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Alginate Synthesis by *Pseudomonas aeruginosa*: a Key Pathogenic Factor in Chronic Pulmonary Infections of Cystic Fibrosis Patients

THOMAS B. MAY, DEAN SHINABARGER, ROMILLA MAHARAJ, JUNICHI KATO,† LEIN CHU, JAMES D. DEVAULT, SIDDHARTHA ROYCHOUDHURY, NICOLETTE A. ZIELINSKI, ALAN BERRY,‡ RANDI K. ROTHMEL,§ TAPAN K. MISRA, AND A. M. CHAKRABARTY*

Department of Microbiology and Immunology, University of Illinois College of Medicine, Chicago, Illinois 60612

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If clinician and scientist were to pool their resources and bring molecular biology to the bedside, it is likely that both the patient and science would benefit.

C. A. Seymour

INTRODUCTION

Pseudomonas aeruginosa is nearly ubiquitous in nature and, in most environments, is quite innocuous. However, *P. aeruginosa* can also cause severe and life-threatening infections in immunosuppressed hosts such as burn patients, patients suffering from respiratory disease, cancer chemotherapy patients, and children and young adults with cystic fibrosis (CF) (67). This opportunistic pathogen produces a number of unique virulence factors that make it particularly adept at infecting specific host tissues. Extracellular toxins, proteases, hemolysins, and exopolysaccharides are a few types of virulence factors that have been implicated in the pathogenicity of *P. aeruginosa* (63, 67, 80, 102, 103, 115, 116, 134, 157, 158). This review will center on the features that make *P. aeruginosa* ideally suited to infect one specific host environment: the CF-affected lung. We will provide details on the synthesis of a single virulence factor, the alginate exopolysaccharide, which allows *P. aeruginosa* to persist in the lungs of CF patients. Although other virulence factors are certainly involved in the initial stages of pulmonary infection by *P. aeruginosa* (67), the production of alginate is ultimately responsible for the poor prognosis for and high mortality rates among CF patients (63, 67, 134). A discussion of the roles of other virulence factors (e.g., toxins, proteases, and hemolysins) in the pathogenicity of this organism will be brief, as there are several excellent reviews covering these topics (80, 102, 103, 115, 116, 157, 158).

P. AERUGINOSA INFECTION IN THE LUNGS OF CF PATIENTS

CF: the Disease

CF, the most prevalent lethal genetic disease among Caucasians, is inherited as an autosomal recessive trait at a rate of 1 in 2,000 live births (39, 67, 134, 167). CF also affects non-Caucasian populations, but to a lesser degree (38, 39, 167). For example, only 1 in 17,000 American black children (93) is affected by the disease. An estimated 5% of the general population are carriers of the CF gene (38). CF heterozygotes (carriers), who are thought to have had a selective advantage in resistance to the bacterial-toxin-mediated diarrhea once prevalent in Europe (4, 70), show none of the clinical symptoms of CF (38). Thus, a major goal of CF research is to screen for potential carriers of the CF gene.

CF is a disease of abnormal electrolyte transport and

* Corresponding author.
† Present address: Department of Fermentation Technology, Faculty of Engineering, Hiroshima University, Saijo-shitami Higashi-Hiroshima, Hiroshima 724, Japan.
‡ Present address: Genencor International, Rochester, NY 14652.
§ Present address: Envirogen, New Brunswick, NJ 08903.

mucous secretion from exocrine glands and secretory epithelia (110, 111). It is best characterized by the triad of (i) chronic pulmonary disease with a persistent cough, (ii) elevated sweat electrolytes, and (iii) pancreatic insufficiencies that result in malabsorption and recurrent diarrhea (18, 28, 38, 167). Therapy for CF patients is generally aimed at improving their nutritional status by exocrine pancreatic enzyme replacement and vitamin supplements and at pulmonary management with increasingly potent antimicrobial agents (18, 167). These efforts alone have raised the average life expectancy of a CF patient from 4 years in 1950 to more than 20 years today (18, 167).

diSant'Agnese et al. (37) first demonstrated the increased salt content of sweat from CF patients, a criterion that is still a key tool for diagnosis of the disease (167). Electrolytes found in increased concentrations in the sweat of CF patients include Na^+, Cl^-, and K^+ (37, 148). As the primary fluid secreted by the sweat coil passes down the sweat duct, NaCl is normally reabsorbed to produce a hypotonic sweat at the skin surface (111). Quinton (131) showed that the sweat duct in CF patients is markedly less permeable to Cl^- and thus less able to reabsorb NaCl. A transducible factor in the sweat of CF patients has also been shown to actively inhibit Na^+ reabsorption by the sweat duct (81, 106, 146). Serous exocrine secretions (sweat) are abnormally concentrated as a result of the low permeability to Cl^- (18) but are otherwise histologically normal (28, 167).

Mucous exocrine secretions, consisting of isotonic bicarbonate-rich fluids containing NaCl and glycoproteins, provide the normal protective mucous layer (110). The mucous exocrine secretions of CF patients, including tracheobronchial secretions, are abnormally thick and are deficient in water and electrolytes (18). The obstruction of organ passages by these abnormal mucous secretions is responsible for most of the clinical manifestations of the disease (28). The appearance of abnormal glycoproteins is associated with the secretory defect (38, 148) and partly accounts for the altered rheological properties of mucous secretions in CF patients (18). Glycoproteins found in the lungs of CF patients, which appear to be altered in the carbohydrate moiety (38) and overall acidity (46), form insoluble complexes with Ca^{2+}. Ca^{2+} ion levels are increased in mucous exocrine secretions of CF patients (28, 38) and result in precipitation of CF-associated glycoproteins in affected organ passages. Bacterial products, leukocytes, and other debris (mainly DNA) also contribute to bronchial thickness of CF patients (38).

As with the sweat ducts, respiratory epithelia in CF patients are less permeable to Cl^- than they are in healthy individuals (53, 89, 90, 162). In contrast with levels in serous secretions, Na^+ levels are abnormally low because of lower permeability to Cl^- (89, 131) and perhaps increased reabsorption of Na^+ (18, 89). Chloride channels have been observed in exocrine tissues from CF patients (53), but the regulation of these channels appears to be defective (53, 97). Hormonal secretagogues, which stimulate increases in cyclic AMP levels that normally open the Cl^- channel, fail to open this channel in CF patients. Furthermore, addition of a cyclic AMP-dependent protein kinase and ATP opens the Cl^- channels in normal cells but not in respiratory cells of CF patients (97). Thus, the defect appears to be either that the channel is altered so that it cannot be phosphorylated or that an associated regulatory protein no longer functions properly (97, 145). It should be noted that the various alterations observed in exocrine gland secretions can be organ dependent (28), making it difficult to identify the primary defect of CF. However, a lower permeability to Cl^- is thought to be the principal result of this defect (90, 131), which in turn causes the dehydrated exocrine secretions observed in these patients (89, 110).

Although considerable progress has been made in understanding the molecular and biochemical bases of CF, only recently has the basic metabolic error been identified. The CF gene, which is located on chromosome 7 close to proto-oncogene *met* (164), has been cloned and sequenced (86, 135, 136). Rommens et al. (136), Riordan et al. (135), and Kerem et al. (86) found that in 70% of patients afflicted with the disease, the CF gene product was likely to be a transmembrane conductance regulator protein lacking a single phenylalanine residue (Phe-508). Other less prevalent mutations in this gene product have subsequently been identified (163). A defective transmembrane conductance regulator protein results in the altered secretions observed in exocrine glands and secretory epithelia (90, 110, 111), and it is now known that this is the primary defect that results in the CF disease state (86, 135, 136). There is hope that identification of the CF gene will allow screening for heterozygote carriers. One limitation of the screening technique is that other less prevalent mutations (30%) would remain undetected. CF gene carriers, however, show a reduced β-adrenergically induced sweat response in exocrine glands (5, 144), which may also provide a useful screen for suspected CF gene carriers.

Clinical Significance of Pulmonary Infections Caused by *P. aeruginosa*

Microbiology of the CF-affected lung. The secretion of a hyperviscous mucus in the CF-affected lung is thought to increase the incidence of bacterial lung infections among CF patients (134). *Staphylococcus aureus*, *Haemophilus influenzae*, and *P. aeruginosa* are most notable in their ability to colonize the CF-affected lung. Although *S. aureus* is usually the first and predominant pulmonary isolate from CF patients, *S. aureus* infection is effectively controlled by treatment with antibiotics (112). *S. aureus*, however, is thought to predispose the CF-affected lung to pseudomonal colonization (112). *H. influenzae* may also assist in this colonization (63) by disturbing respiratory ciliary function (166). *H. influenzae* usually coexists with *P. aeruginosa* (112), but *P. aeruginosa*, being particularly resistant to even the most aggressive antibiotic therapy, gradually dominates the microbial flora within the CF-affected lung (67, 134). *P. aeruginosa* isolated from the respiratory tract of CF patients is initially nonmucoid (40, 41, 72) but switches to a mucoid, alginate-producing form upon progression of the disease (43, 44, 72). Mucoid *P. aeruginosa* isolated from the sputum of CF patients spontaneously reverts to the nonmucoid form upon in vitro culturing (7, 16, 134, 172). Determination of pyocin type (7, 165), phage type (7), and serological group (7, 36) has shown that the mucoid forms are direct variants of the nonmucoid *P. aeruginosa*. In addition, DNA typing has also provided evidence that mucoid and nonmucoid strains are clonal variants (118).

