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The *algR* Gene, Which Regulates Mucoidy in *Pseudomonas aeruginosa*, Belongs to a Class of Environmentally Responsive Genes

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The *Pseudomonas aeruginosa* capsule, composed of polysaccharide alginate, is an important *Pseudomonas* virulence factor encountered primarily in cystic fibrosis. The regulatory *algR* gene positively controls transcription of a key alginate biosynthetic gene, *algD*. The *algR* gene was subcloned and sequenced by creating a set of nested deletions in M13 bacteriophage. DNA sequence analysis of *algR* revealed the homology of its gene product with a recently recognized class of environmentally responsive bacterial regulatory genes, including *ompR*, *phoB*, *sfrA*, *ntrC*, *spo0A*, *dctD*, and *virG*; these transcriptional activators control cellular reactions to osmotic pressure, phosphate limitations, or specific chemical compounds present in the medium or released from wounded host tissue. These findings indicate that novel conditions in lungs affected by cystic fibrosis may be participating in the control of mucoidy.

The primary reason for high mortality and morbidity in cystic fibrosis patients involves pulmonary infections caused by *Pseudomonas aeruginosa* (17). Initially the invading *P. aeruginosa* is nonmucoid, but during the course of the disease it inevitably changes to the mucoid, alginate-producing phenotype. The mucoid capsule of *P. aeruginosa* is a critical virulence determinant, and its appearance is usually associated with the poor prognosis for the disease (17). Mucoidy in *P. aeruginosa* is almost exclusively associated with cystic fibrosis. However, the underlying reasons for this unusual host-pathogen interaction have eluded satisfactory definition. The control of alginate biosynthesis by *P. aeruginosa* can now be analyzed by genetic means (7, 19). We have recently shown that a pivotal step in alginate biosynthesis is the activation in mucoid cells of the *algD* gene encoding GDPmannose dehydrogenase (9) (see Fig. 1A). This enzyme catalyzes a key step in the alginate pathway whereby double oxidation of GDPmannose into GDPmannuronic acid, a precursor for alginate polymerization, channels the pool of sugar intermediates into alginate production. The *algD* gene appears to be positively regulated by the *algR* gene (see Fig. 1B), since mutations in *algR* abolish transcription of *algD* (10). Here we report the sequence of the *algR* gene, which was determined by using a modification of a subcloning and sequencing strategy (6) that permits the stable cloning of large inserts in M13 vectors. The translated sequence of *algR* was found to be homologous to sequences of a class of environmentally responsive regulatory genes (33). This result implies that mucoidy in *P. aeruginosa* is at least partially under environmental control.

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* strains used were 8821 (*his-1* Alg$^+$), 8822 (*his-1* Alg$^-$), and 8852 (*his-1 algR22*) (7). *Escherichia coli* strains used were JM83 (*dlacZ*ΔM15) (40) and M13-sensitive host WB373 (*tra lac*$^+$ Ap^r) (29).

Media and bacterial growth. LB medium was used for *E. coli* and for growing *P. aeruginosa* in liquid culture. When *E. coli* WB373 was used to propagate M13 mTM010 bacteriophage LB medium was supplemented with ampicillin (40 μg/ml). When appropriate, LB medium was supplemented with kanamycin (50 μg/ml) or tetracycline (25 μg/ml). *P. aeruginosa* exconjugants were selected on *Pseudomonas* isolation agar (Difco Laboratories) supplemented with tetracycline (300 μg/ml). All incubations were at 37°C.

Conjugal plasmid transfer and genetic complementation analysis. Transfer of clones made in a broad-host-range subcloning plasmid pVDZ'2 (8) into *P. aeruginosa* were performed by triparental filter matings as described elsewhere (11); helper strain *E. coli* HB101(pRK2013) was used (13). Genetic complementation was scored by observing the mucoid phenotype and by assaying alginate as described previously (11).

Recombinant DNA techniques. Plasmid DNA isolation, restriction endonuclease digestion, M13 preparation, and other manipulations were done as described previously (8, 10, 11, 28, 29).

A modification of the method described by Dale et al. (6) was used for deletion subcloning in mTM010. For this purpose, an oligonucleotide specific for mTM010 M13 vector (29) (5'-TGAATTAATTCCACAAGCTTTTTTTTTT-3') was synthesized by the phosphoramidite method (2). The deletion procedure was essentially as described before (6), except for the step involving *Hin*dIII digestion of the annealed oligonucleotide-template hybrid, which was carried out overnight at 45°C.

