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High Osmolarity Is a Signal for Enhanced *algD* Transcription in Mucoid and Nonmucoid *Pseudomonas aeruginosa* Strains

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Chronic lung infection with mucoid, alginate-producing strains of *Pseudomonas aeruginosa* is a major cause of mortality in cystic fibrosis (CF) patients. Transcriptional activation of the *P. aeruginosa algD* gene, which encodes GDPmannose dehydrogenase, is essential for alginate synthesis. Activation of *algD* is dependent on the product of the *algR* gene. Sequence homology between the *P. aeruginosa algR* gene and the *Escherichia coli ompR* gene, which regulates the cellular response to changes in osmolarity of the growth medium, together with the abnormally high levels of Na^+ and Cl^- in respiratory tract fluid in CF patients suggested that high osmolarity in the lung of the CF patient might be a signal contributing to the induction of alginate synthesis (mucoidy) in infecting *P. aeruginosa*. In both mucoid and nonmucoid *P. aeruginosa* strains (containing a functional *algR* gene), transcriptional activation of *algD* increased as the osmolarity of the culture medium increased. The increased activation of *algD* at high osmolarity was not in itself sufficient to induce alginate synthesis in nonmucoid strains, however, suggesting that other environmental factors are involved in full activation of the alginate genes. The targets of AlgR and OmpR, the *algD* promoter and the *ompC* and *ompF* promoters, respectively, were found to have appreciable sequence homology in the −60 to −110 regions. In *E. coli*, OmpR was capable of activating the *algD* promoter nearly as well as AlgR, but in both cases, activation occurred only under conditions of high osmolarity.

Cystic fibrosis (CF) is an autosomal recessive disease that is characterized by disturbances in electrolyte transport and mucus secretion from exocrine glands and secretory epithelia (27, 28). The abnormalities in exocrine secretions of CF patients include altered electrolyte levels (increased Na^+, Cl^-, and Ca^{2+}) and atypical glycoproteins (25, 40). The hyperviscous nature of secretions of CF patients leads to severe duct obstruction in several organs, including the pancreas, gastrointestinal tract, salivary glands, vas deferens, and lungs (40).

The accumulation of abnormal fluids in the respiratory tract of CF patients appears to predispose the CF patient to bacterial infections. Chronic lung infection with *Pseudomonas aeruginosa* is a major cause of mortality in CF patients. Initially, the *P. aeruginosa* strains recovered from the respiratory tract of CF patients exhibit a typical nonmucoid phenotype, but with prolonged infection, *P. aeruginosa* shifts to a mucoid form that produces large amounts of a slimy exopolysaccharide known as alginate (13, 16). The presence of this extracellular mucus compounds problems associated with the already viscous lung environment of CF patients. Since the first report of the isolation of mucoid *P. aeruginosa* from CF patients (22), it has become increasingly evident that such strains are associated primarily with CF. While the incidence of infection with mucoid *P. aeruginosa* in CF patients reaches as high as 90%, the recovery of mucoid *P. aeruginosa* from patients with other chronic illnesses (but clinically harboring *P. aeruginosa*) is rare (12, 15, 20), exceptions being *P. aeruginosa* infection in chronic obstructive lung disease (approximately 40% incidence of mucoid strains) and chronic *P. aeruginosa* urinary tract infections (up to 10% incidence of mucoid strains) (15). So specific is the relationship between mucoid *P. aeruginosa* and CF that the recovery of mucoid *P. aeruginosa* from a patient with chronic or intermittent lung infection is considered almost diagnostic of CF (38). The pathogenesis of mucoid *P. aeruginosa* in CF was recently reviewed by Govan (15).

