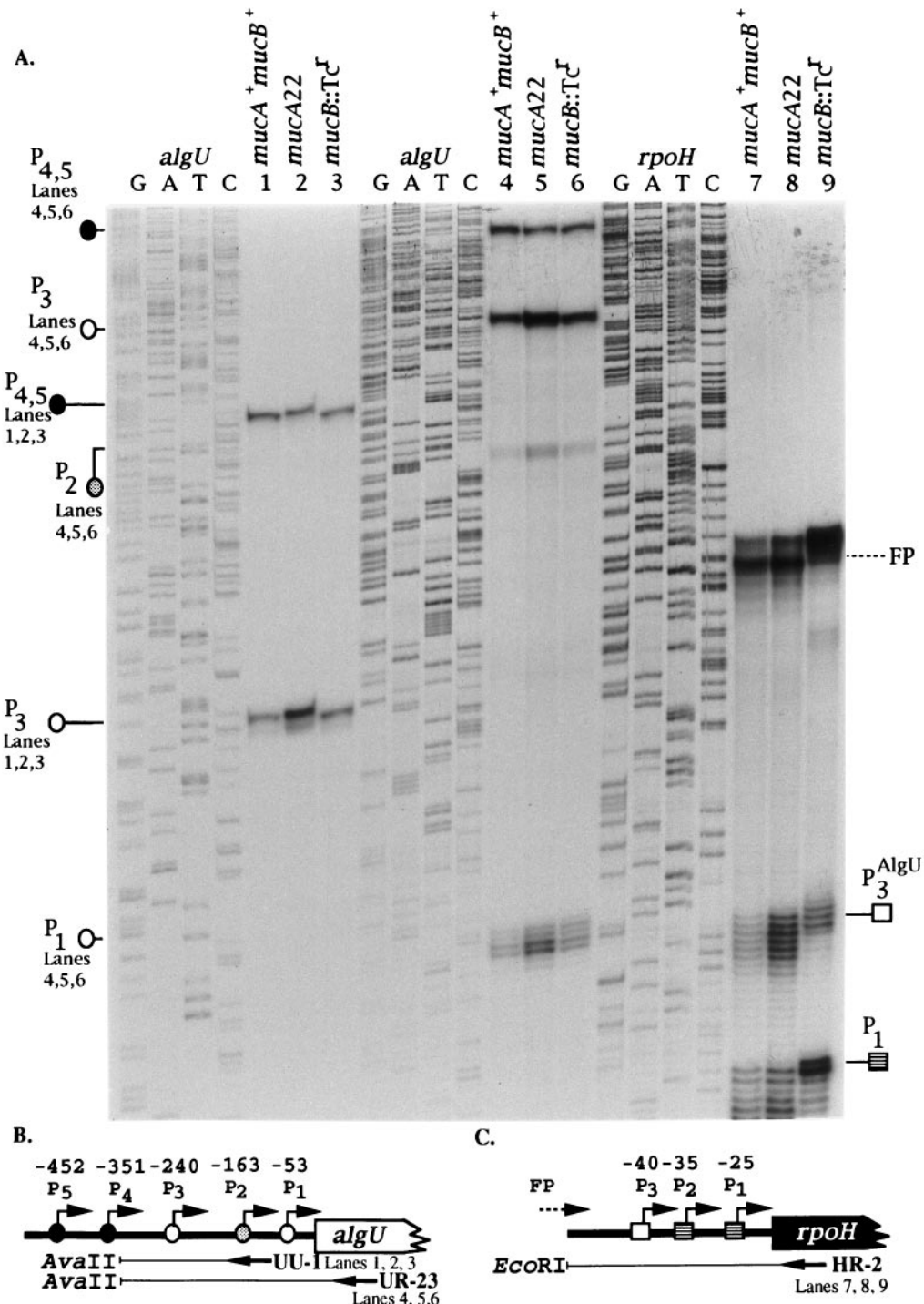
Fig. 4. Induction of AlgU-dependent transcription of *algU* and *rpoH* in *mucA* mutants.
A. S1 nuclease protection analysis. Lanes: 1, PAO381 (*mucA*⁺ *mucB*⁺); 2, PAO578I (*mucA22*); 3, PAO6857 (*mucB*::Tc^R) using an *algU* probe generated with oligonucleotide UU-1; 4, 5 and 6, same as lanes 1, 2 and 3 using an *algU* probe generated with oligonucleotide UR-23; 7, 8 and 9, same as lanes 1, 2 and 3 using an *rpoH* probe generated with oligonucleotide HR-2.
B and C. Schematic representation of probes, oligonucleotides and restriction sites used in S1 nuclease protection analyses. Negative numbers show relative positions of promoters with respect to the initiation codons of *algU* and *rpoH*. Circles represent *algU* promoters, boxes indicate *rpoH* promoters as in (A). Open boxes and circles represent AlgU-dependent promoters; filled or patterned boxes and circles indicate other promoters; FP, band of full protection. All *algU* promoters (P1–P5) shown here have been previously described (Schurr *et al.*, 1995a).
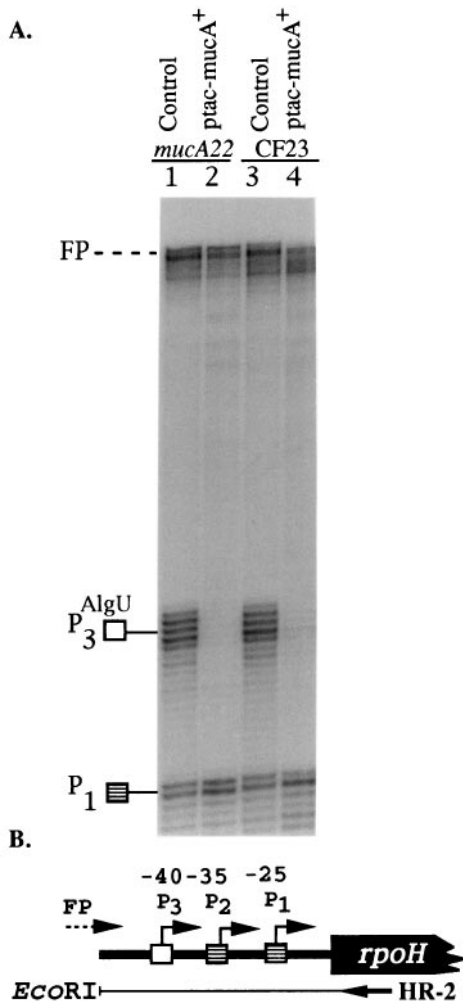
A.