DNA was sequenced by the chain termination method (35), with deoxynucleotide-dideoxynucleotide reaction mixtures adjusted for high G+C DNA as described elsewhere (10). To avoid band compression problems due to the high G+C content, dGTP was substituted for by its analog 7-deaza-dGTP (30). Polymerization reactions were carried out at 42°C. Samples were electrophoresed on 7 M urea–7.5% acrylamide gels in 100 mM Tris–100 mM boric acid–2 mM EDTA (pH 8.3).

Cloning in pVDZ'2 was performed as described previously (8), with *E. coli* JM83 (40) used as a host. The *algR* deletion

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subclones were isolated as *Hin*dIII-*Bgl*II fragments from M13 clones and cloned in the *Hin*dIII and *Bam*HI sites of the pVDZ'2 polylinker inactivating the ability of pVDZ'2 to complement the *dlacZ*ΔM15 allele present in *E. coli* JM83.

RESULTS

Subcloning and sequencing of the regulatory *algR* gene. We have previously shown that the *algD* gene is transcriptionally activated in mucoid *P. aeruginosa* cells (9) and that this activation depends on an unlinked gene, *algR*. This gene has been found to map in a chromosomal region that is capable of inducing mucoidy when chromosomally amplified (Fig. 1B) (11). Furthermore, using an *algD-xylE* transcriptional fusion, we have shown that a mutation in the *algR* gene blocks expression of the *algD-xylE* fusion (10). In the present study, we further subcloned and sequenced the *algR* gene. The *algR* gene was isolated from a genomic library of mucoid *P. aeruginosa* 8821 as a 6.2-kilobase (kb) *Bgl*II DNA fragment complementing the *algR22* mutation. To further delimit the *algR* gene, we used a procedure that produced a series of unidirectional overlapping deletions from both ends of the 6.2-kb *Bgl*II fragment. The intent was to use deletion clones for both subcloning and sequencing of *algR*. A very efficient method has been developed by Dale et al. (6) for introducing deletions in M13 bacteriophage mp-series vectors. However, mp-series vectors do not allow stable maintenance of long DNA fragments, such as the 6.2-kb *Bgl*II fragment containing *algR*. To circumvent this problem, we subcloned the 6.2-kb *Bgl*II fragment into the *Bgl*II site of mTM010 M13 bacteriophage (29). Unlike many other M13 vectors, this derivative allows efficient cloning and stable maintenance of large DNA inserts (29). However, the sequence of the polylinker in mTM010 is different from that for the mp series of M13 vectors. To apply the deletion procedure of Dale et al. (6), we synthesized an oligonucleotide, TGAATTAATTCCAC<u>AAGCTT</u>TTTTTTTTT, partially complementary to the mTM010 sequence 3' to the *Hin*dIII site (underlined). Two unidirectional series of deletions were introduced from both ends of the 6.2-kb *Bgl*II fragment, as described in Materials and Methods. From a single deletion experiment we were able to map the limits of *algR* by subcloning deletion products into the broad-host-range vector pVDZ'2 (8) and complementing the *algR22* mutation (Fig. 1C and Fig. 2). The existence of this collection of overlapping deletions enabled us to precisely determine the 5' and 3' ends of the *algR* gene. The same collection of deletions in M13 was used to determine the *algR* sequence (Fig. 2). The direction of transcription was determined by S1 nuclease protection analysis (data not shown).

***algR* gene sequence analysis: the *algR* gene belongs to a class of positive transcriptional regulators responding to environmental stimuli.** To analyze the characteristics of the *algR* gene, we used its translated sequence to perform a global homology search by using the FASTP computer program (25) and the NBRF protein sequence data base. The only protein sequences that showed similarities to the *algR* gene product sequence were those of the *Klebsiella pneumoniae ntrC* and *E. coli ompR* gene products (Fig. 3). The *ntrC* gene is a regulatory gene controlling a number of operons involved in nitrogen metabolism in response to the intracellular glutamine/2-ketoglutarate ratio, which is a measure of nitrogen availability (21). The *ompR* gene is also a transcriptional regulator controlling the activity of the *ompF* and *ompC* genes in response to changes in osmotic pressure (22). In addition, the *ntrC* and *ompR* gene products have been purified and shown to bind to promoters they regulate (23, 31, 39). The similarity of the *algR* translated sequence to that of the *ntrC* and *ompR* sequences strongly supports our earlier observations suggesting that the *algR* gene is a transcriptional regulator of *algD* (10).