What are the signals in the lungs of CF patients that trigger alginate synthesis by *P. aeruginosa* in this unique environment? Our objective has been to develop nontoxic inhibitors that specifically target the alginate biosynthetic pathway in *P. aeruginosa* as a first step toward eradicating *P. aeruginosa* from the lungs of CF patients. While the alginate biosynthetic enzymes have been our primary focus (14, 39), proteins involved in the regulation of alginate gene expression are equally attractive as targets for inhibition. We demonstrated previously that a number of alginate (*alg*) biosynthetic genes are clustered in the 34-min region of the chromosome of *P. aeruginosa* 8821, a mucoid CF isolate, while an alginate regulatory gene (*algR*) is located at about 10 min (Fig. 1B) (4, 6). (The *P. aeruginosa* PAO chromosome was recently recalibrated [36]. Thus, the map locations of alginate genes reported here, 34 and 10 min, correspond to the previously reported map positions of 45 and 19 min, respectively [4, 6].) The *alg* gene cluster contains a gene, *algD*, that encodes GDPmannose dehydrogenase (Fig. 1A) (9). *algD* is believe to be a primary controlling step in alginate synthesis, since transcriptional activation of *algD* is required for alginate production (9). Both the *algD* gene and its promoter region have been sequenced (10). The promoter that controls *algD* transcription has a novel secondary structure, possessing multiple direct and inverted repeats throughout the −50 to −110 region, as well as in the −35 and −10 regions (10). The *algD* promoter was shown to be under positive control by the *algR* gene product (8, 10). Understanding the regulation of the alginate genes in *P. aeruginosa* is fundamental to achieving our goal of developing inhibitors of this process. In this report, we provide evidence that high osmolarity in the lungs of CF patients is one of the signals that contribute to increased transcription of the *algD* gene in infecting *P. aeruginosa*.

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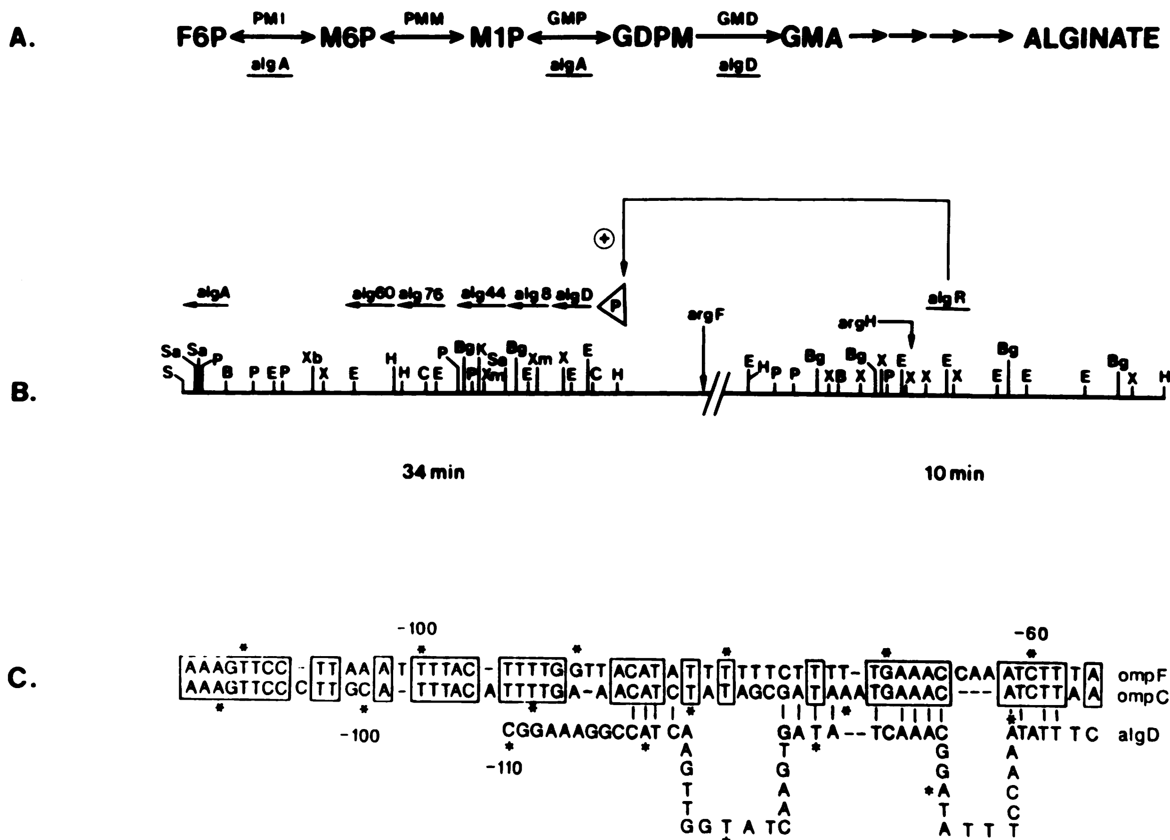