A high incidence of pulmonary infection by mucoid *P. aeruginosa* is found among CF patients (40, 42, 43, 77, 79), but the mucoid form is rarely observed elsewhere in nature (40, 42, 43), even in tissues from outside the respiratory tracts of CF patients or from the lungs of patients suffering from other respiratory diseases (38, 43, 73, 134). In fact, up to 90% of CF patients are infected by mucoid *P. aeruginosa*, whereas less than 2% of non-CF patients are colonized by

the alginate-producing form of the bacterium (40, 42, 63). Furthermore, the severity of the lung infection directly correlates with the presence of mucoid strains (63, 67). Even so, CF-associated lung infection by alginate-producing *P. aeruginosa* is unique in that it usually does not cause the septicemia observed with most pseudomonal infections (63). The occurrence of mucoid *P. aeruginosa* is now so closely associated with CF that it is almost diagnostic for the disease (67, 134). Failure to control mucoid *P. aeruginosa* colonization of the CF-affected lung complicates the already viscous bronchial obstruction (18) and results in the poor prognosis for and high mortality rates among CF patients (67, 134).

Influence of the CF-affected lung on alginate production. The CF-affected lung appears to provide a unique environment for inducing *P. aeruginosa* to mucoidy (40, 43, 61). In fact, CF patients who have undergone lung-heart transplants no longer develop chronic pulmonary infections by mucoid *P. aeruginosa*, apparently because there is no defective (CF) gene in the new lung (141). What then is the nature of the factor(s) in the CF-affected lung that causes this switch from nonmucoid to mucoid, alginate-producing *P. aeruginosa*? For the most part, the factors contributing to this unusual host-pathogen interaction have not yet been determined (171). Several in vitro conditions have been found to either induce or enhance the mucoid mode of growth. For example, changes in the composition of growth media can stabilize alginate production by *P. aeruginosa* (12, 17, 44, 61, 68, 123, 155). Such changes include nutrient limitation and the addition of surfactants. Interestingly, lecithin, which is the major surfactant present in the lung, is one of the surfactants found to enhance alginate production by mucoid *P. aeruginosa* (61). Growth of *P. aeruginosa* in the presence of bacteriophage (61, 108) or selection for antibiotic resistance (64) stimulates nonmucoid forms of *P. aeruginosa* to switch to mucoidy and produce alginate. Høiby et al. (75) have proposed that suboptimal antibiotic concentrations present in the sputum of CF patients may directly select for mucoidy in the secretions of these patients. For several other pseudomonads, growth on subinhibitory concentrations of carbenicillin also induces alginate production (66, 68). Our laboratory has shown that several alginate biosynthetic and regulatory genes are present in a number of pseudomonads (50), which suggests that *P. aeruginosa* is not the only pseudomonad having the genes necessary for alginate biosynthesis (50, 66, 68). Since *P. aeruginosa* is generally found in moist or aquatic environments and since alginate production may be a mechanism for resisting dehydration (67), it is not surprising that the addition of ethanol to solid media causes nonmucoid strains to produce alginate (35). Nonetheless, the correlation (if any) between in vitro induction of alginate synthesis and in vivo response in the CF-affected lung remains unclear.

Effect of mucoidy on host immune reaction to *P. aeruginosa*. The chronic nature of lung infections by mucoid *P. aeruginosa* in CF patients and the exclusive association of the mucoid form of this organism with the disease suggest that alginate provides a selective advantage to *P. aeruginosa* in the CF-affected lung. Govan (61) observed that nonmucoid forms predominate when they are coinoculated with mucoid *P. aeruginosa* in vitro, indicating that the nonmucoid form has a distinct advantage in environments not affected by CF. Current aggressive antibiotic treatment coupled with more-effective pulmonary and nutritional therapies has led to better overall management of lung infections by mucoid *P. aeruginosa* in CF patients. Yet, once acquired, mucoid *P. aeruginosa* is virtually impossible to eliminate from the sputum of CF patients (14, 42, 72, 156, 167), even by intensive pulmonary and antibiotic therapy (36, 41).

The role of the alginate capsule in allowing persistent, chronic infection by *P. aeruginosa* is one of the most intriguing problems of microbial pathogenesis. The alginate layer probably impairs the ability of the immune system to combat *P. aeruginosa* infection of the CF-affected lung. However, CF patients are not deficient in general host immunity (1, 75), since they show normal immune responses to infections outside the respiratory tract (38, 159). CF patients also produce specific serum precipitins against *P. aeruginosa* (13, 36, 41, 72, 75) and maintain increased levels of both circulatory and secretory antibodies (41). Interestingly, the local immune reaction may cause destructive lesions of the respiratory tract (76), which enhances the selection of mucoid variants (72, 75, 76).

The alginate layer of mucoid strains of *P. aeruginosa* appears to prevent antibody coating (107) and thus blocks the immunological determinants required for in vitro opsonic phagocytosis (1, 72, 76, 133, 140, 147). Mucoid strains of *P. aeruginosa* appear to be more resistant to nonopsonic phagocytosis as well (14, 92). Treatment of mucoid strains with alginate lyase to remove the alginate capsule has been shown to enhance phagocytosis (48). Furthermore, Schwarzmann and Boring (147) found that cell washing removed enough of the alginate layer to allow phagocytic killing of mucoid strains equal to that observed for nonmucoid strains. In addition, the alginate polymer has been found to directly inhibit macrophage binding and phagocytosis (92, 122) and may also impede chemotaxis of polymorphonuclear leukocytes (154).

While it is implicit that a viscous polysaccharide like alginate may interfere with phagocytosis, testing this possibility has often led to contradictory results. Several groups found no appreciable effect of alginate encapsulation on the phagocytic killing of mucoid strains of *P. aeruginosa* (6, 11, 113). These opposing views could result from (i) differences between strains used to measure phagocytosis, (ii) rapid reversion of mucoid *P. aeruginosa* to the nonmucoid form, (iii) variability in macrophage and polymorphonuclear leukocyte preparations, (iv) variability in bacterial processing, and (v) differences in the duration of bacterial growth (11, 92). Most important, several groups have identified microcolonies of *P. aeruginosa* associated with the sputum of CF patients (67, 74, 94). Lam et al. (94) found that these fiber-enclosed alginate microcolonies isolated postmortem from CF-affected lungs interfere with pulmonary defense and clearance mechanisms. It seems likely that microcolonies reflect the true in vivo status of *P. aeruginosa* in the lungs of CF patients. In addition, recent evidence demonstrated the presence of antialginate antibody in CF patients harboring only nonmucoid *P. aeruginosa* in their lungs, suggesting that nonmucoid strains produce some alginate in vivo (125). Perhaps even nonmucoid *P. aeruginosa* produces small amounts of alginate in the stressed CF-affected lung to allow the organism to stick to solid surfaces such as the lung itself and to afford initial resistance to phagocytosis. Therefore, we should be cautious in evaluating all of these results, since the environment of the CF-affected lung may be very different from that of in vitro studies and from in vivo animal model systems.

There is also evidence that alginate provides an ionic barrier against penetration of aminoglycoside antibiotics (64, 153). Govan and Fyfe (64) observed that mucoid forms of *P. aeruginosa* are more resistant to carbenicillin, flucloxacillin, and tobramycin than are nonmucoid isolates. However,

Demko and Thomassen (29) observed increased sensitivity of the alginate-producing strains to carbenicillin, tobramycin, and ticarcillin. Thomassen et al. (156) found that mucoid and nonmucoid strains of *P. aeruginosa* show a large degree of variability in antibiotic susceptibility even when isolated from the same individual; however, mucoid isolates were generally more susceptible to gentamicin, carbenicillin, and tobramycin than were nonmucoid isolates. Thus, strain differences and biofilm formation may greatly affect *P. aeruginosa* susceptibility to antibiotics.

Slack and Nichols (153) used antibiotic diffusion through agar as a criterion for direct measurement of the permeability of the alginate layer to antibiotics. They found that, with the exception of β-lactams, alginate did in fact impede the penetration of antibiotics, including aminoglycosides. However, Gordon et al. (59) observed that the ratio of alginate to antibiotic can greatly influence the perceived permeability barrier. When this ratio is high, aminoglycosides (but not β-lactams) are retained in the alginate layer. However, low alginate-to-antibiotic ratios quickly result in disruption of the gel structure and faster penetration of aminoglycosides. They suggested that high levels of antibiotic saturate the negative charge of alginate and result in a breakdown in the permeability layer (59). This ratio could be physiologically important, since antibiotic concentrations in the sputum and pulmonary tracts of CF patients are generally thought to be low. Nichols et al. (117) found that *P. aeruginosa* in biofilms was 1,000-fold less susceptible to tobramycin and cefsulodin than when the organism was dispersed in liquid medium. However, no differences were found in the antibiotic susceptibilities of mucoid and nonmucoid strains when they were in biofilms. It is interesting to note that *P. aeruginosa* has been found to produce 32-fold-more β-lactamase in biofilms than when dispersed (56). This difference could be of physiological importance, since alginate does not appear to impede the permeation of β-lactams (59). However, whether nonmucoid cells produce small amounts of alginate when present in a biofilm and thereby exhibit enhanced antibiotic resistance is not known.