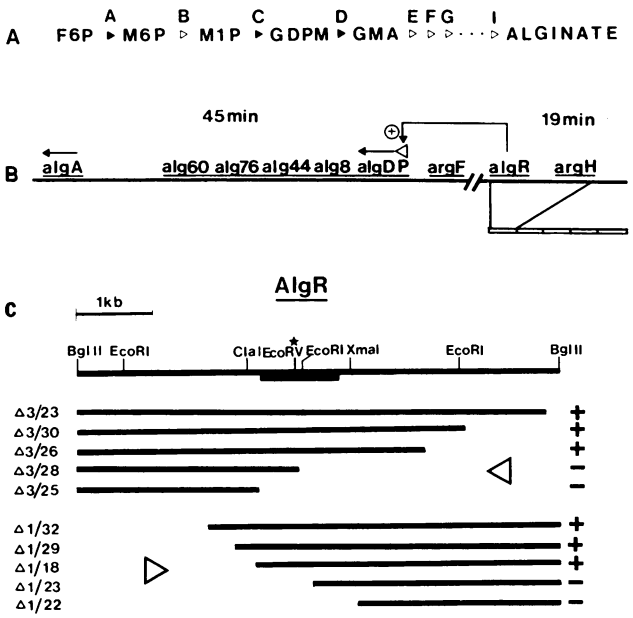

FIG. 1. (A) Alginate biosynthetic pathway. Enzymatic steps are catalyzed by phosphomannose isomerase-GDPmannose pyrophosphorylase, a bifunctional enzyme (step A) (18, 34); phosphomannomutase (step B); phosphomannose isomerase-GDPmannose pyrophosphorylase (step C); and GDPmannose dehydrogenase (step D) (9). Steps E, F, G, and I are putative steps involved in polymerization, epimerization, and export through the cell membrane. Symbols and abbreviations: ▶, characterized enzymatic steps (9, 18, 34); ▷, noncharacterized steps; F6P, fructose-6-phosphate; M6P, mannose-6-phosphate; M1P, mannose-1-phosphate; GDPM, GDPmannose; GMA, GDPmannuronic acid. (B) Alginate genes and regulation. Genes encoding the bifunctional enzyme phosphomannose isomerase-GDPmannose pyrophosphorylase and GDPmannose dehydrogenase are designated *algA* and *algD*, respectively. The *algA* and *algD* genes are at the extreme ends of the alginate gene cluster linked to the *argF* marker. The *algD* gene is under positive control by *algR* (10), a regulatory gene linked to the *argH* gene. Positions of these markers on the *P. aeruginosa* chromosome are given in minutes. The *algR* gene region, when chromosomally amplified (four to six copies) (▭) induces alginate production (11). (C) The 6.2-kb *Bgl*II fragment containing *algR*: restriction map and subcloning strategy. Thick line indicates *algR* coding region. Two *Eco*RV sites are closely linked (15 base pairs apart) (★). The 6.2-kb *Bgl*II fragment was cloned in both orientations in the single *Bgl*II site of mTM010 M13 vector. Two sets of subsequent overlapping deletions were obtained by a modification of the method described by Dale et al. (6). Open triangles denote directions of deletions. Deletion clones (only representative deletions are shown) were subcloned in pVDZ'2 and transferred to the *algR22* mutant strain by triparental bacterial conjugation. Exconjugants were scored for the complementation of the chromosomal *algR* mutation; results are indicated by + or −. Representative deletions used in fine resolution mapping are shown in Fig. 2.

Moreover, the pairwise comparisons of *algR* with the *ntrC* and *ompR* gene products revealed the characteristic pattern recently described for *ompR*, *phoB*, *virG*, *sfrA*, *ntrC*, *spo0A*, *dctD*, and *cheB* (33). Additional analysis with *phoB*, *sfrA*, *virG*, *dctD*, open reading frame 2, *cheB*, *cheY*, *spo0A*, and *spo0F* translated sequences revealed that the N-terminal domains (usually the first 130 to 140 amino acids) of all these

