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Distribution of Alginate Genes in Bacterial Isolates from Corroded Metal Surfaces

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Abstract. The distribution of alginate genes encoding biosynthesis of alginate was examined for bacterial isolates associated with corrosive biofilms recovered from source water, cooling lines, and reactor surfaces of a nuclear power plant. A total of 120 diverse Gram-positive and -negative isolates were obtained. Using DNA:DNA hybridization, 11 isolates were shown to contain sequences homologous to structural (*algD, algG, alg-76*) and/or regulatory (*albB*) alginate biosynthetic genes derived from an alginate-producing cystic fibrosis isolate of *Pseudomonas aeruginosa* (FRD1). Identification of isolates was accomplished by fatty acids methyl esters (FAME) analysis and the Biolog identification system. Nine of the twelve isolates were identified as various *Pseudomonas* spp., and two additional Gram-negative isolates were tentatively identified as *Aeromonas veronii* and *Stenotrophomonas maltophilia*. The remaining isolate was identified as a Gram-positive *Bacillus pumilus*. The results of the investigation extend current knowledge on the distribution of alginate biosynthetic genes in environmental isolates and permits the development of a more environmentally realistic model system to investigate the role of exopolymer production in biofilm formation and biocorrosion processes.

Introduction

Exopolysaccharide (EPS) synthesis is a common characteristic of many bacterial species and is associated with pathogenesis, symbiosis, competition, and survival. EPS's have become important model systems to investigate these interactions as well as molecular aspects of macromolecule assembly, secretion, and gene regulation [2, 11, 13, 17, 19]. The role of EPS is recognized in early stages of microbial adhesion and colonization of surfaces and maturation of biofilm communities [2, 4, 19, 23]. Environmentally, EPS may have important positive and negative conse-

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quences of biofilm formation associated with waste treatment systems, nutrient scavenging, toxicant immobilization, biofouling, and biocorrosion processes [2, 4, 19].

Recently, to extend EPS as a model system in biofilm formation, bioluminescent alginic acid (*alg-lux*) transcriptional gene fusions were created to study the induction of alginate EPS during formation and maturation of microbial biofilms [26]. The alginate model system was developed in a *Pseudomonas aeruginosa* cystic fibrosis isolate with the intent of transferring the reporter gene system to environmentally relevant strains for analysis of the functional role of EPS in biofilm processes.

Alginate is a copolymer of D-mannuronic acid and L-guluronic acid linked by β-1-4 bonds. The alginate exopolymer is anionic and has the ability to bind to metal surfaces and accumulate metal ions into the exopolymer [12,21]. Gum arabic acid, bacterial culture supernatant, *P. atlantica* exopolymer, and alginic acid have been examined for their corrosive properties on copper surfaces [12, 16]. Alginic acid was found to be the most corrosive of the four polymers, and copper ions were incorporated into the polymer matrix [12, 16].

Alginate is naturally produced by a variety of pseudomonads including *P. aeruginosa* [3, 11]. Recent studies have demonstrated the presence of alginate genes in the *Pseudomonas* RNA homology group 1 [9]. In addition to *P. aeruginosa,* many bacterial strains such as *P. glycinea, P. phaseolicola, P. fluorescens, P. cichorii,* and *Azotobacter vinelandii* naturally produce alginate. Other strains of *Pseudomonas* have been reported to produce alginate after exposure to mutagens or repeated transfers in the presence of antibiotics [9]. Therefore, it would appear that many types of bacteria contain some or all the genetic machinery necessary for alginate biosynthesis, however, specific environmental stimuli (stress signals) may be required to induce the production of alginate [1, 8].

The major objectives of this study were (a) to establish possible links between the bacterial exopolymer alginate and adhesion of microbes to metal surfaces and the corrosion of these surfaces, and (b) to identify suitable environmental isolates as future hosts for a *algD-lux* plasmid construction [27]. An environmental strain containing the *algD-lux* plasmid will allow development of an in situ bioluminescent reporter system to examine if and when alginate biosynthesis is induced during bacterial attachment to metal surfaces, and the possible real time assessment of alginate biosynthesis during biocorrosion processes.