Alginate may also promote adherence of mucoid strains to epithelial cells of the pulmonary tract (45, 132, 169), thereby inhibiting pulmonary clearance mechanisms. In vivo experiments using intratracheal inoculation of rat lungs have demonstrated that mucoid variants of *P. aeruginosa* are removed less rapidly from the pulmonary tract than are isogenic nonmucoid strains (62). Adherence to the lung epithelial cells, which is somewhat strain dependent (45), may also be increased by microcolony formation in the CF-affected lung (132).

The effect of alginate on the host immune system is still not completely understood. It appears that the humoral immune mechanism responds to mucoid *P. aeruginosa*, as Pier et al. (126) have recently shown that mice and rats immunized against the mucoid exopolysaccharide have reduced levels of infection after intratracheal challenge, particularly if the trachea is already damaged. However, the cell-mediated response may be inhibited by the alginate layer. It is also possible that alginate encapsulation simply overwhelms the capabilities of the macrophages rather than specifically impeding phagocytosis. In addition, mucoid *P. aeruginosa* generally appears to be more susceptible than the nonmucoid form to antibiotics, with the notable exception of some β-lactams. Possibly the protective microcolony allows the persistence of the "antibiotic-susceptible" mucoid strains within the CF-affected lung (63). The combination of microcolony formation, mechanical obstruction in the CF-affected lung, and perhaps a less-effective cell-mediated immune response may provide the natural selective advantage for mucoid strains (38, 63, 94) and account for the persistence of mucoid *P. aeruginosa* in the CF-affected lung (1).

Nonalginate virulence factors of *P. aeruginosa*. *P. aeruginosa* produces a wide variety of virulence factors (in addition to alginate) that may contribute to the pathogenicity of this bacterium (154). *P. aeruginosa* produces a battery of toxins including cytotoxin (leukocydin) and exotoxins A and S (102, 116, 157). Exotoxins A and S in particular have been shown to play a role in the pathogenicity of the bacterium in chronic lung infections in animal model systems (116). Exotoxin A ADP-ribosylates elongation factor 2, thereby halting protein synthesis and causing cell death (103, 116). Exotoxin S ADP-ribosylates other substrates such as vimentin and GTP-binding protein p21$^{c\text{-}H\text{-}ras}$, but the exact mechanism of toxicity of this enzyme is not known (21, 22). Mutants with defective exotoxin A or exotoxin S have reduced ability to elicit lung damage, although the ability to colonize the lung is not affected by these mutations (116). Several proteases (including collagenase, elastase, and fibrinolysin) are also associated with *P. aeruginosa* virulence in the CF-affected lung (115, 157). *P. aeruginosa* strains with a defective elastase have been shown to have attenuated abilities to damage the host lung in animal model systems (116). The proteases have been observed to evoke mucin release from tracheal epithelium (88, 115) and are generally involved with procurement of nutrients (157). In fact, mucin release may select for mucoid forms by enhancing their growth rate compared with that of nonmucoid *P. aeruginosa* (64). However, Ohman and Chakrabarty (119) demonstrated that protease levels are lower in mucoid strains of *P. aeruginosa* than in nonmucoid forms, indicating that the proteases are probably not a major contributing factor in the later stages of CF-associated pulmonary infections. Alginate may in fact localize the toxins and limit the damage to discrete areas of the CF-affected lung (65). Phospholipase C, a hemolysin, may be one of the most important virulence factors in the initial stages of chronic pulmonary infection by *P. aeruginosa* (157, 158). Phospholipase C degrades lecithin, the major lung surfactant, to phosphorylcholine in response to low-phosphate conditions (102, 103). Associated glycolipids and phosphatases aid in the action of phospholipase C by solubilizing the phospholipids and scavenging the phosphate group from phosphorylcholine, respectively (102). Nearly all CF patients having *P. aeruginosa* pulmonary infections elicit antibody against phospholipase C (157). Unlike that of other pathogenic bacteria, *P. aeruginosa* lipopolysaccharide has a low toxicity and therefore may not play a role in the pathogenicity of this organism in the CF-affected lung as far as direct tissue destruction is concerned (103). *P. aeruginosa* cells also lose the O antigen during infection of the CF-affected lung (69). Although these virulence factors (particularly exotoxin A, exotoxin S, elastase, and phospholipase C) appear to be associated with the pathogenicity of *P. aeruginosa* in the initial stages of CF-associated lung infection, lowering the levels of these potentially more destructive virulence factors while coating itself with a protective alginate layer may allow *P. aeruginosa* to persist in the pulmonary tracts of CF patients (67, 119). However, progressive destruction of the lung still occurs in these patients even though *P. aeruginosa* appears to down regulate these virulence factors and even though the mucoid form is the most pathogenic state in the CF-affected lung. It is entirely possible that the lower levels of these virulence factors

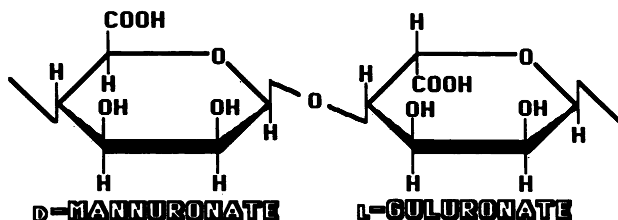

FIG. 1. Alginate structure showing β-1,4-linked D-mannuronic acid and L-guluronic acid. Mannuronic acid residues may be modified with *O*-acetyl groups at position O-2 or O-3 or both, with the O-2 position being preferred (151).

produced by mucoid strains, as measured in vitro, are quite sufficient to make an impact in vivo. Alternatively, the in vitro observations may not reflect the true state of these microorganisms in vivo.

ALGINATE SYNTHESIS BY MUCOID *P. AERUGINOSA*

Biochemistry of Alginate Synthesis

Linker and Jones (100, 101) first reported that the polysaccharide secreted by mucoid *P. aeruginosa* is alginate. The bacterial alginate is similar to the commercially useful polymer typically obtained from marine algae (98) and to the polysaccharide later identified in the slime layer of another bacterium, *Azotobacter vinelandii* (60). Alginate is a linear copolymer of β-1,4-linked D-mannuronic acid and variable amounts of the C-5 epimer L-guluronic acid (Fig. 1; 49). Bacterial alginates differ from the algal polymer in that mannuronate residues may be modified with *O*-acetyl groups (27, 49, 101). Skjak-Braek et al. (151) have shown that acetyl groups are localized predominantly at O-2 but occur also at O-3. In addition, some mannuronate residues are modified at both positions (151).

The alginate biosynthetic pathway of *P. aeruginosa*, as shown in Fig. 2, was initially proposed on the basis of studies by Lin and Hassid (98, 99) with the brown alga *Fucus gardneri* and by Pindar and Bucke (128) with the bacterium *A. vinelandii*. Fructose 6-phosphate was identified as the first alginate precursor for the *P. aeruginosa* biosynthetic pathway and appears to be recruited from the carbohydrate pool via the Entner-Doudoroff pathway (2, 15, 104) and fructose 1,6-bisphosphate aldolase (3). Piggott et al. (127) first demonstrated the presence, albeit at low levels, of the alginate biosynthetic enzymes phosphomannose isomerase (PMI), GDP-mannose pyrophosphorylase (GMP), and GDP-mannose dehydrogenase (GMD). Padgett and Phibbs (124)

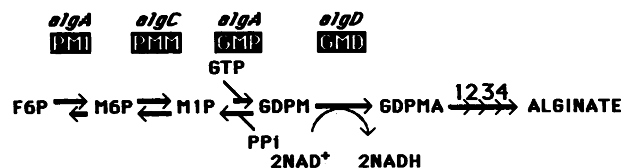

FIG. 2. Alginate biosynthesis pathway. Arrows 1, 2, 3, and 4 indicate the undefined steps of polymerization, acetylation, export, and epimerization. The gene encoding each enzyme is indicated above the enzyme name. Equilibria for the alginate reactions are known for PMI, GMP, and GMD and are indicated by the relative sizes of the arrows for each direction of the reaction. F6P, Fructose 6-phosphate; M6P, mannose 6-phosphate; M1P, mannose 1-phosphate; GDPM, GDP-mannose; GDPMA, GDP-mannuronic acid.

detected another alginate biosynthetic enzyme, phosphomannomutase (PMM), in mucoid strains of *P. aeruginosa*. The activities of these four enzymes (PMI, PMM, GMP, and GMD) are either absent or greatly reduced in nonmucoid strains compared with the low levels in mucoid strains of *P. aeruginosa* (124, 127). Similar results have been obtained by our laboratory (2, 25, 139, 142) and others (129), suggesting that low enzymatic activities are a general characteristic of alginate biosynthetic enzymes even in the most highly mucoid strains of *P. aeruginosa*.