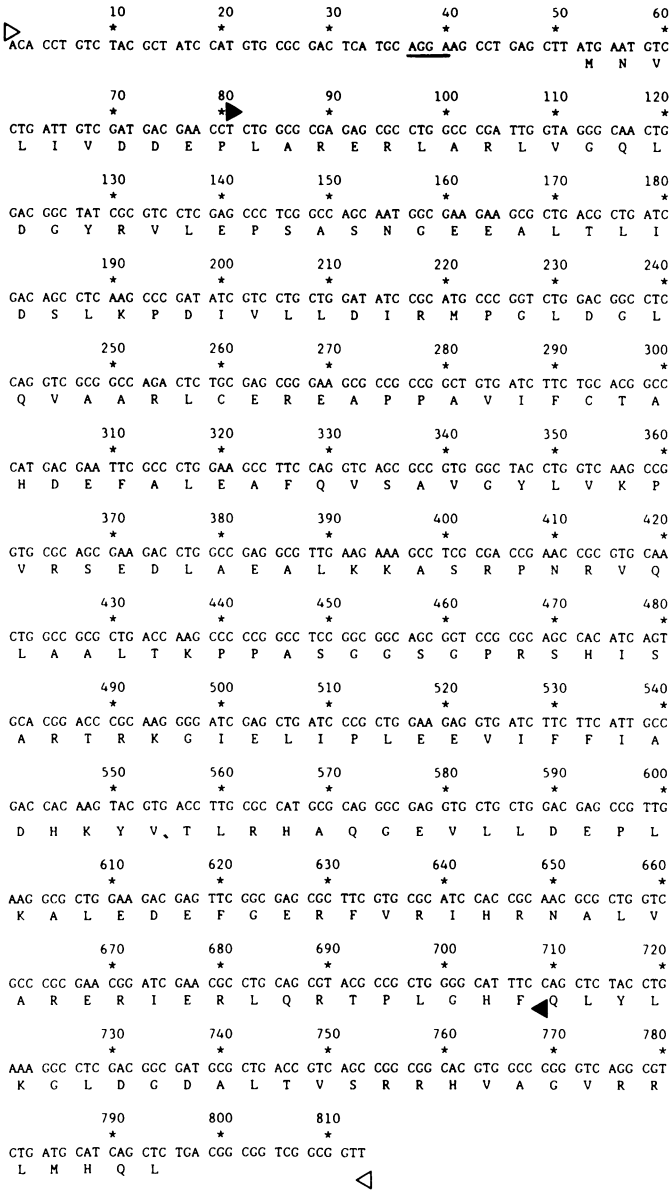

FIG. 2. DNA sequence of the positive regulatory *algR* gene. Triangles denote 5′ endpoints of deletions used for fine subcloning of *algR* with positive complementation (▷) or no complementation (▶) of the *algR*22 mutation. The putative Shine-Dalgarno sequence is underlined. DNA was sequenced by the chain termination method (35). Deletion clones in M13 were used as templates. Reaction mixtures contained the dGTP analog 7-deaza-dGTP to avoid band compression problems on gels (30).

gene products were homologous to the N-terminal half of the *algR* gene product (Fig. 4). This is a common feature of this class of regulatory proteins (33). All genes homologous to *algR* regulate important cellular functions in response to environmental stimuli: *phoB* coordinately regulates a number of genes in the phosphate regulon when the cells are starved for phosphate, *virG* transcriptionally regulates the *vir* loci in the soil pathogen *Agrobacterium tumefaciens* in response to simple phenolic compounds, and other members of this class of proteins regulate environmentally triggered developmental events (*spo0A*) and fast adaptive changes such as those in bacterial chemotaxis (*cheB*). This strongly suggests that *algR* regulates alginate biosynthesis in response to a yet undefined environmental factor(s).

DISCUSSION

In this study we identified the coding region of the *algR* gene and determined its sequence. The subcloning procedure that we used facilitated the sequencing process. By subcloning a large DNA fragment in an M13 vector that supports cloning and maintenance of large DNA inserts, we were able to produce deletion clones which were used for both sequencing and subcloning of the *algR* gene. Our procedure is useful for analyzing new genes that are cloned on large DNA fragments. Prior restriction mapping or unique restriction sites are not needed in this method. One of the advantages of our procedure is the speed with which clones are produced for both delimiting and sequencing genes.