FIG. 1. (A) Pathway of alginate synthesis in *P. aeruginosa*. Abbreviations: F6P, fructose-6-phosphate; M6P, mannose-6-phosphate; M1P, mannose-1-phosphate; GDPM, GDPmannose; GMA, GDPmannuronic acid; PMI, phosphomannose isomerase; PMM, phosphomannomutase; GMP, GDPmannose pyrophosphorylase; GMD, GDPmannose dehydrogenase. The remaining steps of alginate synthesis are polymerization, epimerization, acetylation, and export. The *algA* gene encodes a bifunctional phosphomannose isomerase-GDPmannose pyrophosphorylase enzyme, whereas *algD* encodes GDPmannose dehydrogenase (9, 39). (B) Restriction map showing the alginate gene cluster (at 34 min on the *P. aeruginosa* chromosome) and the *algR* gene (at 10 min). The *algR* gene product acts as a positive regulator of the *algD* promoter (10). The direction of transcription of the alginate genes was established by Wang et al. (43). Abbreviations: S, *Sma*I; Sa, *Sal*I; P, *Pst*I; B, *Bam*HI; E, *Eco*RI; Xb, *Xba*I; X, *Xho*I; H, *Hin*dIII; C, *Cla*I; Bg, *Bgl*II; K, *Kpn*I; Xm, *Xma*I. (C) Nucleotide sequence homology in the −60 to −110 region of the *ompF*, *ompC*, and *algD* genes (10, 30, 31).

MATERIALS AND METHODS

Bacterial strains and plasmid constructions. The bacterial strains and plasmids used are given in Table 1. Plasmid pBR325K1 (Km^r Cm^r Tc^r) was constructed by cloning a kanamycin resistance cassette (as a *Pst*I fragment from pMB2190 [5]) into the unique *Pst*I site of pBR325, thus replacing Ap^r with Km^r. The same method was used to construct plasmid pEW007K (Km^r), in which the Ap^r of pEW007 (32) was replaced by Km^r. This substitution has no effect on the cloned *ompR* gene in pEW007 and was necessary in order to select strains containing both pEW007K and the *ompC* promoter-*lacZ* or the *ompF* promoter-*lacZ* fusion plasmid (pOYL338W and pOY012, respectively, both of which confer Ap^r [37]). Plasmid pDD574 contains the *P. aeruginosa* 8821 *algR* gene cloned on a 6.2-kilobase (kb) *Bgl*II fragment in the pBR322-derived cosmid vector pHC79 (19). Plasmids pAB12A and pAB12B (Km^r Cm^r) also contain the *P. aeruginosa* 8821 *algR* gene, but on a 1-kb *Bam*HI fragment cloned in both orientations into the unique *Bam*HI site of pBR325K1. This provided a selectable marker (Km^r or Cm^r) for strains containing pAB12A or pAB12B in addition to pOY012 of pOYL338W (Ap^r).

Media and growth conditions. *P. aeruginosa* and *Escherichia coli* strains used for determination of *algD* activation were grown at 37°C in YTG medium (5 g of yeast extract, 10 g of tryptone, 2 g of glucose per liter) containing various concentrations of NaCl as specified. Cultures (100 ml for *P. aeruginosa* and 1 liter for *E. coli*) were inoculated with 0.01 volume of overnight starter cultures grown in YTG lacking NaCl and grown for 16 h with shaking at 240 rpm. Cells were then harvested by centrifugation, washed with 0.9% saline, recentrifuged, and stored as frozen pellets at −70°C. *E. coli* strains used for determination of *ompC* or *ompF* activation were grown in M9 medium (26) containing 0.2% glycerol as the carbon source and 50 μg of L-arginine per ml. Antibiotics were used at a final concentration of 50 μg/ml with the exception of tetracycline, which was used at 30 μg/ml.

