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Distribution of Alginate Gene Sequences in the *Pseudomonas* rRNA Homology Group I-*Azomonas-Azotobacter* Lineage of Superfamily B Procaryotes

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Chromosomal DNA from group I *Pseudomonas* species, *Azotobacter vinelandii*, *Azomonas macrocytogenes*, *Xanthomonas campestris*, *Serpens flexibilis*, and three enteric bacteria was screened for sequences homologous to four *Pseudomonas aeruginosa* alginate (*alg*) genes (*algA*, *pmm*, *algD*, and *algR1*). All the group I *Pseudomonas* species tested (including alginate producers and nonproducers) contained sequences homologous to all the *P. aeruginosa alg* genes used as probes, with the exception of *P. stutzeri*, which lacked *algD*. *Azotobacter vinelandii* also contained sequences homologous to all the *alg* gene probes tested, while *Azomonas macrocytogenes* DNA showed homology to all but *algD*. *X. campestris* contained sequences homologous to *pmm* and *algR1* but not to *algA* or *algD*. The helical bacterium *S. flexibilis* showed homology to the *algR1* gene, suggesting that an environmentally responsive regulatory gene similar to *algR1* exists in *S. flexibilis*. *Escherichia coli* showed homology to the *algD* and *algR1* genes, while *Salmonella typhimurium* and *Klebsiella pneumoniae* failed to show homology with any of the *P. aeruginosa alg* genes. Since all the organisms tested are superfamily B procaryotes, these results suggest that within superfamily B, the alginate genes are distributed throughout the *Pseudomonas* group I-*Azotobacter-Azomonas* lineage, while only some *alg* genes have been retained in the *Pseudomonas* group V (*Xanthomonas*) and enteric lineages.

The exopolysaccharide (EPS) alginate is a linear β-1,4-linked copolymer of D-mannuronic acid and its C-5 epimer L-guluronic acid. Alginate is used for a variety of commercial applications (e.g., as a gelling agent and stabilizer in the food and pharmaceutical industries) and is isolated for commercial use from brown seaweeds (23). In addition to brown algae, alginate is produced by some bacterial species, the most widely known being *Pseudomonas* species (18, 19, 26, 29, 33) and *Azotobacter* species (7, 36). Alginate production by *Pseudomonas aeruginosa* infecting the lungs of cystic fibrosis patients leads to severe airway obstruction and thus is a major medical problem confronting these individuals. Alginate-producing (mucoid) strains of *P. aeruginosa* are associated almost exclusively with respiratory tract infection in cystic fibrosis; such strains are rarely recovered from *P. aeruginosa* infection at other tissue sites or from the environment (3, 25, 27). Mucoid strains of *P. aeruginosa* have been isolated from nonmucoid strains in vitro, usually after treatment with mutagens or repeated culturing in the presence of certain antibiotics (13, 26) or by selection of mutants resistant to bacteriophages (32). Using similar techniques, alginate-producing strains of three other *Pseudomonas* species (*P. fluorescens*, *P. putida*, and *P. mendocina*) were isolated in vitro (26). More recently, naturally occurring alginate-producing strains of plant-associated *P. fluorescens* were reported (18). In addition, certain phytopathogenic fluorescent *Pseudomonas* species are occasionally found to produce alginate both in plants and in vitro (18, 19). Thus, it appears that many *Pseudomonas* species are capable of producing alginate but that the genes involved in alginate biosynthesis are not normally expressed.

The pathway of alginate synthesis in *P. aeruginosa* has been partially elucidated (see reference 3 for a review) (Fig. 1). The enzymes catalyzing the first three steps of alginate synthesis, phosphomannose isomerase (PMI), phosphomannomutase (PMM), and GDP-mannose pyrophosphorylase (GMP), are also involved in general carbohydrate metabolism and as such are found in many bacteria. GDP-mannose dehydrogenase (GMD), on the other hand, catalyzes the oxidation of GDP-mannose to GDP-mannuronic acid and is believed to be specific for alginate synthesis (11).