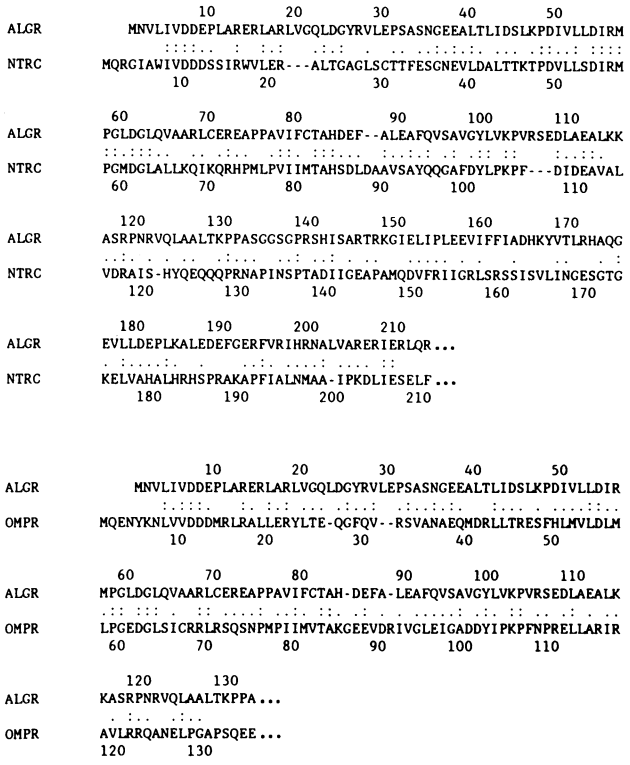

FIG. 3. Homologous N-terminal domains of the *algR*, *ntrC*, and *ompR* gene products. Double dots indicate identities; single dots indicate conserved amino acid substitutions. *P. aeruginosa algR* gene product (ALGR) is the translated sequence of the *algR* coding region (Fig. 2). *K. pneumoniae ntrC* gene product (NTRC) and *E. coli ompR* gene product (OMPR) are the sequences of transcriptional activators regulating central nitrogen metabolism and expression of osmoregulated genes for outer membrane porin proteins (4, 5). *P. aeruginosa algR* gene product and *K. pneumoniae ntrC* gene product are 32% identical, while *P. aeruginosa algR* gene product and *E. coli ompR* gene product show 27% identity (excluding conservative changes); optimized score values were 146 and 137, respectively, and statistical significance values (*z* values) were 17 and 9.7, respectively, as determined by the RDF program (25).

In this report we have analyzed the *algR* gene sequence. *algR* is proposed to be a transcriptional regulator of alginate genes (10). This hypothesis is based on the presence of *algR* in a chromosomal region which when amplified induces mucoidy (11) and on the fact that mutations in this gene abolish transcription of the key structural gene (*algD*) encoding GDPmannose dehydrogenase (10). Our results presented here further support this hypothesis. Moreover, the finding that the *algR* gene product is homologous to a recently recognized class of regulators that respond to environmental stimuli suggests that mucoid capsule production in *P. aeruginosa* is at least in part under the control of yet undefined environmental conditions in the lungs of cystic fibrosis patients.

Recent advancements in genetic analysis of the mechanisms regulating mucoidy in *P. aeruginosa* indicate involvement of several genetic loci. It has been shown in marker transfer experiments that the *muc* locus mapping in the late region of the *P. aeruginosa* chromosome is capable of conferring the mucoid phenotype (16, 26). Recently, isolation of a chromosomal region, termed *algST*, which may correspond to the *muc* locus was reported (14). Although it has been suggested (7, 14) that mucoidy in *Pseudomonas*