Fig. 5. Suppression of AlgU-dependent *rpoH* transcription by *mucA*.
A. S1 nuclease protection analysis of RNA from mucoid strains PAO578I and CF23 with characterized *mucA* mutations (Martin *et al.*, 1993c). Lanes: 1, PAO578I; 2, PAO578I harbouring p*tac-mucA*⁺; 3, CF23; 4, CF23 harbouring p*tac-mucA*⁺.
B. Schematic representation of probe, oligonucleotide (HR-2), and restriction sites used in S1 nuclease protection analysis. Boxes show same promoters as in Fig. 3; FP, band of full protection.

observations and additional considerations: (i) P_3 transcription is absent in *algU* null mutants of *P. aeruginosa*; (ii) P_3 transcription is initiated *in vitro* by AlgU-RNA polymerase holoenzyme; (iii) P_3 is induced under conditions of extreme heat shock; (iv) P_3 expression is increased in *mucA* mutant strains; and (v) the -35 (GAACTT) and -10 (TCaGA) regions of P_3 conform with the AlgU (σ^E) consensus promoter sequence. These findings are also consistent with the interpretation that conversion to mucoidy and transcriptional activation of the heat-shock response in *P. aeruginosa* are co-ordinated and probably occur simultaneously in *mucA* mutants. This is evidenced by: (i) increased *rpoH* P_3 transcription in *mucA* mutant strains; and (ii) suppression of *rpoH* P_3 transcription by

plasmid-borne *mucA*⁺ introduced into laboratory and CF *mucA* mutants. MucA has been demonstrated to negatively regulate AlgU (Fig. 6A) and recent findings support the hypothesis that it does so by binding to AlgU (Martin *et al.*, 1993c; Schurr *et al.*, 1994; 1996; Xie *et al.*, 1996). Since MucA, as shown here, also controls expression of *rpoH* it will be of interest to examine, in future studies, the extent of the effects of *mucA* mutations on the expression of *rpoH*-dependent genes. Such analyses are expected to reveal additional details and uncover the full extent of overlaps between the conversion to mucoidy and heat-shock response in *P. aeruginosa*. It is possible that selection of *mucA* mutants in CF reflects not only the advantages due to the alginate coating but may provide additional protection to the pathogen because of the co-induction of other stress-response systems.