Several alginate (*alg*) genes from *P. aeruginosa* have been cloned, and some of the corresponding gene products have been functionally identified (reviewed in reference 3). Four alginate genes of known function were used in this study. The *algA* gene encodes a bifunctional enzyme having PMI and GMP activities (3, 37), while the *algD* gene encodes GMD (11). Both the *algA* and *algD* genes have been sequenced (8, 12). The *algD* gene is transcriptionally activated in mucoid strains of *P. aeruginosa* (11, 12), and this activation depends on the products of two alginate regulatory genes, *algR1* and *algR2* (2, 15). The *algR1* gene has been sequenced and shows homology to a class of environmentally responsive bacterial regulatory genes (10). Another alginate gene has been cloned that is associated with PMM activity (3), although it is not yet known whether this gene encodes PMM itself or a regulatory protein that activates the PMM structural gene (or its corresponding polypeptide product). For convenience, the latter alginate gene is hereafter referred to in this report as the *pmm* gene.

The ability of some *Pseudomonas* species to produce

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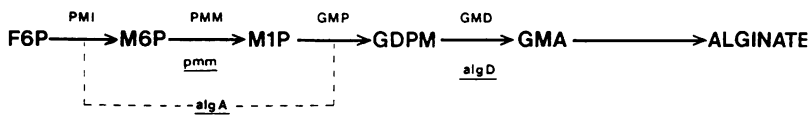

FIG. 1. Pathway of alginate biosynthesis in *P. aeruginosa*. Abbreviations: F6P, fructose-6-phosphate; M6P, mannose-6-phosphate; M1P, mannose-1-phosphate; GDPM, GDP-mannose; GMA, GDP-mannuronic acid. The *algA* gene encodes a bifunctional PMI-GMP enzyme (3, 37). The *algD* gene encodes GMD, while the *pmm* gene is associated with PMM activity (3, 37). The *algR1* gene encodes a regulatory protein that activates the *algD* gene (2, 15). The portion of each *alg* gene used as a probe in this study is given in Results.

alginate in specific environments (e.g., the cystic fibrosis lung, infected plants) or after mutagenesis raises some interesting questions about the molecular evolution of the alginate genes. Are alginate genes present in all *Pseudomonas* species, and if so, are they homologous? If the alginate genes are present in all pseudomonads, are they regulated in response to certain environmental stimuli, similar to the osmotic induction of the *P. aeruginosa algD* gene (2)? On a broader scale, are the alginate genes evolutionarily conserved to a heirarchical level that includes other bacterial genera? The ability of *Azotobacter vinelandii* to produce alginate (36) suggested that this is the case, since the genus *Azotobacter* shares a common lineage with pseudomonads belonging to *Pseudomonas* rRNA homology group I (which includes *P. aeruginosa*) (5, 14). Govan et al. (26) reported that alginate-producing variants of *P. fluorescens*, *P. putida*, and *P. mendocina* could be obtained by selection for carbenicillin resistance. This procedure did not, however, yield alginate-producing mutants of *P. stutzeri*, *P. pseudoalcaligenes*, *P. testosteroni*, *P. acidovorans*, *P. cepacia*, or *P. maltophilia* (*Xanthomonas maltophilia*). This suggests that the latter *Pseudomonas* species lack one or more critical genes (regulatory or structural) involved in alginate synthesis. Since many of the *P. aeruginosa* alginate genes have been cloned, it is now possible to examine genomic DNA from various *Pseudomonas* species and other organisms phylogenetically near *P. aeruginosa* for sequences homologous to the *P. aeruginosa alg* genes to determine the extent to which the presence of *alg* gene sequences parallels phylogenetic relationships. The results of such a study are reported herein.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used in this study are listed in Table 1. All the *Pseudomonas* species, *Escherichia coli* AC80, *Salmonella typhimurium* tyr19, and *Klebsiella pneumoniae* 13883 were grown in L broth plus glucose (10 g of Bacto-Tryptone [Difco Laboratories, Detroit, Mich.], 5 g of Difco yeast extract, 5 g of NaCl, and 1 g of glucose per liter). *Xanthomonas campestris* 12612 was grown in double-strength Difco nutrient broth containing 2 g of glucose per liter. *Azotobacter vinelandii* 478 and *Azomonas macrocytogenes* 12335 were grown in ATCC medium 240 (American Type Culture Collection, ATCC Catalogue of Strains, 17th ed., 1989) containing 20 g of mannitol per liter as the carbon source. The final pH of ATCC medium 240 was 7.6 and 6.0 for *Azotobacter vinelandii* and *Azomonas macrocytogenes*, respectively. *Serpens flexibilis* 29606 was grown in ATCC medium 972. All bacteria were grown at the temperature recommended in the *ATCC Catalogue of Strains* (17th ed., 1989).