The remaining steps of the *P. aeruginosa* alginate pathway have not been elucidated. Lin and Hassid (98) isolated GDP-mannuronic and GDP-guluronic acids from extracts of *F. gardneri* and proposed that these nucleotide sugars are the direct precursors of algal alginate. A C-5 epimerase activity has been observed in extracts of *F. gardneri* and has been found to convert GDP-mannuronic acid to GDP-guluronic acid (99). A second pathway of GDP-guluronate synthesis from sorbitol has also been identified in algae, but the role of this alternate pathway is not understood (130). In contrast, Pindar and Bucke (128) demonstrated that polymannuronic acid is the first polymeric product formed by extracts of *A. vinelandii* and that neither guluronic acid nor GDP-guluronic acid is present at the monomer level. *A. vinelandii* has been shown to produce an extracellular, calcium-dependent C-5 epimerase (71, 95, 96, 152), thereby suggesting that the incorporation of guluronate residues into bacterial alginate occurs at the level of polymannuronate (128). We have demonstrated that GDP-mannuronic acid is a direct precursor of *P. aeruginosa* alginate (139), and Chitnis and Ohman (19) have recently identified a mutant of *P. aeruginosa* that no longer incorporates guluronate into alginate. Thus far, an epimerase activity has not been directly shown for *P. aeruginosa*, and the cellular location of the enzyme is not known. It is therefore not clear whether *P. aeruginosa* utilizes GDP-guluronic acid as a precursor or whether guluronate residues are incorporated into the polymer by an extracellular epimerase. The bacterial polymer differs from algal alginate in that mannuronate residues may be modified with *O*-acetyl groups (27). *O*-Acetyl modification is proposed to regulate the degree of epimerization by shielding mannuronate groups from the epimerase enzyme (155). Alginates from *P. aeruginosa* isolated from the CF-affected lung are highly acetylated (47, 149), which perhaps explains the lack of repeating guluronate block structures in the *P. aeruginosa* polymer (63). However, an *O*-acetylase enzymatic activity has not been directly demonstrated for *P. aeruginosa*. The mechanism for incorporation of the nucleoside diphosphate sugar GDP-mannuronic acid (and perhaps GDP-guluronic acid) into *P. aeruginosa* alginate is also not understood. For that matter, the polymerization process has not been elucidated for *F. gardneri* or *A. vinelandii*. Alginate polymerization, however, is thought to resemble the synthesis of other bacterial cell wall polysaccharides with respect to the involvement of a C_{55}-polyisoprenyl phosphate alcohol carrier lipid and membrane-bound enzymes (139, 155).

Alginate from *P. aeruginosa* is expected to be more elastic in nature as a result of fewer guluronate residues (63). In fact, Doggett et al. (42) have suggested that high levels of Ca^{2+} found in the CF-affected lung may play a role in the regulation of the mannuronate/guluronate ratio. Calcium ions have also been observed to stabilize the alginate gel (67) and cause mucoid *P. aeruginosa* to have a more compact and gelatinous appearance (63). Interestingly, EDTA, which chelates Ca^{2+} and other metals, has been shown to enhance antibiotic effectiveness in vivo, perhaps by reducing the

gelling properties of the alginate polymer (168). In addition, mucoid cells grown in the presence of Ca^{2+} are found to have an overall greater resistance to dehydration than either isogenic nonmucoid strains or mucoid cells grown in the absence of Ca^{2+} (67). Thus it seems that in addition to invoking the synthesis of alginate by nonmucoid strains of *P. aeruginosa*, the CF-affected lung may also control the rheological properties of the polymer.

Molecular and Enzymatic Studies of Alginate Synthesis

The overall goal of our research is to understand the biochemistry and regulation of alginate synthesis in order to identify nontoxic compounds that can effectively interfere with the biosynthesis of alginate by *P. aeruginosa* in the lungs of CF patients. These inhibitors would be clinically beneficial, since *P. aeruginosa* pathogenicity in the CF-affected lung appears to result principally from alginate synthesis.

The inherent instability of the mucoid phenotype observed during routine laboratory propagation of *P. aeruginosa* isolates from CF patients presents a major difficulty in studying both the alginate biosynthetic enzymes and the genes that encode these enzymes. *P. aeruginosa* 8821 was originally isolated from the sputum of a CF patient and, like other mucoid *P. aeruginosa* strains, spontaneously reverts to the nonmucoid form (23). We have designated one nonmucoid isolate strain 8822. Darzins and Chakrabarty (23) used chemical mutagenesis of strain 8822 to produce stable alginate-producing *P. aeruginosa* 8830. Strain 8830 was the parent strain used in our studies on the molecular biology and enzymology of alginate synthesis by *P. aeruginosa*. Darzins and Chakrabarty (23) subsequently isolated a series of nonmucoid (Alg^-) derivatives by further mutagenesis of the stable alginate-producing *P. aeruginosa* 8830. Chromosomal DNA from strain 8830 was digested with the restriction enzyme *Bam*HI and ligated with *Bam*HI-linearized pCP13 (a broad-host-range vector) to generate a library of recombinant plasmids that were then screened for the ability to complement (restore alginate synthesis to) these Alg^- mutants (26). These mutants were divided into seven complementation groups (Fig. 3). Six groups form a cluster at 34 min on the *P. aeruginosa* chromosome and function in alginate biosynthesis, and another group is located at 10 min on the *P. aeruginosa* chromosome and contains alginate regulatory genes (26). The gene for one of the other biosynthetic enzymes (PMM) belongs to an eighth complementation group that appears to map outside of these two gene clusters (Fig. 3; 9, 171). Table 1 summarizes current knowledge about the alginate genes and gene products.

PMI-GMP. Darzins et al. (25) cloned a 6.2-kb *Hin*dIII fragment from an *Escherichia coli* DNA genomic library into a broad-host-range cosmid vector that not only complemented the *manA* (PMI^-) mutant *E. coli* CD1 but also restored alginate synthesis to several of the Alg^- mutants of *P. aeruginosa*. The recombinant plasmid pAD4, which contains a 9.9-kb *Eco*RI-*Bam*HI fragment of *P. aeruginosa* chromosomal DNA (Fig. 3), was likewise found to complement the putative PMI^- Alg^- mutants to PMI^+ Alg^+ (25). pAD4 also complemented the *manA* defect that prevents capsular polysaccharide synthesis by *E. coli* CD1; however, growth on mannose was not restored. The gene encoding PMI activity was designated *algA* and found to reside within a 2.0-kb *Bam*HI-*Sst*I fragment of pAD4. Interestingly, this fragment showed no homology by DNA-DNA hybridization to the cloned *manA* gene from *E. coli* and vice versa. The *algA* gene was mapped to the 20-kb alginate gene cluster at 34 min on the *P. aeruginosa* chromosome (Fig. 3; 26). Darzins et al. (24) determined the nucleotide sequence of the 2.0-kb *Bam*HI-*Sst*I DNA fragment and found a single open reading frame encoding the *P. aeruginosa algA* gene. No significant DNA sequence homology was found between the *P. aeruginosa algA* gene and the *E. coli manA* gene at the nucleotide level; however, DNA-DNA hybridization revealed sequences homologous to the *P. aeruginosa algA* gene in other *Pseudomonas* species and in *A. vinelandii* (24, 50).

Darzins et al. (25) found that both mucoid and nonmucoid strains of *P. aeruginosa* had barely detectable levels of PMI activity, whereas *P. aeruginosa* strains harboring the *manA* gene of *E. coli* showed measurable levels of this enzymatic activity. *P. aeruginosa* containing the plasmid pAD4 still had very low levels of PMI activity. As a result, our laboratory has undertaken a molecular approach to increase the quantities of alginate biosynthetic enzymes of *P. aeruginosa* for subsequent purification, characterization, and inhibitor studies. Darzins et al. (24) constructed the plasmid pAD4033 by cloning the *algA* gene directly downstream of the strong *tac* promoter of a broad-host-range controlled-expression vector, pMMB22, thereby allowing the induction of PMI synthesis by isopropyl-β-D-thiogalactopyranoside (IPTG). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of extracts of *P. aeruginosa* containing pAD4033 demonstrated a 56-kDa polypeptide when cells were induced with IPTG (55). IPTG induction of pAD4033 also resulted in greatly increased levels of PMI enzymatic activity (55).

Sa'-Correia et al. (142) reported that the overexpression of the *algA* gene in *P. aeruginosa* resulted not only in the appearance of PMI activity but also in an increase in the activities of PMM and GMP. In contrast, introduction of the *E. coli manA* gene into *P. aeruginosa* led to an increase in PMI and PMM activities but not in GMP activity. The converse experiment, in which the *P. aeruginosa algA* gene was introduced into *E. coli manA* mutant strain CD1, resulted in the appearance of both PMI and GMP activities. Both activities are normally undetectable in extracts of this strain of *E. coli* (142). Furthermore, column chromatographic fractionation of extracts of *P. aeruginosa*, which were induced for overexpression of the *algA* gene product, clearly separated PMI and PMM activities but not PMI and GMP activities (9, 142). These results suggested that the *algA* gene, contained within the 2.0-kb *Bam*HI-*Sst*I fragment, encodes a single polypeptide that has two enzymatic activities: PMI and GMP (Table 1; 142).