Two of the three mapped *rpoH* promoters (P_1 and P_2) are initiated by σ^{70} as shown in this study. A previous
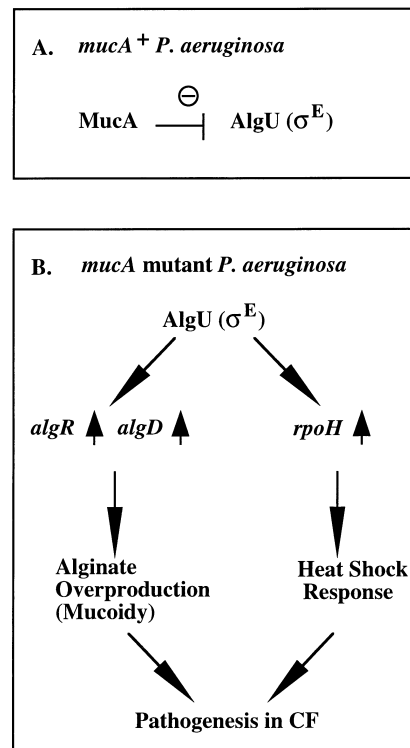

Fig. 6. Proposed model of co-induction of mucoidy and the heat-shock response in *mucA* mutants of *P. aeruginosa*.
A. MucA is a negative regulator of AlgU. In cells with a functional *mucA* gene, AlgU activity is inhibited and colonies have non-mucoid morphology.
B. In strains carrying *mucA* mutations, AlgU activity is increased. This stimulates downstream events such as increased alginate production (conversion to mucoidy) and increased transcription of the heat-shock sigma-factor gene *rpoH*. This may cause induction of heat-shock response genes in *P. aeruginosa*, which encode highly conserved antigens as suggested by Jensen *et al.* (1993; 1995), that may contribute to the immunopathology in CF.

report (Naczynski *et al.*, 1995) examined transcription of *P. aeruginosa rpoH* in the heterologous host *E. coli*. One of the mRNA 5′ ends observed in our study (P$_1$ at −25) matches with the initiation start sites reported by Naczynski *et al.* (1995). However, the P$_3$ transcriptional start site, mapped in our study in five strains to the same position (−41), did not match any of the other bands reported by Naczynski *et al.* (1995) for plasmid-borne *P. aeruginosa rpoH* expressed in *E. coli*. A third band corresponding to full probe protection (Fig. 4A, lanes 7–9; FP) was also observed in our studies, suggesting the existence of at least one additional transcription initiation site. Such a transcript has not been reported by Naczynski *et al.* (1995). These and additional discrepancies are best explained by potential expression differences of the *P. aeruginosa rpoH* gene in *E. coli* and *P. aeruginosa*. The transcription from P$_2$ observed in this study was detectable only on minimal media, which is reminiscent of medium-dependence for expression of one of the *E. coli rpoH* promoters (Nagai *et al.*, 1990). It is also worth noting that it was difficult to detect P$_2$ activity *in vivo* unless AlgU-dependent transcription from P$_3$ was absent. At present, we do not know whether the physical proximity of the canonical −10 and −35 regions for the P$_2$ and the P$_3$ promoters or some other factors may be the basis for this apparent interference. However, as the two promoters are active under different physiological conditions, the occupancy of P$_2$ and P$_3$ may be mutually exclusive. Interestingly, the intensity of P$_1$ and P$_2$ transcripts *in vitro* (Fig. 3B) was in favour of P$_2$ in contrast to the relative intensity *in vivo* (Fig. 2B). However, the intensity of signals obtained *in vitro* may also reflect transcript size. It is also important to note that P$_3$ transcription was stronger than P$_2$ transcription under all conditions when both promoters were active.

Several studies have implicated stress proteins from different bacteria in the induction of autoimmune disease (Lamb *et al.*, 1989; Oldstone, 1987; Young, 1990). Heat-shock proteins of many pathogenic bacteria (e.g. *Salmonella* spp., *E. coli*, *Chlamydia trachomatis*, *Mycobacterium* spp., and *Brucella* spp.) are recognized by the immune system (Cameron *et al.*, 1994; Cerrone *et al.*, 1991; Elzer *et al.*, 1994; Johnson *et al.*, 1991; Roop *et al.*, 1994; Tatum *et al.*, 1994; van Eden *et al.*, 1988) and have been implicated in bacterial virulence or in immunopathology associated with the sequelae of bacterial infections (Lamb *et al.*, 1989; van Eden *et al.*, 1988; Young, 1990). One of the recently studied proteins that belongs in this category is HtrA (DegP) (Lipinska *et al.*, 1990; Strauch and Beckwith, 1988). HtrA is important for virulence of *Salmonella* (Johnson *et al.*, 1991) and appears to play a role in the virulence of *Brucella abortus* (Elzer *et al.*, 1996) and *Yersinia enterolitica* (Li *et al.*, 1996). In *P. aeruginosa*, this factor has two homologues, *algW* and *mucD* (Boucher *et al.*,