DNA isolation and preparation of probe DNA. Total bacterial DNA was isolated from the organisms listed in Table 1 by the method of Goldberg and Ohman (24). Total DNA (50 μg) was digested to completion with appropriate restriction endonucleases (obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md.), and the fragments were separated by electrophoresis in 0.7% agarose gels (Tris-acetate buffer system). The gel was stained with ethidium bromide, and the bands were visualized with a UV transilluminator. After being photographed, the gels were blotted as described below.

Probe DNA was prepared by digesting cesium chloride-purified plasmid DNA with appropriate restriction endonucleases, followed by electrophoresis under the same conditions described above for total DNA. After the gel was photographed, the desired fragment containing all or part of the specified *alg* gene was excised from the gel and eluted from the agarose slice with a Gene Clean kit (Bio 101). The isolated fragments were then labeled with [α-^{32}P]dCTP (Amersham Corp., Arlington Heights, Ill.) with a nick translation kit (Bethesda Research Laboratories). Unreacted nucleotides were removed from the reaction mixture with Gene

TABLE 1. Bacterial strains used in this study

Bacteria[a]	Alginate production	Source or reference
Pseudomonas aeruginosa 8830	+	8
Pseudomonas aeruginosa PAO1 (ATCC 15612)	+[b]	28
Pseudomonas fluorescens (ATCC 13525)	+[b]	ATCC
Pseudomonas putida (ATCC 12633)	+[b]	ATCC
Pseudomonas mendocina (ATCC 25411)	+[b]	ATCC
Pseudomonas stutzeri JM300	+[b]	6
"*Pseudomonas glycinea*" 2159	+	19
"*Pseudomonas glycinea*" A-29-2	+	19
"*Pseudomonas phaseolicola*" race 2	+	19
Pseudomonas marginalis PF-05-2	−	18
Pseudomonas marginalis (ATCC 10844)	−	ATCC
Pseudomonas marginalis HT041B	−	34
Pseudomonas fluorescens W4F1607	+	18
Pseudomonas cichorii P36	+	19
Pseudomonas species (ATCC 31461)	−	I. Sá-Correia
Escherichia coli AC80	Not known	8
Salmonella typhimurium tyr-19	Not known	9
Klebsiella pneumoniae (ATCC 13883)	Not known	ATCC
Azotobacter vinelandii (ATCC 478)	+	ATCC
Azomonas macrocytogenes (ATCC 12335)	Not known	ATCC
Xanthomonas campestris (ATCC 12612)	Not known	ATCC
Serpens flexibilis (ATCC 29606)	Not known	ATCC

[a] "*P. glycinea*" 2159 and A-29-2, "*P. phaseolicola*" race 2, *P. marginalis* PF-05-2, ATCC 10844, and HT041B, and *P. cichorri* P36 are all known phytopathogens (18, 19, 34).

[b] Alginate production by these *Pseudomonas* species is only observed after exposure to certain antibiotics and/or mutagens (see Introduction).