Shinabarger et al. (150) have recently purified the protein encoded by *P. aeruginosa algA*. The N-terminal amino acid sequence of the purified polypeptide matched the sequence predicted by DNA sequence analysis. PMI-GMP appeared to have a subunit molecular mass of 56,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, while the DNA sequence showed a polypeptide with an apparent molecular mass of 53,000. Gel filtration chromatography indicated that the native PMI-GMP is a monomeric protein. In addition, the purified protein was found to catalyze two independent catalytic steps (Fig. 2): the isomerization of fructose 6-phosphate to mannose 6-phosphate and the synthesis of GDP-mannose and PP_i from GTP and mannose 1-phosphate. Interestingly, the PMI reaction appeared to highly favor the synthesis of mannose 6-phosphate (forward direction), while the GMP reaction favored the formation of mannose 1-phosphate and GTP (reverse direction). This result may well explain why the *P. aeruginosa*

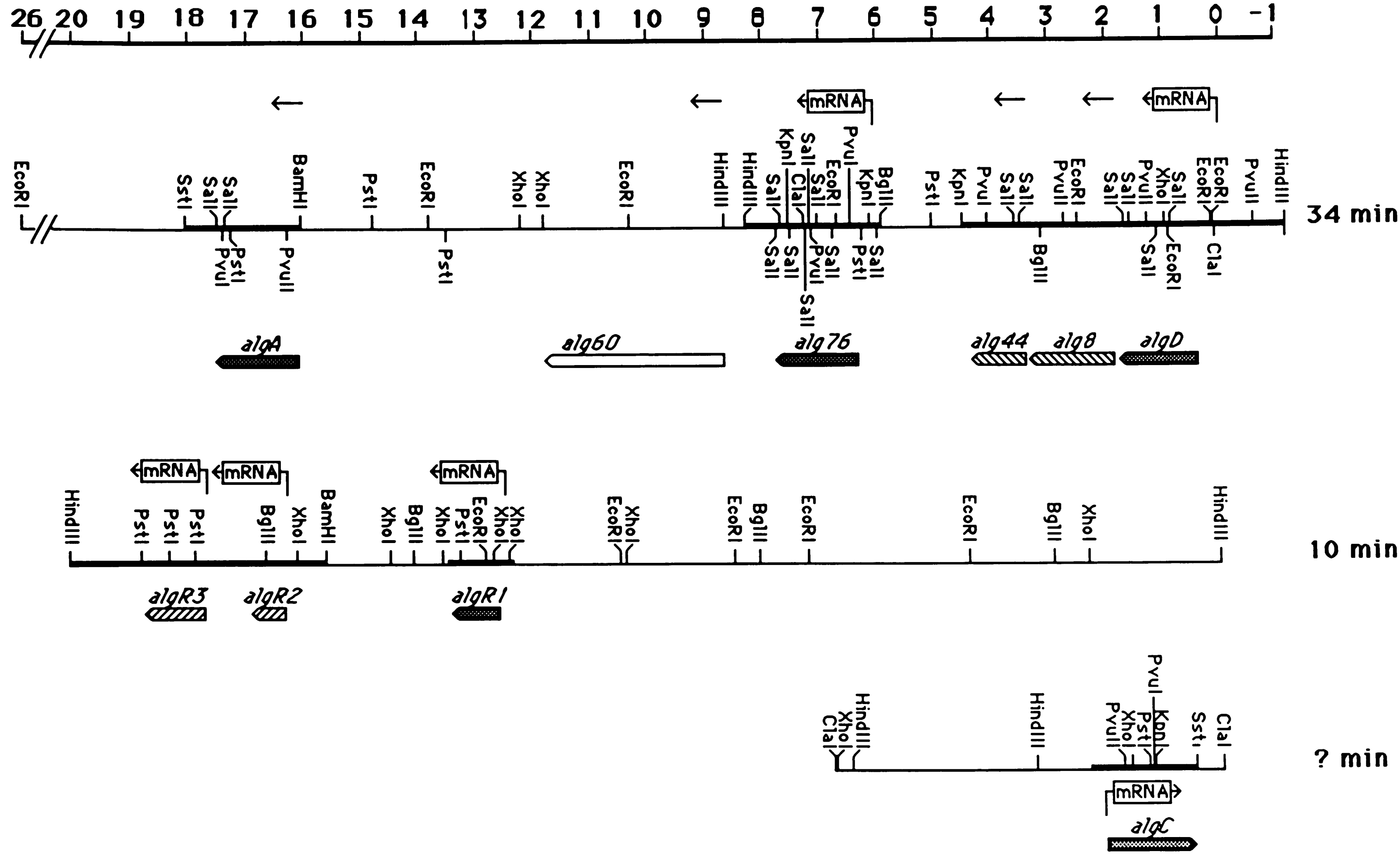

FIG. 3. Genetic map of alginate gene clusters. The locations of the three gene clusters on the *P. aeruginosa* chromosome are indicated in minutes. The location of the *algC* gene is not known and therefore is indicated by a question mark. The *P. aeruginosa* chromosome has been recalibrated so numbers represent the revised map positions. Restriction enzyme sites are indicated by vertical lines. The scale above the maps is in nucleotides (kilobases). ▬, Sequenced DNA; ——, unsequenced regions; ——→, direction of transcription; ⟵mRNA┐, direction of transcription and transcriptional start; ◀▆, protein encoded by the indicated gene and confirmed by N-terminal amino acid sequencing; ◀▨, protein-coding region predicted from DNA sequence analysis of the indicated gene; ◁▭, protein encoded by the indicated gene for which the exact coding region has not been determined; — // —, DNA regions extending to the *Eco*RI site at 26 kb. *algG*, encoding an enzyme involved in the synthesis of guluronate residues, has been mapped between the *alg-60* and *alg-76* genes by Chitnis and Ohman (19) but is not shown here. The *algS*, *algT*, and *algN* genes, which control switching and map at 68 min (51, 52, 57), and the *algB* gene, which enhances alginate production and maps at 13 min (58, 120), are also not shown.

TABLE 1. Alginate genes and enzymes

Gene	Promoter[a]	Polypeptide (size)	Function[b]	Reference(s)
algA	—	PMI-GMP (53 kDa)	Biosynthesis	24, 25, 55, 142, 150
algC	σ^{54}	PMM (51 kDa)	Biosynthesis	10, 170, 171
algD	σ^{54}	GMD (48 kDa)	Biosynthesis	31, 32, 34, 138, 139
alg-8	—	Alg8	Unknown	105, 160, 161
alg-44	—	Alg44 (41 kDa)	Unknown	109, 160, 161
alg-76	σ^{54}	Alg76 (54 kDa)	Unknown	20, 160, 161
alg-60	—	Alg60	Unknown	109, 160, 161
algG[c]	—	Epimerase	Biosynthesis	19
algR1	σ^{54}	AlgR1 (27.5 kDa)	Regulation	23, 30, 34, 82, 87
algR2 (*algQ*)	σ^{70}	AlgR2 (18 kDa)	Regulation	34, 83, 84, 91
algR3 (*algP*)	σ^{70}	AlgR3 (39 kDa)	Regulation	85, 91
algS	—	AlgS	Switch	51, 52
algT	—	AlgT	Switch	51, 52
algN	—	AlgN	Switch	120
algB	—	AlgB	Regulation	57, 58

[a] —, Uncharacterized promoters. The assignment of σ^{54}-recognizing promoters is based on the presence of a GG-N_{10}-GC motif and is only tentative.

[b] The genes of unknown function are thought to encode proteins involved in polymerization (biosynthesis). The switch function differs from the regulatory function in that the switch genes control the genotypic conversion to mucoidy and the regulatory genes control the levels of enzymes for alginate production once the switch has been turned on.

[c] *algG* has been defined only as a gene controlling the insertion of guluronate residues into the alginate polymer.

algA gene does not confer on the *manA* mutant strain of *E. coli* the ability to readily grow on mannose as the sole source of carbon. In addition, this result suggested that PMI could trap fructose 6-phosphate from the carbohydrate pool while other enzymes, particularly GMD, pull the biosynthetic reactions towards alginate synthesis. The two enzymatic activities may reside within different catalytic domains, since the substrates or products of one activity did not inhibit the other activity. In addition, these two enzymatic activities showed different requirements with respect to metal cofactors and reducing agents. A mutant *algA* gene has been sequenced and found to encode a polypeptide defective in both enzymatic activities. We are currently cloning the defective gene from other *algA* mutants in an attempt to determine whether PMI and GMP are contained on two independent enzyme domains.