1996). The *mucD* gene is believed to be under the control of AlgU. These relationships and the increased sensitivity to heat killing of *algU* mutants (Yu *et al.*, 1995) have provided the basis for suggestions that the control of mucoidy and the heat-shock response may be intimately associated in *P. aeruginosa*. Such views are now corroborated in this study and extended to include the major heat-shock sigma-factor gene *rpoH*. Several general properties of the heat-shock response in *P. aeruginosa* have been investigated (Allan *et al.*, 1988). It has been reported that 17 proteins with apparent molecular masses ranging from 15.7–103.4 kDa are induced in response to temperature shifts from 37°C to 42°C. It has also been shown that two of these proteins (61 kDa and 76 kDa) are immunologically similar to *E. coli* GroEL and DnaK, respectively (Allan *et al.*, 1988). These studies have also confirmed that GroEL is one of the predominant proteins responding to heat shock in *P. aeruginosa*. GroEL (Hsp 60) is a highly immunodominant molecule and is frequently recognized by antibodies in bacterial infections (Cerrone *et al.*, 1991; van Eden *et al.*, 1988). It is worth mentioning that a vigorous immune response to GroEL has been noted as a result of bacterial infections in CF (Jensen *et al.*, 1993).

There are several lines of evidence indicating that an exuberant but apparently ineffective activation of the host immunological response contributes to the extensive tissue damage observed in chronic infections by *P. aeruginosa* in CF lungs (Boat *et al.*, 1989). Furthermore, autoimmune pathology and rheumatoid abnormalities such as episodic or chronic erosive arthropathy, hypertrophic pulmonary osteoarthropathy, rheumatoid arthritis and diabetes mellitus have been associated with the chronic disease in CF (Coffey *et al.*, 1989). While these processes are not understood at present, questions associated with these phenomena may be central to the issues of inflammation and associated morbidity and mortality in CF. It has been proposed that the highly conserved stress antigens of *P. aeruginosa* such as heat-shock proteins may play an important role in the immunopathology seen in CF (Jensen *et al.*, 1995; Jensen *et al.*, 1993). Thus it is possible that the putative induction of the heat-shock response, as a collateral effect of conversion to mucoidy (Fig. 6B) or as a part of selection for strains with upregulated stress systems during chronic colonization of the lung, may contribute to such processes in CF. Further *in vitro* and *in vivo* investigations of the *rpoH*-dependent regulon, which with a few exceptions (Fujita *et al.*, 1993; Jensen *et al.*, 1995) remains to be characterized in *P. aeruginosa*, and antigens whose production may be induced in *mucA* mutants may reveal important targets or modulators of the immune response in the context of the hypersensitivity reactions and inflammation leading to various manifestations of immune pathology in CF.

Experimental procedures

Bacterial strains, plasmids and growth conditions

PAO1 is the standard genetic *P. aeruginosa* strain (Holloway, 1955). PAO6852 is an *algU*::Tc^R derivative of PAO1 described previously (Martin *et al.*, 1994). PAO6857 is a *mucB*::Tc^R derivative of PAO1 described previously (Schurr *et al.*, 1996). CF23 and CF1 are *mucA* CF patient isolates that have been previously described (Martin *et al.*, 1993c). p*tac-mucA*$^+$ is a pVDtac24 derivative carrying *mucA* under the control of the *tac* promoter as previously described (Martin *et al.*, 1993c). pRK2013 is the plasmid that contains the *tra* functions used in triparental conjugations (Figurski and Helinski, 1979). pETU-1610 is the AlgU expression clone that produced the purified AlgU as previously described (Schurr *et al.*, 1995b). *P. aeruginosa* was grown in *Pseudomonas* isolation agar (PIA) supplemented with 300 µg of carbenicillin or 300 µg of tetracycline when required. *E. coli* was grown in Luria–Bertani (LB) medium supplemented with 25 µg ml^{-1} kanamycin, 30 µg ml^{-1} chloramphenicol and 40 µg ml^{-1} ampicillin as needed. Polymerase chain reaction (PCR) fragments were subcloned into the Invitrogen pCRII vector when cloned. *E. coli* BL21(DE3) pLysS (Studier and Moffat, 1986) was used for the overproduction and purification of AlgU.