Genetic procedures. Transformations, plasmid isolation, cloning, and other DNA manipulations were carried out by standard methods (26). Transfer of plasmids from *E. coli* to *P. aeruginosa* was performed by triparental filter matings (11).

Extract preparation and enzyme assays. Crude extracts of *P. aeruginosa* or *E. coli* to be used for catechol 2,3-dioxygenase (C23O) assays were prepared by thawing cell pellets in 5 ml of 50 mM potassium phosphate buffer (pH 7.5) and sonicating the suspension three times for 30 s at 100 W each time. The suspensions were then centrifuged at 40,000

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties[a]	Reference or source
E. coli		
JM83	*ara* Δ(*lac-pro*) *rpsL* (Str^r) *thi* φ80 d*lacZ* Δ*M15*	42
MC4100 *recA*	Nal^r *recA* F$^-$ *araD139* Δ(*argF-lac*) *U169 rpsL150 relA1 fibB25 ptsF25 deo-1*	3
MH1160 *recA*	MC4100 *recA ompB101 ompR1*	17
P. aeruginosa		
PAO1	Prototroph, *chl-2*	21
8821	*his-1* Alg$^+$, mucoid CF isolate (unstable)	4
8822	*his-1 alg-1*, spontaneous nonmucoid revertant derived from 8821	4
8830	*his-1* Alg$^+$, stable mucoid derivative of 8822	4
Plasmids		
pVD2X	Tc^r, *algD-xylE* transcriptional fusion	9
pBR325K1	Km^r Cm^r Tc^r derivative of pBR325	This study
pEW007	Ap^r, 3.2-kb *Eco*RI-*Bam*HI fragment containing *ompR* cloned in pBR322	32
pEW007K	Km^r derivative of pEW007	This study
pOY012	Ap^r, *ompF* promoter-*lacZ* fusion (pSC101 replicon)	37
pOYL338W	Ap^r, *ompC* promoter-*lacZ* fusion (pSC101 replicon)	37
pDD574	6.2-kb *Bgl*II fragment containing *algR* cloned in pHC79	R. Dikshit
pAB12A	1-kb *Bam*HI fragment containing *algR* cloned in pBR325K1	This study
pAB12B	pAB12A, reverse orientation of *algR*	This study

[a] Ap, Ampicillin; Km, kanamycin; Cm, chloramphenicol; Tc, tetracycline.

× *g* for 30 min (4°C). The supernatant was then either used directly for C230 assays (*E. coli*) or subjected to acetone fractionation (*P. aeruginosa*). Acetone fractionation was performed as described by Nozaki (35), but with omission of the DNase and dialysis steps. C230 was assayed as previously described (35). Protein concentrations were determined by the method of Bradford (1). β-Galactosidase activity in *E. coli* was assayed by the procedure of Miller (29).

RESULTS AND DISCUSSION

Recent cloning and nucleotide sequencing of the *algR* gene revealed homology with a number of regulatory genes that are known to respond to environmental stimuli, including *phoB*, *ntrcC*, *spo0A*, and interestingly, *ompR* (7). In *E. coli* K-12, the product of the *ompR* gene activates the promoters for two outer membrane protein genes, *ompC* and *ompF* (34). Expression of the *ompC* and *ompF* genes, whose products control the passive diffusion of small hydrophilic molecules across the cell membrane, is affected in a reciprocal manner by osmolarity (e.g., NaCl concentration) of the culture medium (24). As osmolarity increases, the *ompC* gene is preferentially expressed while *ompF* expression decreases. The expression of *ompC* and *ompF* is also linked to another gene, *envZ*, whose product is believed to sense osmolarity, relaying the information to OmpR, which in turn activates the appropriate (*ompC* or *ompF*) promoter (41).