Materials and Methods

Bacterial Strains, Media, and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Table 1 and Table 2, respectively. Cells were routinely cultured in complex medium that contained the following per liter distilled water: Tryptone, 10 g; yeast extract, 5 g; and sodium chloride, 5 g. The complex medium (Luria broth or agar) was used to grow large quantities of cells for the purpose of DNA isolation. Liquid media were solidified by the addition of 1.5% (w/v) Bacto agar. Media were sterilized by autoclaving at 15 psi for 30 min. Liquid cultures of cells were grown at 28°C in Erlenmeyer flasks, fitted with gas permeable tops, which contained culture volumes not exceeding one-tenth the nominal flask volume, and aerated by rotation at 240 rpm.

Table 1. Plasmids used in this study

Plasmid	Vector	Insert	Source/reference
pKK61	pLAFR1	Alginate biosynthetic gene cluster	D. Ohman [29]
pKK223-3	pJG1	1.74 kb *EcoR*1-*Xho*1 insert of *algB*	D. Ohman [29]
pUTK55	Bluescript[a]	1.35 kb *EcoR*1-*Cla*1 insert of *algB* gene	This study
pUTK56	Bluescript	0.6 kb *Hind*III-*Hind*III insert of *algG* gene	This study
pUTK57	Bluescript	1.8 kb *Hind*III-*EcoR*1 insert of *alg-76* gene	This study
pUTK58	Bluescript	2.2 kb *Xho*1-*Hind*III insert of *algD* gene	This study

[a] Stratagene, La Jolla, Calif.

Table 2. Tentative identification and source of environmental bacterial strains demonstrating DNA homology with alginate genes from *P. aeruginosa*

Bacteria[a]	Similarity index[b]	Source
Pseudomonas aureofaciens	0.821 (F)	Essential electrical cooling water drain water
Bacillus pumilus	0.531 (F)	Reactor tubercle (interior)
Pseudomonas putida	0.796 (F)	Reactor vessel (solid debris)
Pseudomonas putida	0.408 (F)	Water storage tank
Pseudomonas fluorescens C	0.811 (B)	Tubercle attached to weld
Pseudomonas fluorescens E	0.575 (B)	Reactor vessel (solid debris)
Stenotrophomonas maltophilia	0.810 (B)	Raw cooling water header (dark colored)
Pseudomonas syringae	0.795 (F)	Raw cooling water header (light colored)
Aeromonas veronii	0.928 (B)	Water pipe inlet nodules
Pseudomonas aeruginosa	0.724 (B)	Pipe section (6″) with tubercles at site of weld
Pseudomonas alcaligenes	0.940 (B)	Tubercles on carbon steel
Pseudomonas alcaligenes	0.940 (B)	Pipe section (surface)

[a] One-hundred-twenty bacterial isolates from various locations at the Watts Bar Nuclear Power Plant facility were screened by colony hybridization with the *algD* gene probe; only 11 strains demonstrated DNA homology with *algD*

[b] Similarity indexes were determined using FAME analysis [26] and the Biolog Identification System (Hayward, Calif.). Similarities showing the best match are shown here. FAME and Biolog Identification System are abbreviated by F and B, respectively

Identification of Bacterial Isolates

Identification of bacterial strains was accomplished by fatty acids methyl esters (FAME) analysis and the Biolog Identification System. Analytical Services (Essex Junction, Vt.) performed the Biolog identifications.

DNA Isolation and Quantitation

The procedure employed for total DNA isolation was as described by Golberg and Ohman [15]. The spectrophotometric method of DNA quantitation has been previously described [24]. The amounts of DNA were standardized and all methods were identical for preparation and probing each sample, therefore band intensities suggest the degree of DNA homology between alginate genes from environmental isolates and *P. aeruginosa*.