RNA isolation and S1 nuclease protection assay

RNA was isolated as previously described (Schurr *et al.*, 1995a). Uniformly labelled single-stranded probes were generated from M13 derivatives carrying the appropriate promoter region (*algU* or *rpoH*). The *algU* probe has been described previously (Martin *et al.*, 1994; Schurr *et al.*, 1995a). The *P. aeruginosa rpoH* gene was cloned as a 967 bp PCR fragment using oligonucleotides HF-1 (5′-AGGCGGATCAC-GACCG-3′, −109 to −94 relative to the start codon) and HR-1 (5′-CGATCAGCCGAGAATC-3′, +941 to +958 relative to the ATG) and cloned into pCRII and termed pCR*rpoH*. The *rpoH* promoter region was subcloned from pCR*rpoH* into M13mp18 as an *Xmn*I/*Eco*RI 385 bp fragment. The oligonucleotide HR-2 (5′-CGAGTGCACGTAGGCTTCCA-3′, +50 to +69 relative to the start codon) was used to generate a 188-nucleotide uniformly labelled α-^{32}P probe as previously described (Schurr *et al.*, 1995a). This probe contained 10 bp from the pCRII vector. The probe was hybridized to 50 µg of RNA and treated with S1 nuclease as previously described (Martin *et al.*, 1994).

In vitro *transcriptional run-off assay*

The *in vitro* run-off assay using AlgU with the P$_1$ promoter of *algU* has been described previously (Schurr *et al.*, 1995b). His$_{10}$-AlgU was renatured by dilution (1:10) into renaturation buffer (total volume 30 µl) consisting of 100 mM Tris-HCl (pH 7.5), 33% glycerol, 10 mM KCl, 10 mM MgCl$_2$, 0.3 mM ATP, 0.33 mg ml^{-1} GroEL and 0.2 mg ml^{-1} GroES (Epicentre Technologies) and incubated for 20 min at 25°C. A standard sigma-factor/core RNA polymerase preincubation reaction (6 µl) consisted of 3 µl with 0.6 pmol core RNA polymerase (Epicentre Technologies) freshly diluted in 10 mM Tris-HCl (pH 8.0), 10 mM KCl, 10 mM β-mercaptoethanol, 1 mM EDTA,

0.4 mg ml^{-1} bovine serum albumin, 0.1% Triton X-100 and 0.72 pmol His$_{10}$-AlgU in 3 µl of renaturation buffer. After 10 min on ice, DNA template (0.1 pmol) was added to the RNA polymerase/sigma-factor mixture and incubated in transcription buffer (40 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl$_2$, 1 mM DTT) for 10 min at 37°C. Transcription was carried out in a final volume of 20 µl and was initiated by addition of ATP, CTP and GTP (200 µM each), 20 µM [α-^{32}P]-UTP (8 Ci mmol^{-1}) to the DNA template/core RNA polymerase/sigma-factor mix and samples incubated for 15 min at 37°C. The reaction was stopped and products separated on a sequencing gel. The *rpoH* P$_{1-4}$ 47 bp DNA template used in the *in vitro* run-off assay was generated by PCR using the oligonucleotides HF-1 and HR-3 (5′-GGAAC-CAAGGCATGTACAGG-3′, +19 to +38 relative to the *rpoH* start codon).

AlgU purification

AlgU was purified from *E. coli* BL21(DE3) pLysS (Studier and Moffat, 1986) using the expression vector pETU-1610 as previously described (Schurr *et al.*, 1995b). *E. coli* BL21(DE3) pLysS cells containing pETU-1610 were grown in LB supplemented with 30 µg of chloramphenicol and 40 µg of ampicillin to an OD$_{590}$ of 0.4. AlgU production was induced by the addition of 1 mM IPTG and incubated for 1 h. The cells were collected and lysed in metal chelate affinity chromatography buffer containing 6 M urea and 80 mM imidiazole. The cell-free extract was chromatographed on an Ni-NTA agarose column washed with 100 mM imidiazole. AlgU was eluted from the column by the addition of 200 mM imidiazole. Fractions containing AlgU were pooled and AlgU was renatured prior to use as described above.

References

Allan, B., Linseman, M., MacDonald, L.A., Lam, J.S., and Kropinski, A.M. (1988) Heat shock of *Pseudomonas aeruginosa*. *J Bacteriol* **170**: 3668–3674.

Benvenisti, L., Koby, S., Rutman, A., Giladi, H., Yura, T., and Oppenheim, A.B. (1995) Cloning and primary sequence of the *rpoH* gene from *Pseudomonas aeruginosa*. *Gene* **155**: 73–76.

Boat, T.F., Welsh, M.J., and Beaudet, A.L. (1989) Cystic fibrosis. In *The Metabolic Basis of Inherited Disease.* Scriver, C.R., Beaudet, A.L., Sly, W.S., and Valle, D. (eds). New York: McGraw-Hill, pp. 2649–2680.