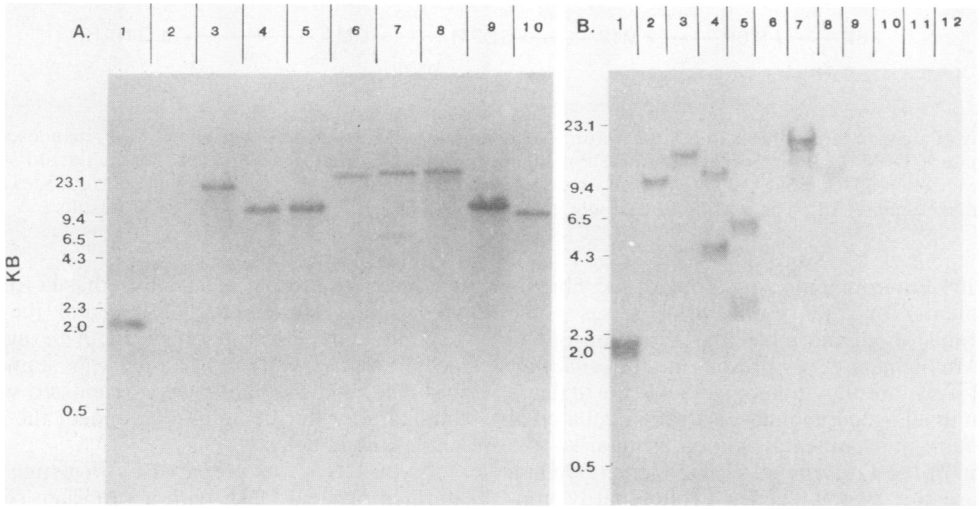

FIG. 2. Hybridization of the *P. aeruginosa algA* gene with *Bam*HI-*Sst*I-digested chromosomal DNA from the organisms listed in Table 1. The organisms are in lanes numbered as follows: A1, *P. aeruginosa* 8830; A2, *E. coli* AC80; A3, "*P. glycinea*" 2159; A4, "*P. glycinea*" A-29-2; A5, "*P. phaseolicola*" race 2; A6, *P. marginalis* PF-05-2; A7, *P. marginalis* 10844; A8, *P. marginalis* HT041B; A9, *P. fluorescens* W4F1607; A10, *P. cichorii* P36; B1, *P. aeruginosa* PAO1; B2, *P. fluorescens* 13525; B3, *P. putida* 12633; B4, *P. mendocina* 25411; B5, *P. stutzeri* JM300; B6, *Pseudomonas* species strain 31461; B7, *Azotobacter vinelandii* 478; B8, *Azomonas macrocytogenes* 12335; B9, *Salmonella typhimurium* tyr-19; B10, *Serpens flexibilis* 29606; B11, *X. campestris* 12612; B12, *K. pneumoniae* 13883.

Clean. Probe DNA was denatured just before hybridization by heating at 100°C for 5 min and cooling rapidly on ice.

Blotting. Transfer of DNA to nitrocellulose and subsequent hybridizations were done by the method of Southern (38) as outlined by Maniatis et al. (31). Agarose gels containing total DNA from the organisms listed in Table 1 were incubated at room temperature in 0.25 N HCl without agitation for 20 min. The gels were then treated with denaturing solution (1.5 M NaCl in 0.5 N NaOH) followed by neutralizing solution (1.5 M NaCl in 1 M Tris chloride, pH 8.0), each treatment performed at room temperature for 1 h with constant agitation. The DNA was transferred from each gel to nitrocellulose filters (Gene-Screen Plus, BA85; 0.45-μm pore size; Schleicher & Schuell, Inc., Keene, N.H.) (31). After transfer of DNA, the filters were removed from the gel and washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (31) with constant agitation for 20 min to remove adherent gel fragments. The filters were placed between sheets of Whatman 3MM paper. The sheets were then placed between glass plates and dried at 80°C under vacuum.