The sequence homology between the *algR* gene (which controls positively the expression of the *algD* gene [8, 10]) and the *ompR* gene (7), together with the fact that the respiratory tract fluid of CF patients contains high levels of Na^+ and Cl^- ions (90 and 80 mM, respectively) (25, 40), suggested that alginate gene expression may be linked to osmolarity. We tested this hypothesis in both mucoid and nonmucoid *P. aeruginosa* strains containing plasmid pVD2X, an *algD-xylE* transcriptional fusion plasmid constructed in such a way that the level of *algD* gene expression can be conveniently measured by assaying for activity of the *xylE* gene product, C230 (9, 10). In both mucoid (8821) and nonmucoid (PAO1) *P. aeruginosa* strains, the level of *algD* expression increased as the medium osmolarity (i.e., NaCl concentration) increased (Table 2). Maximum activation was achieved at 0.35 M NaCl; higher concentrations of NaCl resulted in decreased activation. It should be noted that increased levels of C230 resulting from growth in a highly osmotic environment is not a general phenomenon, since the levels of enzymes such as glucose-6-phosphate dehydrogenase did not change in response to increased medium osmolarity (data not shown). When KCl was substituted for NaCl at concentrations giving equal ionic strength (measured as conductance), results similar to those shown in Table 2 were obtained (J. D. DeVault, A. Berry, T. K. Misra, A. Darzins, and A. M. Chakrabarty, Bio/Technology, in press). Under conditions of high osmolarity, strain PAO1 still failed to produce detectable levels of alginate, suggesting that the NaCl-dependent activation of the *algD* gene was not in itself sufficient to cause alginate synthesis.

The homology between *algR* and *ompR* (7) raised the interesting question of whether AlgR could activate the *ompC* or *ompF* promoters or OmpR could activate the *algD* promoter and whether such activation would be dependent on osmolarity. The *ompC* and *ompF* genes, as well as their promoters, have considerable sequence homology, suggest-

TABLE 2. Activation of the *algD* promoter in *P. aeruginosa* grown under conditions of high osmolarity[a]

Concn of NaCl (M)	C230[b] activity (mU/mg of protein) in:	
	PAO1(pVD2X) (nonmucoid)	8821(pVD2X) (mucoid)
0	1,129	7,654
0.1	1,284	19,547
0.2	1,653	26,364
0.35	3,302	31,255
0.5	1,833	30,002

[a] Cultures were grown in YTG medium (plus tetracycline) as described in Materials and Methods. The growth rates of PAO1 and 8821 in YTG medium were not affected by the addition of NaCl at concentrations of 0 to 0.3 M.

[b] No background C230 activity was detected in controls lacking either substrate or enzyme.

TABLE 3. Activation of the *algD* promoter by AlgR and OmpR in *E. coli* grown under conditions of high osmolarity[a]

Strain(plasmid[s])	Relevant properties	C230 activity[b] (mU/mg of protein) with 0.3 M NaCl
JM83(pVD2X)	*ompR*$^+$ *algD-xylE*	1.650
MH1160(pVD2X)	*ompR algD-xylE*	0.141
MH1160(pVD2X, pEW007)	*ompR algD-xylE ompR*$^+$ (plasmid)	3.100
MH1160(pVD2X, pDD574)	*ompR algD-xylE algR*$^+$	5.155
MH1160(pVD2X, pAB12A)	*ompR algD-xylE algR*$^+$	3.073
MH1160(pVD2X, pAB12B)	*ompR algD-xylE algR*$^+$	0.679

[a] Cultures were grown in YTG medium plus appropriate antibiotics as described in Materials and Methods.