Boucher, J.C., Martinez-Salazar, J.M., Schurr, M.J., Mudd, M.H., Yu, H., and Deretic, V. (1996) Two distinct loci affecting conversion to mucoidy in *Pseudomonas aeruginosa* in cystic fibrosis encode homologues of the serine protease HtrA. *J Bacteriol* **178**: 511–523.

Cameron, R.M., Stevenson, K., Inglis, N.F., Klausen, J., and Sharp, J.M. (1994) Identification and characterization of a

putative serine protease expressed *in vivo* by *Mycobacterium avium* subsp. paratuberculosis. *Microbiology* **140:** 1977–1994.

Cerrone, M.C., Ma, J.J., and Stephens, R.S. (1991) Cloning and sequence of the gene for heat shock protein 60 from *Chlamydia trachomatis* and immunological reactivity of the protein. *Infect Immun* **59:** 79–90.

Coffey, M., Hassan, J., Feighery, C., Fitzgerald, M., and Bresnihan, B. (1989) Rheumatoid factors in cystic fibrosis: associations with disease manifestations and recurrent bacterial infections. *Clin Exp Immunol* **77:** 52–57.

Deretic, V., Gill, J.F., and Chakrabarty, A.M. (1987) Gene *algD* coding for GDPmannose dehydrogenase is transcriptionally activated in mucoid *Pseudomonas aeruginosa*. *J Bacteriol* **169:** 351–358.

Deretic, V., Schurr, M.J., Boucher, J.C., and Martin, D.W. (1994) Conversion of *Pseudomonas aeruginosa* to mucoidy in cystic fibrosis: environmental stress and regulation of bacterial virulence by alternative sigma factors. *J Bacteriol* **176:** 2773–2780.

DeVries, C.A., and Ohman, D.E. (1994) Mucoid-to-nonmucoid conversion in alginate-producing *Pseudomonas aeruginosa* often results from spontaneous mutations in *algT*, encoding a putative alternate sigma factor, and shows evidence for autoregulation. *J Bacteriol* **176:** 6677–6687.

van Eden, W., Thole, J.E.R., van der Zee, R., Nordzij, A., van der Embden, J.D.A., Hensen, E.J., and Cohen, I.R. (1988) Cloning of the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis. *Nature* **331:** 171–173.

Elzer, P.H., Phillips, R.W., Kovach, M.E., Peterson, K.M., and Roop, R.M.I. (1994) Characterization and genetic complementation of a *Brucella abortus* high-temperature-requirement A (*htrA*) deletion mutant. *Infect Immun* **62:** 4135–4139.

Elzer, P.H., Phillips, R.W., Robertson, G.T., and Roop, R.M.I. (1996) The HtrA stress response protease contributes to resistance of *Brucella abortus* to killing by murine phagocytes. *Infect Immun* **64:** 4838–4841.

Erickson, J.W., Vaughn, V., Walter, W.A., Neidhart, F.C., and Gross, C.A. (1987) Regulation of the promoters and transcripts of *rpoH*, the *Escherichia coli* heat shock regulatory gene. *Genes Devel* **1:** 419–432.

Erickson, J.W., and Gross, C.A. (1989) Identification of the σ^E subunit of *Escherichia coli* RNA polymerase: a second alternate σ factor involved in high-temperature gene expression. *Genes Devel* **3:** 1462–1471.

Figurski, D.H., and Helinski, D.R. (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. *Proc Natl Acad Sci USA* **76:** 1648–1658.

Fujita, N., Nomura, T., and Ishihama, A. (1987) Promoter selectivity of *Escherichia coli* RNA polymerase: purification and properties of holoenzyme containing the heat-shock σ subunit. *J Biol Chem* **262:** 1855–1859.

Fujita, M., Tanaka, K., Takahashi, H., and Amemura, A. (1993) Organization and transcription of the principle σ gene (*rpoDA*) of *Pseudomonas aeruginosa* PAO1: involvement of a σ^{32}-like RNA polymerase in *rpoDA* core expression. *J Bacteriol* **175:** 1069–1074.

Fyfe, J.A.M., and Govan, J.R.W. (1980) Alginate synthesis in mucoid *Pseudomonas aeruginosa*: a chromosomal locus involved in control. *J Gen Microbiol* **119:** 443–450.

Goldberg, J.B., Gorman, W.L., Flynn, J., and Ohman, D.E. (1993) A mutation in *algN* permits *trans* activation of alginate production by *algT* in *Pseudomonas* species. *J Bacteriol* **175:** 1303–1308.

Govan, J.R.W., and Deretic, V. (1996) Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev* **60:** 539–574.

Grossman, A.D., Erickson, J.W., and Gross, C.A. (1984) The *htpR* gene product of *E. coli* is a sigma factor for heat-shock promoters. *Cell* **38:** 383–390.