PMM. As mentioned above, hyperproduction of the *algA* gene also results in increased levels of PMM activity. Berry et al. (9) used this observation to identify mutants defective in PMM activity by overexpressing the *algA* gene in other Alg$^-$ mutants and screening for the absence of PMM enzyme activity. One mutant, strain 8858, no longer demonstrated the concomitant increase in PMM activity upon IPTG induction of the *algA* gene. A *Bam*HI genomic-DNA library of the stable mucoid strain 8830 was then screened for recombinant plasmids that complemented strain 8858 to Alg$^+$ PMM$^+$. The plasmid pAB8, which contains the *algC* gene encoding PMM enzymatic activity, was found to complement *P. aeruginosa* 8858 (9). The gene encoding PMM activity resides within a 2.6-kb *Hin*dIII-*Sst*I fragment of pAB8 and encodes a single polypeptide with a subunit molecular mass of 51,000 (Fig. 3, Table 1; 10, 171). The *algC* mutant 8858 is not complemented by any of the alginate genes located at 34 and 10 min on the *P. aeruginosa* chromosome (171). Both the wild-type and mutant *algC* genes have recently been sequenced, and the transcriptional initiation site has been determined (170). The *algC* promoter contains a σ^{54} recognition (RpoN) sequence for RNA polymerase binding (Fig. 4, Table 1). The implications for this type of promoter will be discussed later. In addition, DNA-RNA hybridizations showed that *algC* is transcribed in both mucoid and nonmucoid strains of *P. aeruginosa*. We are currently purifying and characterizing the PMM enzyme for future studies aimed at identifying nontoxic inhibitors of PMM enzymatic activity. We will also study the regulation of this enzyme, since a high level of PMI-GMP appears to induce PMM activity. One interesting possibility is that mannose 6-phosphate, the product of the PMI reaction, acts as an inducer of PMM in *P. aeruginosa* (142).

GMD. Darzins and Chakrabarty (23) isolated the recombinant plasmid pAD2, which contains a 9.5-kb *Hin*dIII fragment that complements a number of the other Alg$^-$ mutants in the gene cluster located at 34 min on the *P. aeruginosa* chromosome (Fig. 3). Deretic et al. (31) subsequently cloned a 3.2-kb *Cla*I-*Bgl*II fragment of pAD2 under control of the *tac* promoter of the broad-host-range controlled-expression vector pMMB24. IPTG induction of this plasmid (pVD211) resulted in the synthesis of a 48-kDa polypeptide that corresponded to an increase in GMD enzymatic activity (Table 1; 31). DNA sequencing of *algD* and its promoter revealed multiple direct repeats extending 500 bp upstream of the translational start site (32). The *algD* promoter also contains a σ^{54} recognition (RpoN) sequence for RNA polymerase binding (Fig. 4, Table 1; 34). Transcription of the *algD* gene appears to be environmentally activated and controlled by at least two alginate regulatory genes,

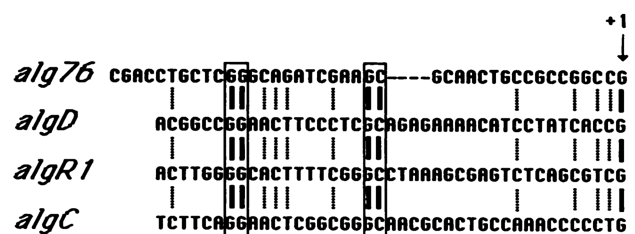

FIG. 4. Comparison of alginate gene promoter regions. The promoter DNA sequences are as indicated. Symbols: ❙, exact matches of the four promoters; ⁞, matches of three promoters; +1, mRNA start site; boxes, RpoN conserved sequence with 10 nucleotides between; ----, gaps introduced to align the *alg-76* mRNA start site with the consensus sequences.

algR1 and *algR2*. The regulation of this enzyme will be discussed in more detail below.

Roychoudhury et al. (139) have purified and characterized GMD, an NAD^+-dependent four-electron transfer dehydrogenase that catalyzes the oxidation of GDP-mannose to GDP-mannuronic acid (Fig. 2). GMD is of particular importance in alginate biosynthesis, since in addition to being subject to transcriptional regulation, it plays a pivotal role in alginate synthesis by catalyzing the mainly unidirectional conversion of GPD-mannose to GDP-mannuronate. The purified protein was found to be a hexamer. Kinetic analysis showed K_ms of 14.9 μM for GDP-mannose and 185 μM for the NAD^+ cofactor. A low K_m appears to be a general feature of alginate biosynthetic enzymes and may allow the low enzyme levels required for alginate biosynthesis by heavily mucoid strains of *P. aeruginosa*. Guanosine 5′-monophosphate was shown to be a potent competitive inhibitor of GMD activity (K_i, 22.7 μM), whereas mannose had no effect on enzymatic activity. Roychoudhury et al. (139) suggested that the guanosine moiety of GDP-mannose must bind first, thereby allowing the mannose moiety to enter the catalytic pocket. ATP was also found to inhibit GMD activity. Although the mode of ATP inhibition is not yet understood, it is interesting that the amino acid sequence of GMD revealed a potential ATP-binding site at the N-terminal end of the polypeptide (139). Roychoudhury et al. (138) have recently used site-directed mutagenesis to change cysteine 268, an amino acid residue thought to be part of the catalytic site, to serine. This alteration in the GMD polypeptide virtually abolished enzymatic activity, thereby substantiating the involvement of cysteine 268 in GMD enzymatic activity. Limited proteolytic cleavage of the wild-type GMD protein showed that the N-terminal part of GMD folds into a structural domain (M_r, 27,000) that is enzymatically inactive but relatively stable towards further proteolysis. The C-terminal domain (M_r, 16,000), containing the catalytic site, was also shown to be enzymatically inactive. In addition, the C-terminal domain was degraded to smaller peptide fragments by extended proteolysis. Stability of the enzyme towards proteolysis was markedly increased by GDP-mannose and guanosine 5′-monophosphate. This suggested the possibility that substrate (or inhibitor) binding leads to a conformational change that limits the accessibility of the cleavage sites to protease (138). Further mutagenesis of GMD will be used to identify the GDP-mannose- and NAD^+-binding sites. These experiments will aid in defining the various protein domains involved in GMD enzymatic activity.

Alginate polymerization. For the most part, the process by which GDP-mannuronate is incorporated into the alginate polymer has not been elucidated. We have obtained some evidence that a small polypeptide associated with the membrane of *P. aeruginosa* 8821 participates in polymer formation by binding GDP-mannuronate (109, 171). We are currently attempting to identify the protein associated with this activity. Unfortunately, development of an in vitro polymerization assay has eluded us. Much of the problem may be that the polymerase enzymes, like every other alginate biosynthetic enzyme studied thus far, are produced in extremely low levels even in *P. aeruginosa* strains that produce copious amounts of the polymer. The *alg-8*, *alg-44*, *alg-76*, and *alg-60* genes are found in the biosynthetic enzyme gene cluster located at 34 min on the *P. aeruginosa* chromosome (Fig. 3) and are most likely to encode the proteins responsible for polymerization of mannuronate residues and subsequent export of the polymer from the cell (Fig. 2, Table 1).

The *alg-8* gene, which resides within a 1.9-kb *Sal*I fragment (Fig. 3), was recently sequenced (105). DNA sequence analysis indicated that the gene product could be 55.5 kDa in size. The translational start site remains to be confirmed by N-terminal amino acid sequence analysis. The polypeptide predicted from the *alg-8* DNA sequence appears to be extremely hydrophobic (49% hydrophobic amino acid residues), indicating that Alg8 is most likely a membrane-bound protein. However, the Alg8 protein has not been directly demonstrated either by maxicell analysis or by hyperproduction of the protein.

The *alg-44* gene resides within a 1.9-kb *Bgl*II-*Pst*I fragment (Fig. 3). Maxicell analysis demonstrated that the *alg-44* gene encodes a 41-kDa polypeptide (161), and DNA sequence analysis predicted a protein having 44% hydrophobic amino acid residues (160). We recently demonstrated that the hyperexpression of *alg-44* from the *tac* promoter resulted in the appearance of a 41-kDa membrane-bound protein, as was expected from analysis of the DNA sequence (109). Enzymes involved in the polymerization of most bacterial polysaccharides are generally located in the cell membrane (155). Since the Alg8 and Alg44 proteins are both located in the membrane fraction, we believe that these proteins are probably involved in the alginate polymerization process.

Chu et al. (20) have recently determined the nucleotide sequence of a 2.4-kb *Bgl*II-*Hin*dIII fragment (Fig. 3) containing the *alg-76* gene. The transcription of *alg-76* appears to initiate from its own promoter. Interestingly, the promoter sequence was found to be similar in many respects to that of the *algD* promoter, suggesting that *alg-76* transcription may be regulated in a similar fashion (Fig. 4; see below). The Alg76 protein was hyperproduced from the *tac* promoter and found to be a 54-kDa polypeptide associated with the cell membrane fraction. N-terminal amino acid sequence analysis of the purified protein matched the sequence predicted by DNA sequence analysis. The Alg76 polypeptide appears to contain a 33-amino-acid signal sequence that is cleaved during export of the protein from the cytoplasm. Preliminary results from cellular fractionation studies suggested that Alg76 is located in the periplasmic space yet is tightly associated with the exterior of the cytoplasmic membrane. We speculate that Alg76 may function in later stages of polymerization or in export of the polymer or both. We are currently developing an assay system for testing this possibility.