Hybridization. The baked membranes were prehybridized by incubating them for 7 h at 42°C in 50% deionized formamide–5× SSC–5× Denhardt reagent (31)–100 μg of denatured salmon sperm DNA per ml–10 mM sodium phosphate buffer (pH 6.5). The prehybridization buffer was then removed, and the filters were incubated overnight at 42°C in 50% deionized formamide–5× SSC–1× Denhardt reagent–100 μg of denatured salmon sperm DNA per ml–10 mM sodium phosphate buffer (pH 6.5)–10% dextran sulfate–1 μg of labeled probe DNA. After hybridization, the filters were rinsed for 15 min in 2× SSC containing 0.1% sodium dodecyl sulfate (repeated four times) with constant agitation at room temperature, followed by two washes (40 min each) in 0.1% SSC containing 0.1% sodium dodecyl sulfate at 65°C. The filters were air dried and then exposed to Kodak SB-100 X-ray film at −80°C.

RESULTS

The *P. aeruginosa algA* gene encoding PMI-GMP (Fig. 1) is located on a 2-kilobase (kb) *Bam*HI-*Sst*I chromosomal DNA fragment (8). To determine whether sequences homologous to the *algA* gene were present in other pseudomonads and organisms phylogenetically related to *P. aeruginosa*, total DNA from the organisms listed in Table 1 (digested with *Bam*HI and *Sst*I) was hybridized with a 1.2-kb segment of the *P. aeruginosa algA* gene. This fragment contains about 40 base pairs upstream of the translational start site of *algA* as well as most of the *algA* coding sequence (8). The *P. aeruginosa algA* gene hybridized with DNA from all the *Pseudomonas* species tested in this study (except *Pseudomonas* species strain ATCC 31461), as well as with *Azotobacter vinelandii* and *Azomonas macrocytogenes*. The *algA* gene did not hybridize with DNA From *E. coli*, *K. pneumoniae*, *Salmonella typhimurium*, *X. campestris*, or *Serpens flexibilis*, suggesting a nonhomologous gene encoding PMI or the absence of such a gene altogether. It is interesting that for all *Pseudomonas* species with the exception of *P. aeruginosa* PAO1 (ATCC 15612), the *algA* gene hybridized with chromosomal fragments of different sizes than 2 kb (i.e., the size of the chromosomal *Bam*HI-*Sst*I fragment that contains the *P. aeruginosa algA* gene). For *P. stutzeri*, *P. mendocina*, and *P. marginalis* 10844, the *algA* probe hybridized with two chromosomal fragments, suggesting that the *algA* homologs in these species contain a *Bam*HI or an *Sst*I restriction site.

The *P. aeruginosa pmm* gene was cloned on a 2.5-kb *Hin*dIII-*Sst*I DNA fragment and is associated with PMM activity (Fig. 1) (3). This fragment containing *pmm* hybridized with chromosomal DNA from all the pseudomonads tested (Fig. 3), but again the sizes of the fragments containing *pmm* homologs varied. The *pmm*-containing fragment also hybridized with DNA from *Azotobacter vinelandii*, *Azomonas macrocytogenes*, *X. campestris*, and *Serpens flexibilis* but not with DNA from *E. coli*, *Salmonella typhimurium*, or *K. pneumoniae*.