Hershberger, C.D., Ye, R.W., Parsek, M.R., Xie, Z.-D., and Chakrabarty, A.M. (1995) The *algT* (*algU*) gene of *Pseudomonas aeruginosa*, a key regulator involved in alginate biosynthesis, encodes an alternative σ factor (σ^E). *Proc Natl Acad Sci USA* **92:** 7941–7945.

Hiratsu, K., Amemura, M., Nashimoto, H., Shinagawa, H., and Makino, K. (1995) The *rpoE* gene of *Escherichia coli*, which encodes σ^E, is essential for bacterial growth at high temperature. *J Bacteriol* **177:** 2918–2922.

Holloway, B.W. (1955) Genetic recombination in *Pseudomonas aeruginosa*. *J Gen Microbiol* **13:** 572–581.

Jensen, P., Formsgaard, A., Shand, G., Hindersson, P., and Høiby, N. (1993) Antigenic analysis of *Pseudomonas aeruginosa* and *Pseudomonas cepacia* GroEL proteins and demonstration of a lipopolysaccharide-associated GroEL fraction in *P. aeruginosa*. *APMIS* **101:** 621–630.

Jensen, P., Fomsgaard, A., Høiby, N., and Hindersson, P. (1995) Cloning and nucleotide sequence of the *groE* operon of *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *APMIS* **103:** 113–123.

Johnson, K., Charles, I., Dougan, G., Pickard, D., O'Gaora, P., Ali, T., Miller, I., and Hormaeche, C. (1991) The role of stress-response protein in *Salmonella typhimurium* virulence. *Mol Microbiol* **5:** 401–407.

Lamb, J.R., Mendez-Samperio, P., Mehlert, A., So, A., Rothbard, J., Jindal, J., Young, R.A., and Young, D.B. (1989) Stress proteins may provide a link between the immune response to infection and autoimmunity. *Int Immunol* **1:** 191–196.

Li, S., Dorrell, N., Everest, P.H., Dougan, G., and Wren, B.W. (1996) Construction and characterization of a *Yersinia enterolitica* O:8 high-temperature requirement (*htrA*) isogenic mutant. *Infect Immun* **64:** 2088–2094.

Lipinska, B., Zylicz, M., and Georgopoulos, C. (1990) The HtrA (DegP) protein, essential for *Escherichia coli* survival at high temperature, is an endopeptidase. *J Bacteriol* **172:** 1791–1797.

Lonetto, M.A., Brown, K.L., Rudd, K.E., and Buttner, M.J. (1994) Analysis of the *Streptomyces coelicolor sigE* gene reveals the existence of a subfamily of eubacterial RNA polymerase σ factors involved in the regulation of extracytoplasmic functions. *Proc Natl Acad Sci USA* **86:** 7573–7577.

Martin, D.W., Holloway, B.W., and Deretic, V. (1993a) Characterization of a locus determining the mucoid status of *Pseudomonas aeruginosa*: AlgU shows sequence similarities with a *Bacillus* sigma factor. *J Bacteriol* **175:** 1153–1164.

Martin, D.W., Schurr, M.J., Mudd, M.H., and Deretic, V.

(1993b) Differentiation of *Pseudomonas aeruginosa* into the alginate-producing form: inactivation of *mucB* causes conversion to mucoidy. *Mol Microbiol* **9:** 497–506.

Martin, D.W., Schurr, M.J., Mudd, M.H., Govan, J.R.W., Holloway, B.W., and Deretic, V. (1993c) Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. *Proc Natl Acad Sci USA* **90:** 8377–8381.

Martin, D.W., Schurr, M.J., Yu, H., and Deretic, V. (1994) Analysis of promoters controlled by the putative sigma factor AlgU regulating conversion to mucoidy in *Pseudomonas aeruginosa*: relationship to stress response. *J Bacteriol* **176:** 6688–6696.

Naczynski, Z.M., Meuller, C., and Kropinski, A.M. (1995) Cloning the gene for the heat shock response positive regulator (sigma 32 homolog) from *Pseudomonas aeruginosa*. *Can J Microbiol* **41:** 75–87.

Nagai, H., Yano, R., Erickson, J.W., and Yura, T. (1990) Transcriptional regulation of the heat shock regulatory gene *rpoH* in *Escherichia coli*: involvement of a novel catabolite-sensitive promoter. *J Bacteriol* **172:** 2710–2715.

Oldstone, M.B. (1987) Molecular mimicry and autoimmune disease. *Cell* **50:** 819–820.