The *alg-60* gene, which resides within a 3.2-kb *Xho*I-*Hin*dIII fragment (Fig. 3), was found to encode a 55-kDa polypeptide (161). Interestingly, hyperexpression of the *alg-60* gene resulted in a 3.5-fold increase in GMD activity. This suggested that the Alg60 protein either directly regulates GMD expression or catalyzes a biosynthetic step that utilizes a feedback inhibitor of GMD enzymatic activity as a substrate. We favor the latter possibility, since the *alg-60* gene lies within a gene cluster that has been found to encode alginate biosynthetic enzymes (Fig. 3). However, hyperproduction of the Alg60 protein has been found to inhibit the mucoid mode of growth by strains 8821 and 8830 (109, 161). Only further analysis of the *alg-60* gene and the corresponding gene product will determine which possibility is correct.

Regulation of Alginate Synthesis

Regulatory genes. It has become apparent in recent years that a distinct set of genes respond to the environment of the

CF-affected lung, cause normally nonmucoid *P. aeruginosa* to switch to a form that produces alginate, and also cause an increase in the levels of key alginate biosynthetic enzymes. Fyfe and Govan (54) identified a regulatory region (*muc*) located at 68 min on the *P. aeruginosa* chromosome that contains the alginate conversion genes controlling the spontaneous switch between the mucoid and nonmucoid phenotypes. Flynn and Ohman (51) subsequently identified two tightly linked genes, designated *algS* and *algT*, within the *muc* region. The cloned *algS* gene was found to complement the spontaneous *algS* mutants only when integrated into the chromosome (i.e., *cis* complementing), whereas a plasmid-borne *algT* gene was capable of complementing *algT* mutants (i.e., *trans* complementing). *algS* is thought to be the genetic switch for induction of alginate synthesis by *P. aeruginosa* within the CF-affected lung, where *algS* activates *algT*, resulting in spontaneous alginate conversion (Table 1; 52). Another gene, *algN*, prevented *trans* complementation by *algT* and is thought to be a negative regulator of alginate synthesis (Table 1; 120). The conversion mechanism is not clear. Alginate conversion has been shown to be *recA* independent (121), and there is no evidence for gross rearrangements of the *algS* gene when mucoid and nonmucoid forms of *P. aeruginosa* are compared (52). This may suggest that subtler changes in *algS* are involved in the switching mechanism. Goldberg and Ohman (57, 58) have also isolated a temperature-sensitive *alg* mutant (Alg$^+$ at 37°C, Alg$^-$ at 42°C) that could be complemented by a gene, designated *algB*, located at 13 min on the *P. aeruginosa* chromosome. The *algB* gene may be involved in the production of high levels of alginate (Table 1), although it is obviously not essential for alginate synthesis, since low levels of alginate biosynthesis are observed in *algB* mutants (58).

Darzins and Chakrabarty (23) isolated a recombinant plasmid, pAD1, containing a 20-kb *Hin*dIII fragment of *P. aeruginosa* 8830 chromosomal DNA that slowly induced alginate synthesis in the spontaneous nonmucoid strains 8822 as well as in other nonmucoid laboratory strains of *P. aeruginosa*. The plasmid pAD1 was also found to complement both the *alg-22* and *alg-52* mutations of Alg$^-$ strains 8852 and 8882, respectively. The 20-kb *Hin*dIII fragment of pAD1 was mapped to the 10-min region of the *P. aeruginosa* chromosome (Fig. 3). Deretic et al. (33) found that growth of a *rec-2* strain of *P. aeruginosa* on subinhibitory concentrations of kanamycin not only induced alginate synthesis but also resulted in a four- to sixfold amplification of this putative regulatory region. Spontaneous nonmucoid revertants showed a reduction in the copy number of the amplified region, thereby suggesting a direct relationship between amplification of the regulatory region and the induction of alginate synthesis (33).

The *algD* gene, which codes for the GMD protein, has been shown to be transcriptionally activated in mucoid, but not nonmucoid, strains of *P. aeruginosa* (8, 31). Thus, transcriptional activation of *algD* is necessary for alginate production. Deretic et al. (32) went on to show that the *algD* promoter region contains multiple direct and inverted repeats upstream of the transcriptional and translational start sites that could be involved in *algD* regulation. A transcriptional fusion plasmid pVD2X was used to screen the various Alg$^-$ mutants for a positive regulatory gene for *algD* transcription. The transcriptional fusion system used was constructed by cloning the promoterless *xylE* gene, encoding catechol 2,3-dioxygenase activity (detected colorimetrically), downstream of the *algD* promoter (31). The *alg-22* mutant strain 8852 containing pVD2X showed greatly reduced levels of *algD* transcription (32). This suggested that the plasmid pAD1, which complements the 8852 mutation, contained a positive regulatory gene(s) required for alginate biosynthesis.

The regulatory gene that complemented the 8852 mutation is contained within an internal 6.2-kb *Bgl*II fragment of pAD1 (Fig. 3, Table 1) and was designated *algR1* (originally *algR*; 23, 30). Hyperexpression of the *algR1* gene from the *tac* promoter resulted in the synthesis of a 27.5-kDa polypeptide (82). The nucleotide sequence of *algR1* was recently determined (30, 34). DNA sequence analysis suggested that the *algR1* gene belongs to a class of environmentally responsive bacterial regulatory genes including *ompR*, *phoB*, *sfrA*, *ntrC*, *spo0A*, *dctD*, and *virG* (137). These transcriptional activators control the cellular responses to osmotic pressure, phosphate limitation, or chemical compounds found in a particular environmental niche (137). The *algR1* gene product appears to be in the regulatory (receiver) class of proteins rather than the sensory (transmitter) class of the two-component sensory transduction systems (34). Furthermore, the *algR1* promoter was found to contain a σ^{54} recognition (RpoN) sequence for RNA polymerase binding (Fig. 4, Table 1). Transcription of *algR1* was very low in RpoN mutant strains (87), although the involvement of RpoN in the activation of alginate promoters has recently been disputed (114). The presence of an RpoN-like promoter in *algR1* and *algD* suggests that the unique environmental conditions of the CF-affected lung may participate in the regulation of mucoidy via transcriptional activation of both *algR1* and *algD* (34, 87). Perhaps other *alg* promoters (i.e., those having RpoN-like sequences) are also transcriptionally activated in response to the environment of the CF-affected lung.

An additional regulatory gene, designated *algR2* (Table 1), was localized to a 4.4-kb *Hin*dIII-*Bam*HI fragment of pAD1 and was found to complement the mutation in strain 8882 (Fig. 3; 34, 84). The AlgR2 protein appears to function as an attenuator of alginate biosynthesis by acting cooperatively with the AlgR1 protein to increase the level of *algD* activation (84). Overexpression of the *algR2* gene from the *lac* promoter resulted in the synthesis of an 18-kDa polypeptide in *E. coli* (83). When cloned on a moderate-copy-number vector (pMMB66EH), the *algR2* gene induced the nonmucoid strain 8822 to switch back to mucoidy (84). Other nonmucoid strains of *P. aeruginosa* do not respond to *algR2* in this fashion (84). A third regulatory gene, *algR3* (Table 1), was also identified within the 4.4-kb *Hin*dIII-*Bam*HI fragment of pAD1 on the basis of a somewhat inefficient complementation of strain 8882 (Fig. 3; 85). DNA nucleotide sequence analysis predicted that the *algR3* gene product would be a highly basic regulatory protein, and an in vitro-coupled transcription-translation system showed that *algR3* encoded a 39-kDa polypeptide (85). Interestingly, the predicted amino acid sequence is quite similar (44% identity) to that of the histone H1 protein from the sea urchin *Lytechinus pictus* (85). This is the first report of a bacterial histonelike protein that has significant homology to eukaryotic histone H1. Hulton et al. (78) recently showed that the *osmZ* gene encodes a histone H1-like protein that may regulate changes in DNA supercoiling in response to environmental signals such as osmolarity. It will be interesting to find out what role the AlgR3 histonelike protein plays in alginate synthesis and whether it, too, activates the *algD* promoter by controlling the degree of supercoiling, especially since supercoiling is necessary for activation of the

algD promoter (34, 35). Both *algR2* and *algR3* were found to contain σ^{70} RNA polymerase recognition sequences and are constitutively expressed in both mucoid and nonmucoid strains of *P. aeruginosa* (84, 85). However, the expression of these two regulatory genes was higher in mucoid strains than in nonmucoid strains (84, 85). Konyecsni and Deretic (91) subsequently described the regulatory genes *algP* and *algQ*. Comparison of the restriction enzyme maps and the nucleotide sequences containing *algQ-algP* and *algR2-algR3* shows that *algQ* and *algP* are analogous to *algR2* and *algR3*, respectively. We will refer to these regulatory genes as *algR2* and *algR3* to prevent confusion. It is important to note that neither AlgR2 nor AlgR3 shows homologies to the transmitter (sensory) class of proteins of the two-component sensory transduction system. The environmental sensor(s) for alginate synthesis remains undefined to date.