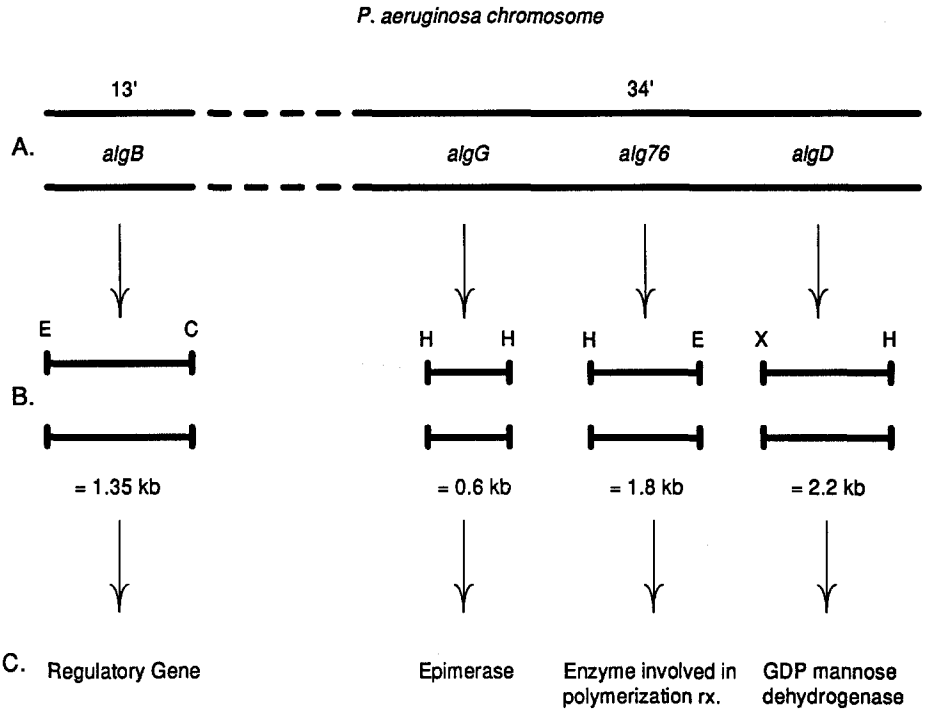

Fig. 1. **A** Location of the alginate genes on the *Pseudomonas aeruginosa* chromosome. **B** restriction fragments isolated and used to probe the genomic DNA of environmental bacterial isolates. Restriction enzyme abbreviations are as follows: E = *EcoR1*, C = *ClaI*, H = *Hind*III, X = *Xho*I. **C** Gene function.

DNA Probes

The *algB* regulatory gene and the 23 kb DNA region containing the biosynthetic gene cluster were provided courtesy of Dennis Ohman. In this study, specific DNA regions containing a particular gene function were isolated to use as gene probes (Fig. 1). The *algD* probe was a 2.2 kb *Xho*1-*Hind*III DNA fragment which contained part of the GDP mannose dehydrogenase structural gene and the far upstream regulatory region of the *algD* gene [20]. The *algG* gene probe was composed of a 0.6 kb *Hind*III-*Hind*III fragment internal to the *algG* gene. The *alg-76* gene probe was a 1.8 kb *EcoR*1-*Hind*III fragment containing most of the alg-76 gene. The *algB* gene probe was a 1.35 kb *EcoR1*-*Cla*1 fragment containing ~97% of the *algB* regulatory gene. Each DNA probe construction is schematically depicted in Fig. 1.

Preparation of Probe DNA

Plasmid DNA was purified in cesium chloride density gradients as previously described [24]. Purified plasmid DNA was digested with the appropriate restriction enzymes, after which the restriction mixture was electrophoresed on agarose gels. Specific gene fragments were isolated by excising the DNA fragment of interest from the gel and purifying the fragments using either the Stratagene gene clean system [24] or digestion of agarose with agarose [24].

Nick Translation Procedures

DNA probes were labelled [^{32}P]CTP using the BRL (Gaithersburg, Md.) procedure described by the manufacturer.