[b] Activities were measured in at least three separate experiments with essentially identical results. The lower limit of detectability of C230 activity was 0.033 mU/mg. Activity measured with no NaCl, with 0.3 M NaCl plus nalidixic acid, and with 0.3 M NaCl plus novobiocin was <0.033. Nalidixic acid and novobiocin were present at final concentrations of 5 μg/ml.

ing a common evolutionary origin (30, 31). OmpR binds to the *ompF* and *ompC* sequences at the −60 to −105 and −78 to −102 regions, respectively (23, 34), while AlgR is believed to bind at the −60 to −110 region of the *algD* promoter (8). We compared these regions of the *ompC* and *ompF* promoters with the *algD* promoter. Figure 1C demonstrates the presence of four discrete regions (−60, −75, −80, and −100) of the *algD* promoter that show sequence homology (13 of 54 base pairs) with the *ompC* and *ompF* promoters. It is noteworthy that these areas of homology are in the regions that are homologous between *ompC* and *ompF* (Fig. 1C).

We tested the ability of the *ompR* gene product to activate the *algD* promoter in *E. coli* under conditions of both low and high osmolarity. Since *E. coli* lacks a functional *algR* gene and since AlgR is required for activation of the *algD* promoter (10), we expected to see no activation of *algD* in *E. coli*. To our surprise, when plasmid pVD2X (the *algD-xylE* transcriptional fusion [9, 10]) was introduced into *E. coli* JM83, activation of *algD* (measured as C230 activity) was observed, but only when cells were grown under conditions of high osmolarity (Table 3). Since JM83 is *ompR*$^+$, this suggested that OmpR might be activating *algD*. When pVD2X was introduced into the *ompR* mutant *E. coli* MH1160, very little activation of *algD* occurred even under conditions of high osmolarity. When the cloned *ompR* gene (pEW007) or *algR* gene (pDD574, pAB12A) was introduced into the MH1160(pVD2X) background, *algD* activation at high osmolarity was restored (Table 3). The level of activation of *algD* by OmpR under conditions of high osmolarity was comparable to the level of activation caused by AlgR under similar conditions (Table 3).

Since activation of *algD* is dependent on a functional *algR* (or *ompR*) gene, as well as on high osmolarity, and since there is appreciable sequence homology between *algR* and *ompR* and the *algD*, *ompC*, and *ompF* promoters, it seems very unlikely that the observed activation of *algD* (i.e., C230 activity) could be a result of vector sequences (or sequences created during construction of pVD2X) that have promoter activity. Mutagenesis of plasmid pVD2X will define the nucleotides within the *algD* promoter that are essential for activation of *algD*.

We then tested whether the *algR* gene product could activate the *ompC* or *ompF* promoters. The *algR* gene (cloned on a 1-kb *Bam*HI fragment in the modified pBR325-based vector pBR325K1 [Table 1]) was introduced into the *ompR* mutant *E. coli* MH1160 (17) containing either the *ompC* promoter-*lacZ* or the *ompF* promoter-*lacZ* transcriptional fusion (plasmids pOYL338W and pOY012, respectively) (37). The presence of *algR* had no significant effect on the level of transcription of *ompC* or *ompF* (tested under conditions of low and high osmolarity) in *E. coli* MH1160 (Table 4). The lack of activation of *ompC* and *ompF* is not due to the lack of expression of the *P. aeruginosa algR* gene in *E. coli*, since, as shown above, the presence of *algR* resulted in activation of the *algD* promoter in *E. coli* MH1160. The presence of a functional *ompR* gene (either chromosomal or plasmid borne) caused dramatic activation of both the *ompC* and *ompF* promoters, as was previously reported (37), and the level of activation of *ompC* or *ompF* by OmpR was dependent on osmolarity of the growth medium (Table 4). Although *algR* did not activate *ompC* or *ompF*, we did observe that introduction of *algR* into *E. coli* strains containing the *ompC* promoter-*lacZ* or the *ompF*

TABLE 4. Effect of OmpR and AlgR on the expression of *ompC* and *ompF* in *E. coli* MH1160 (*ompR* mutant) grown in various concentrations of NaCl[a]