Environmental activation. The *algD* promoter region shows homology to a number of bacterial promoters that are induced in response to environmental stress. These include the osmoregulated promoters for the *E. coli* outer membrane porin proteins OmpF and OmpC (8, 87). In addition, the *algD* promoter has been found to contain a σ^{54} (RpoN) recognition sequence (87) as well as two putative AlgR1-binding sites in the far-upstream region (82). Likewise, the AlgR1 protein shows homology to environmentally responsive regulatory proteins such as OmpR, the regulator of *ompC* and *ompF* gene expression (8, 30, 34). The *algR1* promoter quite interestingly shows a distinct similarity to the upstream promoter region of *algD* (34) as well as to the *ompF* and *ompC* promoters (87). The σ^{54} RNA polymerase recognition sequence (GG-N_{10}-GC) may be a feature common to many *P. aeruginosa* alginate promoters, since the *algC* and *alg-76* promoters also contain the putative σ^{54} sequence (Fig. 4, Table 1). One consistent deviation from the σ^{54} consensus sequence of enteric bacteria (CT<u>GG</u>YAYR-N_4-TT<u>GC</u>A, where <u>GG</u> and <u>GC</u> residues are at positions −24 and −12) is that *P. aeruginosa alg* σ^{54} promoters are shifted several base pairs upstream of their normal locations (−33 and −21) relative to the mRNA transcriptional start site.

Since the *algD* promoter showed similarities with those of environmentally regulated genes, we introduced the *algD-xylE* transcriptional fusion vector (pVD2X) into OmpR⁻ *E. coli* harboring plasmids containing *ompR*, *algR1*, *algR2*, or both *algR1* and *algR2* and then tested the effect of NaCl on transcriptional activation of the *algD* promoter (8, 9, 84). Growth in 300 mM NaCl was found to result in a significant increase (>4-fold) in *algD* promoter activity compared with that of cells grown in the absence of added salt, and *algD* activation was 10- to 20-fold higher in the presence of these regulatory proteins than in their absence. Most surprisingly, OmpR was capable of activating the *algD* promoter nearly as well as AlgR1 did in *E. coli*. This was an interesting demonstration of crosstalk between environmentally controlled regulatory proteins (8). The presence of both the *algR1* and *algR2* gene products further stimulated *algD* activation by threefold and indicated that the AlgR1 and AlgR2 proteins act cooperatively in the induction of *algD* expression (84). Furthermore, subinhibitory concentrations of the DNA gyrase inhibitors nalidixic acid and novobiocin were found to reverse *algD* activation by NaCl in *E. coli* (8, 9, 34), indicating that negative supercoiling of the promoter DNA also plays a role in the activation of *algD* transcription (34). Kimbara and Chakrabarty (87) constructed an *algR1-xylE* promoter fusion and showed that *algR1* is also transcriptionally activated by NaCl in *E. coli*.

Transcriptional activation of *algD* was also observed when mucoid (8821) and nonmucoid (PAO1) forms of *P. aeruginosa*, both containing pVD2X, were grown in media of increasing osmolarity (8). The degree of *algD* activation was the same when these strains were grown in 0.44 M sucrose or in an iso-osmotic concentration of NaCl or KCl. Expression of genes encoding nonrelated enzymes such as glucose 6-phosphate dehydrogenase was unaffected by medium osmolarity. These results demonstrated that the response of *algD* to these environmental stimuli was osmolarity specific. Curiously, increased osmolarity did not induce alginate biosynthesis in nonmucoid strains of *P. aeruginosa*, although alginate production by mucoid strain 8821 was twofold greater (8, 9). Phosphate and nitrogen limitation conditions have also been found to activate *algD* in a similar fashion (34).

Alginate production is thought to be a mechanism by which *P. aeruginosa* resists dehydration in the CF-affected lung (67). Thus, DeVault et al. (35) tested the effect of a known dehydrating agent, ethanol, on the transcription of *algD* and alginate biosynthesis. The addition of up to 1% ethanol to the growth media stimulated *algD* transcription, as measured by plasmid pVD2X, in both mucoid and nonmucoid strains of *P. aeruginosa*. *P. aeruginosa* containing a plasmid with the *xylE* gene under control of the *tac* promoter was unresponsive to ethanol, clearly showing that ethanol-induced activation was specific for *algD*. In addition, neither ethanol, phosphate, nor nitrogen was able to activate *algD* transcription in *E. coli* strains containing pVD2X. A possible explanation is that *E. coli* lacks a specific sensory protein(s) present in *P. aeruginosa* (35). Interestingly, growth of the nonmucoid strains 8822 and PAO1 on nutrient agar plates containing 3 to 5% ethanol resulted in mucoid variants after extended growth periods (6 to 14 days). However, the spontaneous nonmucoid revertant strain 8822 switched to alginate production in response to ethanol at a substantially greater frequency (10^{-4}) than was observed for PAO1. Ethanol-induced mucoidy appeared to be fairly stable in the absence of the dehydrating agent (i.e., the organisms were genotypically mucoid), and any nonmucoid variants that were obtained reverted to mucoidy upon passage on ethanol-supplemented plates. Other environmental conditions tested (anaerobiosis, pH extremes, and temperature) had only a marginal effect on both *algD* promoter activation and conversion to mucoidy (35). Strain 8830 isolates that were selected for resistance to high levels of nalidixic acid lost the ability to synthesize alginate. Furthermore, *algD* activation in response to ethanol was greatly reduced by DNA gyrase inhibitors (like nalidixic acid) and in *P. aeruginosa* PAO515 containing an altered DNA gyrase activity. This further supported the evidence that a functional DNA gyrase is required for activation of *algD* transcription and subsequent alginate production (35). We speculate that the requirement of a functional DNA gyrase and an RpoN-recognizing promoter in the transcription of *algD*, as well as binding of AlgR1 at sites considerably distant (−382- and −458-bp regions) from the RNA polymerase-binding site (−21 to −33 bp), may in turn require DNA looping for initiation of *algD* transcription. It appears that promoters, such as the *algD* promoter, require activators that act at a distance (143). A model for AlgR1 activation of the *algD* promoter via DNA looping is presented in Fig. 5. It is also interesting to note that the 14-mer AlgR1 binding sequence (CCGTTCGTCN$_5$; 82) occurs upstream of the *algC* gene (−91-bp region). *algC* gene expression, as determined by *algC-lacZ* fusions, is substantially reduced in an AlgR1⁻ mutant background

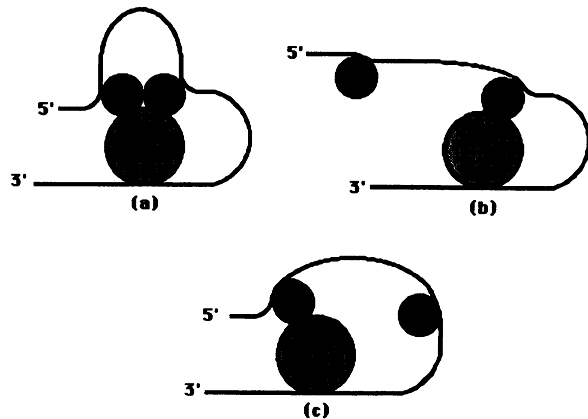

FIG. 5. DNA looping model of *algD* promoter activation by AlgR1. AlgR1 binds to two far-upstream sites (−382- and −458-bp regions) of the *algD* promoter. Symbols: ●, AlgR1 polypeptides; ●, RNA polymerase-σ^{54} complex. The binding of two AlgR1 molecules is additive, allowing maximal activation of the *algD* promoter (structure a). Binding of a single AlgR1 molecule at either of the upstream sites (structures b and c) allows a somewhat less effective contact with the RNA polymerase-σ^{54} complex that results in reduced activation of the *algD* promoter.

(170), thereby suggesting that AlgR1 may be a common activator of *algC*, *algD*, and perhaps other alginate genes.

CONCLUSIONS

The last decade has seen a great increase in our understanding of the molecular biology and biochemistry of alginate synthesis by *P. aeruginosa*. We and other workers have cloned and sequenced many of the alginate genes and have purified a number of the biosynthesis enzymes. Our unraveling of the regulatory mechanisms governing alginate biosynthesis has shown that the regulation of alginate synthesis is complex. Most important, we have identified two potential conditions in the CF-affected lung (high NaCl, dehydration) that activate the *algD* promoter and may participate in the transition of the organism to mucoidy (alginate production). However, other promoters (particularly the RpoN-like promoters) may play an important role in this transition. We have yet to reach our primary goal of understanding the basic mechanisms involved in the induction, regulation, and biosynthesis of alginate in the CF-affected lung. Our hope is to find inhibitors that interfere with the regulation and biosynthesis of alginate by *P. aeruginosa* that are both nontoxic and able to effectively reach the site of infection in the lung. Although this is a formidable task, the potential benefit to patients afflicted with CF is great. Elimination of the protective alginate layer of *P. aeruginosa* might render the bacterium more susceptible to attack by the host's immune system and to antibiotic therapy. Pier et al. (126) have taken a slightly different approach by eliciting antibodies (immunizing) against the alginate capsule. Their promising results suggest that these antibodies protect rats and mice against intratracheal inoculation of mucoid *P. aeruginosa*. It is hoped that *P. aeruginosa* infection of the CF-affected lung will soon be eradicated. These approaches to studying the role of alginate biosynthesis in the lungs of CF patients and finding new ways to eliminate the protective barrier may truly bring molecular biology to the bedside in the near future.

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