Colony Hybridizations

Bacterial strains were transferred from agar petri plates onto nylon filters. The membrane-attached colonies were then lysed by placing the filter colony-side-up in pools of 0.5 *M* NaOH for 1 min. The filters were then neutralized in 1.5 *M* NaCl, 1.0 *M* Tris-HCl pH 8.0, were allowed to air dry, and then baked for 1 h at 80°C. Nick translated DNA probes were added to membranes and incubated 10–12 h at 65°C. Nylon filters were then washed (high stringency) three times at 65°C and dried, and Kodak (New Haven, CT) imaging film was placed over filters and incubated 12 h at minus 80°C [25].

Slot Blot Techniques

Purified DNA samples were added to nitrocellulose membranes while under vacuum filtration. The nitrocellulose filters were baked at 80°C for 1 h to fix the DNA to the membrane. The hybridizations, washes, and film development were as described above for the colony hybridizations. Autoradiograms were quantified using a Bioimage Visage 110 analytical imager (Millipore, Ann Arbor, Mich.). A *Hafnia* strain was included as a negative control for the hybridization studies. *Hafnia* is known to produces copious amounts of non-alginate exopolymer. *P. aeruginosa* FRD1, the host strain for the isolation of gene probes, was used as the positive control.

Results

The 120 environmental organisms analyzed in this study were previously recovered from a variety of environments within a nuclear power plant facility. Organisms were isolated from source water, cooling lines, and reactor surfaces (Table 2). In addition, the attached organisms were taken from surfaces that ranged from extremely corroded (tubercles) to surfaces with only minor corrosion (Table 2). The *algD* probe, which contains the regulatory region and a portion of the structural gene GDP mannose dehydrogenase (Fig. 1), was used to screen 120 environmental isolates by colony hybridization. Of the 120 bacteria screened, 11 isolates demonstrated homology to the *algD* gene probe. The *algD* probe was chosen because it is unique to the alginate biosynthetic pathway and is the key step in committing the cell to alginate biosynthesis [6,7]. Initial characterization of the 11 isolates indicated that 10 isolates were Gram-negative, dominated by *Pseudomonas* spp. but including *Stenotrophomonas* spp. and *Aeromonas* spp. (Table 2). The remaining isolate showing homology to *algD* was found to be a Gram-positive organism most closely resembling *Bacillus pumilus,* and to our knowledge represents the first Gram-positive organism to demonstrate homology to the biosynthetic *algD* gene.

Bacterial isolates that demonstrated homology to *algD* were further examined by slot blot hybridization methods. The *alg-76* and *algG* DNA probes were used to determine if other biosynthetic genes were present in these strains. The *algB* gene probe was selected to determine if the environmental strains demonstrated homology with a regulatory gene in addition to genes that encode for enzymatic reactions.

Table 3. Comparison of quantitative DNA blot hybridization analysis of environmental isolates treated with probes and giving positive results with structural or regulatory alginate genes

Bacteria	DNA probes optical density[a]			
	algD	*algG*	*alg-76*	*algB*
Pseudomonas aeruginosa (FRD1)	218	170	55	143
Hafnia	0	0	0	0
Pseudomonas aureofaciens	5	39	28	142
Bacillus pumilus	6	19	4	0
Pseudomonas putida	187	1	0	54
Pseudomonas putida	74	8	67	29
Pseudomonas fluorescens C	128	307	116	124
Pseudomonas fluorescens E	0	0	0	57
Stenotrophomonas maltophilia	21	19	7	22
Pseudomonas syringae	38	111	2	43
Aeromonas veronii	20	0	0	7
Pseudomonas aeruginosa	200	105	15	150
Pseudomonas alcaligenes	123	53	22	17
Pseudomonas alcaligenes	34	17	3	27

[a] DNA probes optical density: as integrated optical density obtained by scanning DNA blots with a Bioimage Visage 110 (Ann Arbor, Mich.)

In *P. aeruginosa, algB* is a regulatory gene that interacts with *algD* to control the amount of alginate produced [14, 29].