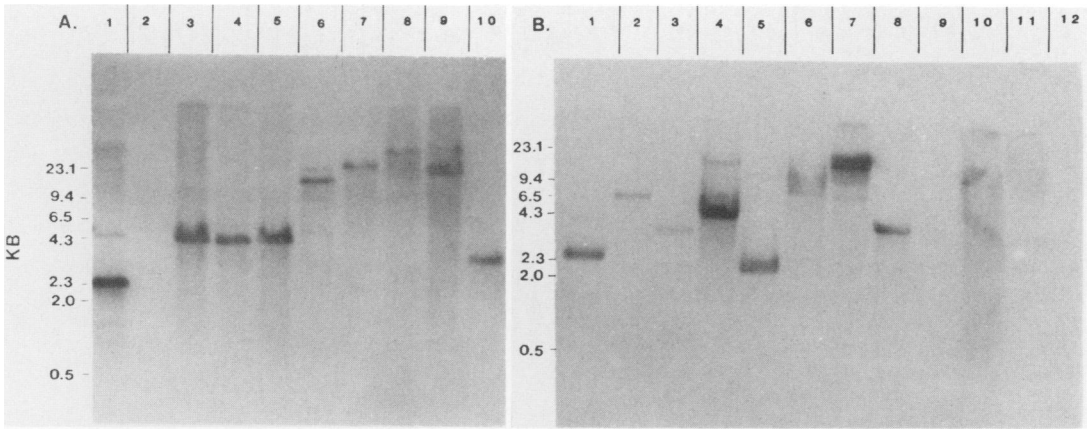

FIG. 3. Hybridization of the *P. aeruginosa pmm* gene with *Hin*dIII-*Sst*I-digested chromosomal DNA from the organisms listed in Table 1. Lanes are as in Fig. 2.

In contrast to PMI and PMM, which are involved in general carbohydrate metabolism, the *algD* gene is believed to be specific for alginate synthesis (11). A 1.2-kb *Bam*HI-*Xho*I fragment containing the *algD* promoter, the leader sequence, and part of the *algD* coding region was used to probe *Cla*I-*Bgl*II-digested chromosomal DNA from the organisms listed in Table 1 (Fig. 4). The *algD* gene hybridized with DNA from all the *Pseudomonas* species tested with the exception of *P. stutzeri* and *Pseudomonas* species strain ATCC 31461. The *algD* gene also hybridized with DNA from *Azotobacter vinelandii* and *E. coli*. DNA from *Salmonella typhimurium*, *K. pneumoniae*, *X. campestris*, *Serpens flexibilis*, and *Azomonas macrocytogenes* did not hybridize with *algD*. The "*P. glycinea*" strains and "*P. phaseolicola*" contained a band that hybridized with *algD* and was of the same size, although each strain also contained smaller fragments that hybridized with *algD*. In addition, the *P. marginalis* strains exhibited two bands that hybridized with *algD*.

The 744-base-pair *algR1* gene is located within a 6.2-kb *Bgl*II-*Bgl*II fragment in the *P. aeruginosa* chromosome (10). A 1-kb *Bam*HI-*Bam*HI fragment containing *algR1* was used to probe *Bgl*II-digested chromosomal DNA from the organisms listed in Table 1 for sequences homologous to *algR1*. *algR1* hybridized with DNA from all the pseudomonads tested (except *Pseudomonas* species strain ATCC 31461), as well as with *Azotobacter vinelandii*, *Azomonas macrocytogenes*, *X. campestris*, *E. coli*, and *Serpens flexibilis* (Fig. 5). The *algR1* gene did not hybridize at all with DNA from *Salmonella typhimurium* or *K. pneumoniae*. Again, in most cases the size(s) of the band(s) hybridizing with *algR1* varied, even in the *Pseudomonas* species.

DISCUSSION

The results shown in Fig. 2 to 5 are summarized in Table 2. These data are strictly qualitative, and no attempt is made to compare the degree of sequence homology (i.e., compare the relative intensity of bands observed after autoradiography).

Acidic EPS are known to be involved in *Rhizobium* species-plant nodulation (30) and *Erwinia stewartii* plant infections (16). Fett and colleagues (17–19) reported that many fluorescent phytopathogenic *Pseudomonas* species also produce EPS under certain conditions and that the EPS is in some cases alginate. It was proposed that alginate produced by phytopathogenic pseudomonads is a virulence factor, possibly playing a role in adherence of bacteria to plant hosts and subsequently providing the encapsulated bacteria with a protective environment. This may enable the bacteria to produce extracellular enzymes and other products that alter the host to favor bacterial multiplication (19). It is clear from the results presented here that all the