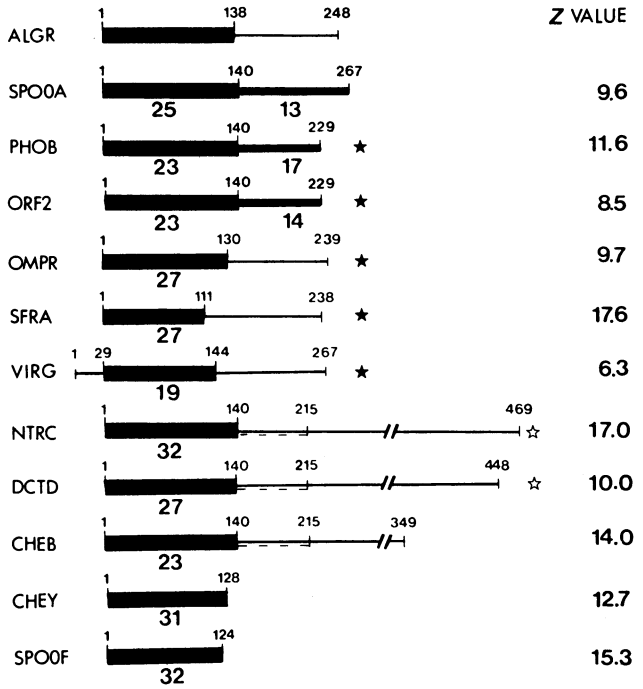

FIG. 4. Summarized homologies of the *algR* gene product (ALGR) with the members of a class of environmentally responsive regulatory genes. Thick lines represent regions of strong similarities observed in pairwise comparisons of ALGR with individual members of the group. Medium lines represent regions with lower-level homologies. Thin lines represent regions with little or no similarity; thin lines underlined by a dashed line were still picked by the FASTP program. Numbers below the sequences indicate the percentages of identical residues (excluding conservative amino acid substitutions). Numbers above the lines indicate amino acid residues; for ALGR, the end of homologies is taken as the most frequent end of the optimized alignments. Closed and open stars indicate groups of sequences which show a higher level of similarity among themselves than with other sequences. The *z* value, a measure of the significance of a similarity, was obtained by using the RDF program (25), which compares the query sequence with 20 randomly permuted versions of related sequences. The result is expressed as the number of times the difference between the similarity score and the mean of random scores exceeds the standard deviation of random scores. Values greater than 6 are considered probably significant, while values greater than 10 are positively significant (25). SPO0A and SPO0F are the products of sporulation positive regulatory genes (*spo0A* and *spo0F*) in *Bacillus subtilis* (38). PHOB, the *phoB* gene product, is the positive regulatory element for the phosphate regulon of *E. coli* (27). ORF2 is an open reading frame found immediately upstream of the *phoM* gene, which is also involved in the positive regulation of the phosphate regulon in *E. coli* (1). OMPR is the *E. coli ompR* gene product and NTRC is the *K. pneumoniae ntrC* gene product. SFRA is the gene product of *sfrA* (*dye*), which regulates several envelope proteins which affect resistance to dyes and sex factor expression (12); it also modulates repression of aerobic pathways in *E. coli* (24). VIRG, the product of *virG*, is the positive regulator of virulence genes in *A. tumefaciens* which are activated in response to simple phenolic compounds released from wounded plant tissues (41). DCTD, the *dctD* gene product, is a positive regulatory element that controls C_4-dicarboxylate transport in *Rhizobium leguminosarum* (32). CHEB and CHEY are the only proteins from this group that are not identified as transcriptional activators. They are the gene products of the *cheB* and *cheY* genes, which regulate chemotaxis in *Salmonella typhimurium* (36).

species could be regulated by a flip-flop or gene conversion mechanism, no DNA rearrangements, which are usually associated with such phenomena, have been observed (15). The role of the *muc* region therefore remains unclear (presumably the *muc* region [16, 26] is identical or similar to the *algST* region [14, 15]). Our results point to the importance of another genetic locus, *algR*, which is linked to markers different from those for the *muc* region (7, 16, 26).

Thus, current evidence indicates the involvement of several genetic elements controlling expression of alginate genes. Although it is difficult at present to assign a precise role to the *muc* (*algST*) region, it is clear that *algR* is a transcriptional regulator. As to how the entire system might be operating, a comparison with the regulation of colanic acid capsule synthesis in *E. coli* might be illuminating. It has recently been shown that several positive and negative regulatory loci are involved in the regulation of colanic acid biosynthesis (20). Mucoidy in *E. coli* K-12 has been associated with the *lon* mutations which are now known to stabilize the *rcsA* gene product, a positive transcriptional regulator of colanic acid synthesis (37). However, even in the *lon* mutant cells, another positive regulator, *rcsB*, is required for the mucoid phenotype (3). It has been hypothesized that this second positive regulator is involved in transmitting environmental signals into transcriptional regulatory events (3). Likewise, the alginate synthesis in *P. aeruginosa* could be responding to environmental changes via *algR*, not excluding additional regulatory networking such as the *muc* or *algST* locus or both.

It is clear that alginate biosynthesis is a complexly regulated phenomenon. In future studies it will be of prime importance to define not only the regulatory genes but also the environmental factors to which these regulators respond. These studies will contribute to our overall knowledge of the factors that regulate polysaccharide capsule synthesis in bacteria. More important, such analysis could be critical for future treatments of the infections in cystic fibrosis.

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