The *algD* gene probe hybridized strongly to environmental isolates *P. putida, P. fluorescens, P. syringae, P. aeruginosa, P. alcaligenes, Aeromonas veronii,* and *Stenotrophomonas maltophilia.* A weak hybridization signal was observed with strains *P. aereofaciens* and *B. pumilus* (Table 2, Fig. 2). The other structural gene probes *algG* and *alg-76* demonstrated similar results as *algD* with the following exceptions: one strain of *P. putida* demonstrated very weak homology with *algG* and no homology to *alg-76. A veronii* demonstrated no homology with *algG* and *alg-76* DNA probes. One of the *P. alcaligenes* isolates also demonstrated a weak hybridization signal with the *alg-76* gene probe (Table 2, Fig. 2). The *algB* probe hybridized with DNA from all strains, including DNA from *P. fluorescens E* which had not shown any DNA homology with the *algG* or *alg-76* gene probes (Table 2, Fig. 2).

Discussion

Past investigations have suggested alginate production by *P. aeruginosa* was unique to the environment of the cystic fibrosis lung [1, 6, 7, 8, 9, 18]. However, it is very doubtful if bacteria, which are found predominantly in the soil and water, developed this energy-expensive and highly regulated pathway for alginate biosynthesis to inhabit the lungs of humans. It is more likely that bacteria evolved alginate production as a survival mechanism to the harsh environmental conditions that usually prevail in the soil and water environments. Several environmental stress signals which induce alginate synthesis in *P. aeruginosa* have been identified.