Strain(plasmid[s])	Relevant properties	β-Galactosidase activity[b] with NaCl at:		
		0	0.15 M	0.3 M
MH1160	*ompR*	5	0	0
MH1160(pOY012)	*ompR ompF-lacZ*	67	66	38
MH1160(pOY012, pEW007K)	*ompR ompF-lacZ ompR*$^+$ (plasmid)	12,068	9,279	6,200
MH1160(pOY012, pAB12A)	*ompR ompF-lacZ algR*$^+$	81	70	74
MH1160(pOY012, pAB12B)	*ompR ompF-lacZ algR*$^+$	77	41	24
MH1160(pOYL338W)	*ompR ompC-lacZ*	69	107	104
MH1160(pOYL338W, pEW007K)	*ompR ompC-lacZ ompR*$^+$ (plasmid)	1,377	1,628	1,683
MH1160(pOYL338W, pAB12A)	*ompR ompC-lacZ algR*$^+$	78	75	117
MH1160(pOYL338W, pAB12B)	*ompR ompC-lacZ algR*$^+$	152	116	102
MC4100	*ompR*$^+$ (chromosomal)	12	0	0
MC4100(pOY012)	*ompR*$^+$ *ompF-lacZ*	11,219	7,361	4,938
MC4100(pOYL338W)	*ompR*$^+$ *ompC-lacZ*	138	229	247

[a] Cultures were grown in 5 ml of M9 medium (26) containing 0.2% glycerol and 50 μg of L-arginine per ml, plus appropriate antibiotics.

[b] β-Galactosidase was assayed by the method of Miller (29).

promoter-*lacZ* fusion led to the recovery at a low frequency of mucoid colonies. Such mucoid *E. coli* colonies were never observed in the absence of the *algR* gene. We have observed that introduction of *algR* into nonmucoid strains of *P. aeruginosa* also leads to the recovery of mucoid colonies at a low frequency (unpublished observations). The ability of *algR* to induce mucoidy in *E. coli* suggests similarities in the regulation of exopolysaccharide synthesis in *E. coli* and *P. aeruginosa*.

The osmolarity-dependent activation of the *algD* promoter is similar to that of other osmotically regulated genes. In *E. coli* and *Salmonella typhimurium*, expression of the *proU* gene increases more than 100-fold in response to increased medium osmolarity (2). Higgins et al. (18) provided evidence that DNA supercoiling plays a role in osmotic induction of *proU* transcription. Nalidixic acid and novobiocin, which reduce DNA supercoiling by inhibiting DNA gyrase activity, were also found to reduce expression of *proU* at high osmolarity (18). We found that both nalidixic acid and novobiocin abolished activation of the *algD* promoter in *E. coli* grown under conditions of high osmolarity (Table 3). When similar experiments were carried out with a *tac* promoter-*xylE* fusion plasmid, neither novobiocin nor nalidixic acid had any inhibitory effect on transcription (data not shown), indicating that the inhibition of *algD* activation by the gyrase inhibitors is not a general phenomenon. While this does not prove that DNA supercoiling actually occurs at the *algD* promoter itself, at the very least it indicates that in *E. coli* the mechanisms of promoter activation that involve DNA supercoiling are also responsible for activation of the *algD* promoter under conditions of high osmolarity.

The analogy between the *algR-algD* and the *ompR-ompC* and *ompR-ompF* systems, based on sequence homologies and functional interchangeability of AlgR and OmpR, suggests that in *P. aeruginosa* there may be an osmolarity-sensing gene similar to the *E. coli envZ* gene. The product of such a gene might activate *algR* in a manner analogous to *envZ*-mediated activation of OmpR (41). Ninfa et al. (33) recently showed that sensory transduction in the Ntr and Che systems involves a common protein phosphotransfer system based on the cross-specificities of the homologous modulator proteins NR_{II} and CheA; each of these proteins was able to function in heterologous modulator-effector pairs. A family of related modulator-effectors pairs that together coordinate cellular responses to environmental stimuli was proposed (33). Experiments are now in progress to determine if an *envZ*-like gene exists in *P. aeruginosa* and to identify other environmental factors (specifically those features characteristic of the lung environment of the CF patient) that contribute to the activation of the alginate genes in *P. aeruginosa*.

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