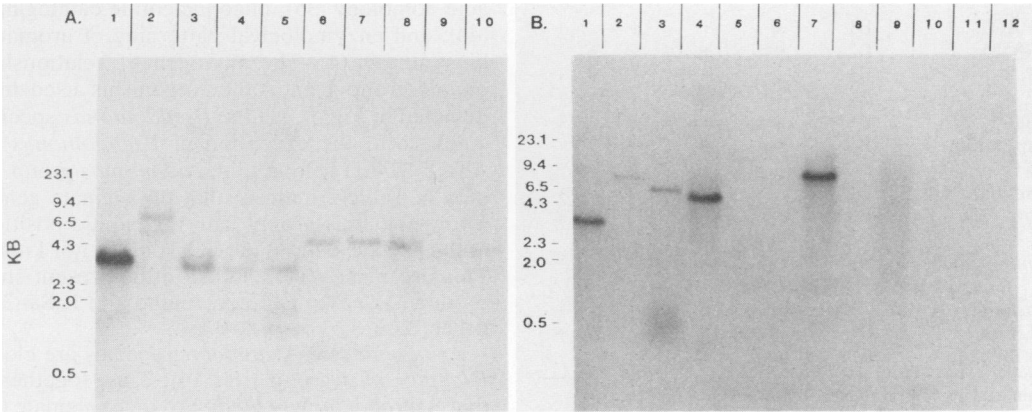

FIG. 4. Hybridization of the *P. aeruginosa algD* gene with *Cla*I-*Bgl*II-digested chromosomal DNA from the organisms listed in Table 1. Lanes are as in Fig. 2.

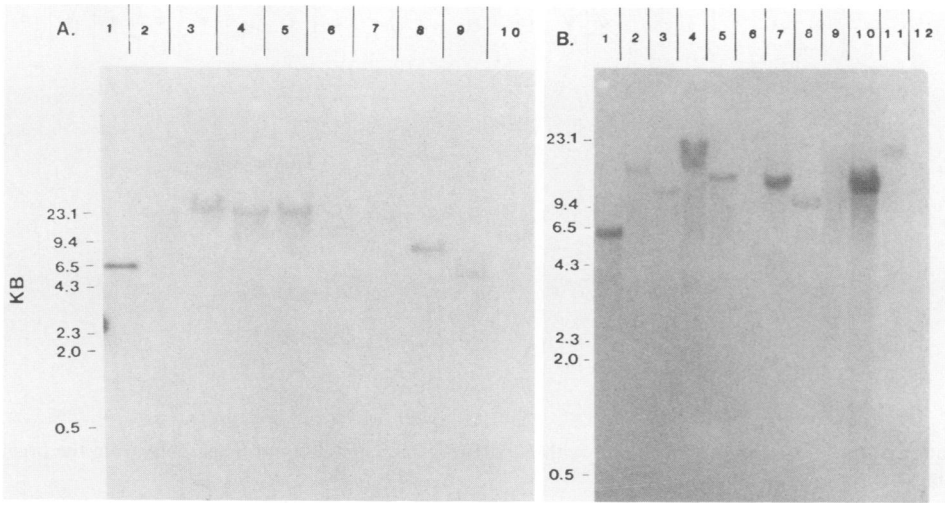

FIG. 5. Hybridization of the *P. aeruginosa algR1* gene with *Bgl*II-digested chromosomal DNA from the organisms listed in Table 1. Lanes are as in Fig. 2.

phytopathogenic pseudomonads tested (both alginate producers and nonproducers) possess genetic sequences homologous to the *P. aeruginosa* alginate genes, although the variation in banding patterns suggests some evolutionary divergence of alginate gene sequences. It would be interesting to generate alginate-negative (Alg^-) mutants of the phytopathogenic pseudomonads by gene replacement techniques, replacing chromosomal *alg* genes with *P. aeruginosa* homologs that been rendered inactive by mutation. Such Alg^- mutants could then be used to evaluate the role of alginate in virulence.