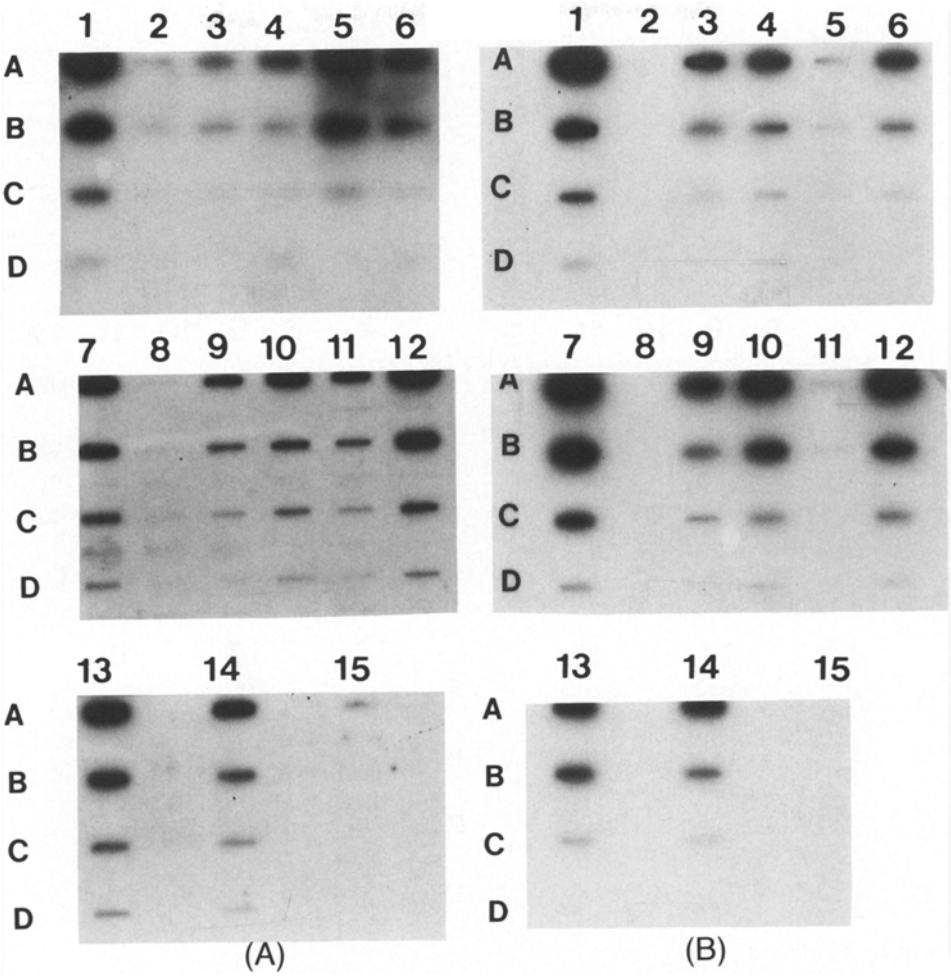

Fig. 2. DNA from environmental isolates probed with: (A) *AlgD* probe; (B) *AlgG* probe; (C) *Alg-76* probe; and (D) *AlgB* probe. Total DNA from the following isolates was loaded as follows: *Pseudomonas aeruginosa* FRD1, lane 1; *Hafnia,* lane 2; *Pseudomonas aureofaciens,* lane 3; *Bacillus pumilus,* lane 4; *Pseudomonas putida,* lane 5; *Pseudomonas putida,* lane 6; *Pseudomonas fluorescens C,* lane 7; *Pseudomonas fluorescens E,* lane 8; *Stenotrophomonas maltophilia,* lane 9; *Pseudomonas syringae,* lane 10; *Aeromonas veronii,* lane 11; *Pseudomonas aeruginosa,* lane 12; *Pseudomonas alcaligenes,* lane 13; *Pseudomonas alcaligenes,* lane 14. The letters indicate the amounts of DNA loaded on to nylon filter as follows: **a** 1 μg; **b** 0.33 μg; **c** 0.11 μg; **d** 0.037 μg; **e** 0.012 μg. (Lane 15, Blank)

Stress signals, such as membrane dehydration and osmolarity changes have been shown to induce alginate biosynthesis [1, 8].

The genetic pathway and biochemistry of alginate biosynthesis in *P. aeruginosa* has been studied because of alginate's role in the cystic fibrosis patient [1, 8]. Excellent studies have located both the regulatory and structural genes for alginate biosynthesis [3, 13, 18, 20, 22, 28]. The biosynthetic pathway for alginate funnels carbohydrates into the formation of the exopolymer. In the first reactions, *algA*