Raina, S., Missiakas, D., and Georgopoulos, C. (1995) The *rpoE* gene encoding the σ^E (σ^{24}) heat shock sigma factor of *Escherichia coli*. *EMBO J* **14:** 1043–1055.

Roop, R.M.I., Fletcher, T.W., Sriranganathan, N.M., Boyle, S.M., and Schuring, G.G. (1994) Identification of an immunoreactive *Brucella abortus* HtrA stress response protein homolog. *Infect Immun* **62:** 1000–1007.

Rouvière, P.E., De Las Penas, A., Mecsas, J., Lu, C.Z., Rudd, K.E., and Gross, C.A. (1995) *rpoE*, the gene encoding the second heat-shock sigma factor, σ^E, in *Escherichia coli*. *EMBO J* **14:** 1032–1042.

Roychoudhury, S., May, T.B., Gill, J.F., Singh, S.K., Feingold, D.S., and Chakrabarty, A.M. (1989) Purification and characterization of guanosine diphospho-D-mannose dehydrogenase. A key enzyme in the biosynthesis of alginate by *Pseudomonas aeruginosa*. *J Biol Chem* **264:** 9380–9385.

Schurr, M.J., Martin, D.W., Mudd, M.H., Hibler, N.S., Boucher, J.C., and Deretic, V. (1993) The *algD* promoter: regulation of alginate production by *Pseudomonas aeruginosa* in cystic fibrosis. *Cell Mol Biol Res* **39:** 371–376.

Schurr, M.J., Martin, D.W., Mudd, M.H., and Deretic, V. (1994) Gene cluster controlling conversion to alginate-overproducing phenotype in *Pseudomonas aeruginosa*: functional analysis in a heterologous host and role in the instability of mucoidy. *J Bacteriol* **176:** 3375–3382.

Schurr, M.J., Yu, H., Boucher, J.C., Hibler, N.S., and Deretic, V. (1995a) Multiple promoters and induction by heat shock of the gene encoding the alternative sigma factor AlgU (σ^E) which controls mucoidy in cystic fibrosis isolates of *Pseudomonas aeruginosa*. *J Bacteriol* **177:** 5670–5679.

Schurr, M.J., Yu, H., Martinez-Salazar, J.M., Hibler, N.S., and Deretic, V. (1995b) Biochemical characterization and posttranslational modification of AlgU, a regulator of stress response in *Pseudomonas aeruginosa*. *Biochem Biophys Res Commun* **216:** 874–880.

Schurr, M.J., Yu, H., Martinez-Salazar, J.M., Boucher, J.C., and Deretic, V. (1996) Control of AlgU, member of the σ^E-like family of stress sigma factors, by the negative regulators MucA and MucB and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. *J Bacteriol* **178:** 4997–5004.

Strauch, K., and Beckwith, J. (1988) An *Escherichia coli* mutation preventing degradation of abnormal periplasmic proteins. *Proc Natl Acad Sci* **85:** 1576–1580.

Studier, F.W., and Moffat, B.A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol* **189:** 113–130.

Tatum, F.M., Cheville, N.F., and Morfitt, D. (1994) Cloning, characterization and construction of *htrA* and *htrA*-like mutants of *Brucella abortus* and their survival on BALB/c mice. *Microb Pathogen* **17:** 23–26.

Wang, Q., and Kaguni, J.M. (1989) A novel sigma factor is involved in expression of the *rpoH* gene of *Escherichia coli*. *J Bacteriol* **171:** 4248–4253.

Wozniak, D.J., and Ohman, D.E. (1994) Transcriptional analysis of the *Pseudomonas aeruginosa* genes *algR*, *algB*, and *algD* reveals a hierarchy of alginate gene expression which is modulated by *algT*. *J Bacteriol* **176:** 6007–6014.

Xie, Z.D., Hershberger, C.D., Shankar, S., Ye, R.W., and Chakrabarty, A.M. (1996) Sigma factor–anti-sigma factor interaction in alginate: inhibition of AlgT by MucA. *J Bacteriol* **178:** 4990–4996.

Young, R.A. (1990) Stress proteins and immunology. *Annu Rev Immun* **8:** 401.

Yu, H., Schurr, M.J., and Deretic, V. (1995) Functional equivalence of *Escherichia coli* σ^E and *Pseudomonas aeruginosa* AlgU: *E. coli rpoE* restores mucoidy and reduces sensitivity to reactive oxygen intermediates in *algU* mutants of *P. aeruginosa*. *J Bacteriol* **177:** 3259–3268.

Yura, T., Nagai, H., and Mori, H. (1993) Regulation of the Heat-shock response in bacteria. *Annu Rev Microbiol* **47:** 321–350.