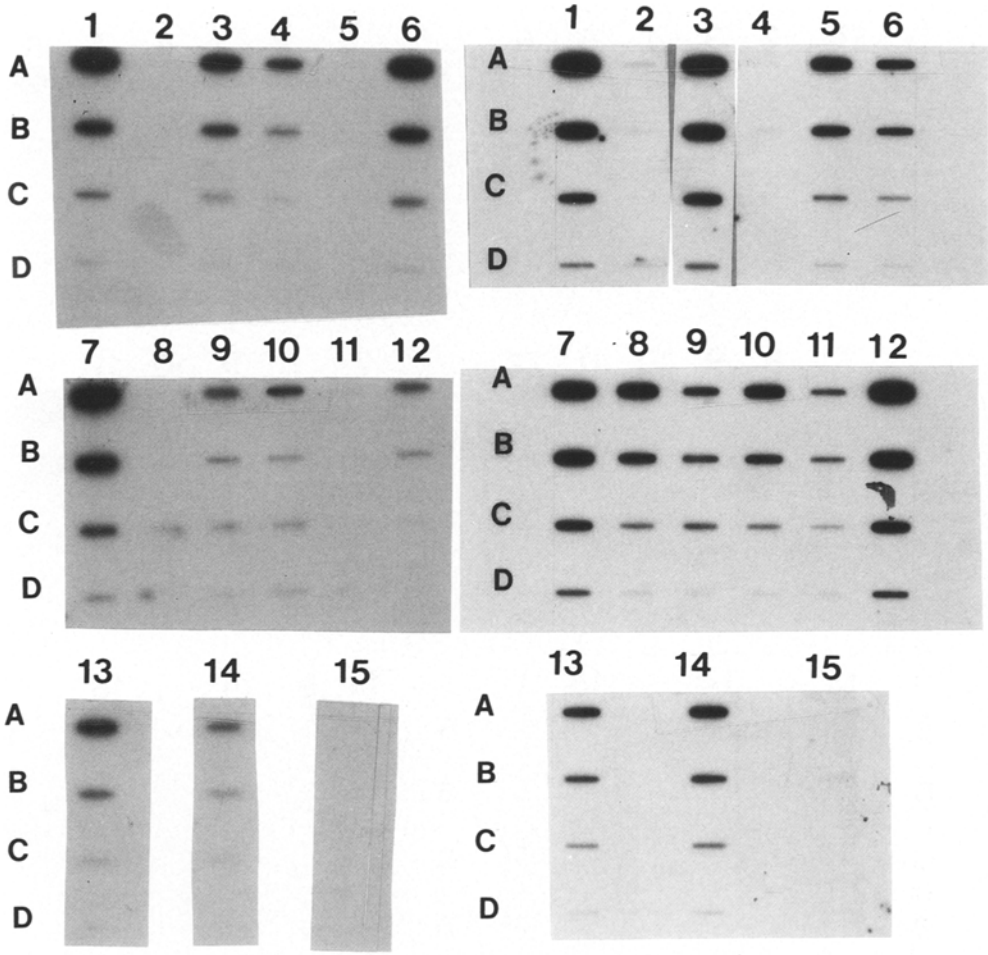

Fig. 2. Continued.

encodes for both phosphomannose isomerase (PMI) and GDP-mannose pyrophosphorylase (GMP), *pmm* encodes for phosphomannomutase activity. Both of these reactions are common to other pathways for carbohydrate metabolism. Once production in the bacterial cell has been induced and there is expression of GDP-mannose dehydrogenase (product of *algD*), a significant amount of intracellular carbohydrate pools are converted into alginate. The GDP mannose dehydrogenase and the enzymatic steps beyond this key step are unique to alginate biosynthesis. Some of the downstream reactions have not been characterized as well as the upstream reactions. However, the downstream reactions in the biosynthetic pathway are anticipated to be specific for alginate.

It has been suggested that this alginate biosynthetic pathway is similar to the alginate biosynthetic pathway in *Azotobacter vinelandii* [11, 28]. In *A. vinelandii* an extracellular, Ca^{2+}-dependent C-5 epimerase converts some of the mannuronate residues to guluronate residues [28]. The final product is a mixed copolymer of

guluronate and mannuronate residues. Genetic evidence exists that suggests the reactions are very similar in *P. aeruginosa* and that the *algG* gene is involved in the epimeration of d-mannuronate to a L-guluronate [28]. There is some evidence that the *alg-76* gene is involved in the polymerase reaction in the formation of alginate (A. Chakrabarty, personal communication) in diverse organisms.

This study was designed to examine bacteria comprising biofilms on metal surfaces where corrosion was occurring to determine if the biofilm members had the genetic machinery necessary to alginate biosynthesis. To our knowledge this study is the first to establish the presence of both structural and regulatory genes for alginate biosynthesis in bacteria isolated from corroding metal surfaces. These results also indicate that the alginate genes may be broadly distributed among environmental bacterial genera not included in the RNA homology group 1.

The library of organisms from biofilm/biocorrosion surfaces examined in this study were not systematically collected to demonstrate alginate production. Consequently, from the fact that 9% of the isolates demonstrated homology to *algD* it may be argued that this is the random frequency of occurrence of the alginate pathway in metal surface biofilm microbial assemblages. More extensive random sampling would be required to verify this frequency.

These experiments were also designed to identify a host bacterium for studies with an *algD*-bioluminescent reporter plasmid constructed in our laboratory. This reporter gene construction will allow in situ monitoring of environmental factors that induce alginate biosynthesis via the *algD* gene.

The results of this investigation suggest the possible extension of the alginate model system to the study of exopolymer biosynthesis in biocorroding biofilms as well as other biofilm microbial communities, which may demonstrate alginic acid biosynthetic genes. In this regard, it is of interest to determine the dynamic role of alginate in the establishment, colonization, and maturation of biofilm communities [5]. Of particular relevance is the elucidation of environmental parameters influencing alginate biosynthesis and the temporal and spatial production of EPS in biofilm formation. Preliminary studies using *algD-lux* reporter plasmids in the strains developed in this study have validated both existence of the alginate pathway and expression in response to environmental stimuli in select isolates [10]. Such experiments will provide information about environmental stimuli and stress signals that induce alginate biosynthesis under conditions far removed from the cystic fibrosis lung environment. This information should be of importance in further studies of bacterial attachment, biocorrosion, and biofilm processes.

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