TABLE 2. Hybridization of *P. aeruginosa* alginate gene probes to DNA from organisms used in this study

Bacteria[a]	Lane[b]	Hybridization with probe[c]:			
		algA	*pmm*	*algD*	*algR1*
P. aeruginosa 8830	A1	+	+	+	+
E. coli AC80	A2	−	−	+	+
"*P. glycinea*" 2159	A3	+	+	+	+
"*P. glycinea*" A-29-2	A4	+	+	+	+
"*P. phaseolicola*" race 2	A5	+	+	+	+
P. marginalis PF-05-2	A6	+	+	+	+
P. marginalis 10844	A7	+	+	+	+
P. marginalis HT041B	A8	+	+	+	+
P. fluorescens W4F1607	A9	+	+	+	+
P. cichorii P36	A10	+	+	+	+
P. aeruginosa PAO1	B1	+	+	+	+
P. fluorescens 13525	B2	+	+	+	+
P. putida 12633	B3	+	+	+	+
P. mendocina 25411	B4	+	+	+	+
P. stutzeri JM300	B5	+	+	−	+
Pseudomonas sp. strain 31461	B6	−	+	−	−
Azotobacter vinelandii 478	B7	+	+	+	+
Azomonas macrocytogenes 12335	B8	+	+	−	+
Salmonella typhimurium tyr-19	B9	−	−	−	−
Serpens flexibilis 29606	B10	−	+	−	+
X. campestris 12612	B11	−	+	−	+
K. pneumoniae 13883	B12	−	−	−	−

[a] Complete strain designations are given in Table 1.
[b] A and B refer to panels A and B of Fig. 2 to 5.
[c] The fragments of each alignate gene used as a probe are described in the Results.

Although *P. marginalis* makes novel acidic EPS (18, 34; S. F. Osman and W. F. Fett, unpublished data), it has not been reported to make alginate. However, *P. marginalis* does contain sequences homologous to the *P. aeruginosa* alginate genes (Table 2). Thus, it is possible that in plants or under certain in vitro conditions, *P. marginalis* makes alginate in addition to or instead of other acidic EPS. In support of this notion, Cote and Krull (7) reported that *Azotobacter chroococcum* produces both alginate and a different acidic EPS in batch cultures.

The genus *Pseudomonas* is now recognized as being an extremely heterogeneous taxonomic group. Species belonging to *Pseudomonas* actually compose five distinct groups whose phylogenetic relatedness extends beyond the familial level (35, 40). Thus, species belonging to *Pseudomonas* rRNA homology group I are actually more closely related to enteric bacteria (*E. coli*, *K. pneumoniae*) than to *Pseudomonas* species belonging to *Pseudomonas* rRNA homology groups II, III, and IV (40). *Pseudomonas* homology group I is composed of two subgroups, the nonfluorescent (Ia) subgroup and the fluorescent (Ib) subgroup, based on nucleic acid homology (35), oligonucleotide cataloging of 16s rRNA (40), and enzymological patterning of aromatic amino acid biosynthesis (4). The phylogenetic relationship of *Pseudomonas* group I and other organisms used in this study is depicted in Fig. 6. All the *Pseudomonas* species used in this study (with the exception of *Pseudomonas* species strain ATCC 31461) belong to *Pseudomonas* group I (35), and the data in Table 2 indicate that the alginate genes are present throughout this group. The taxonomic position of *Pseudomonas* species strain ATCC 31461 is not known at present. This organism was included in the present study because it produces a novel EPS (A. Fialho and I. Sá-Correia, unpublished data).

Azomonas and *Azotobacter* species are closely related to *Pseudomonas* group I (5, 14). This, together with the fact that *Azotobacter vinelandii* produces alginate (36), suggested that the *P. aeruginosa alg* gene sequences are present in *Azomonas* and *Azotobacter* species. The data in Table 2

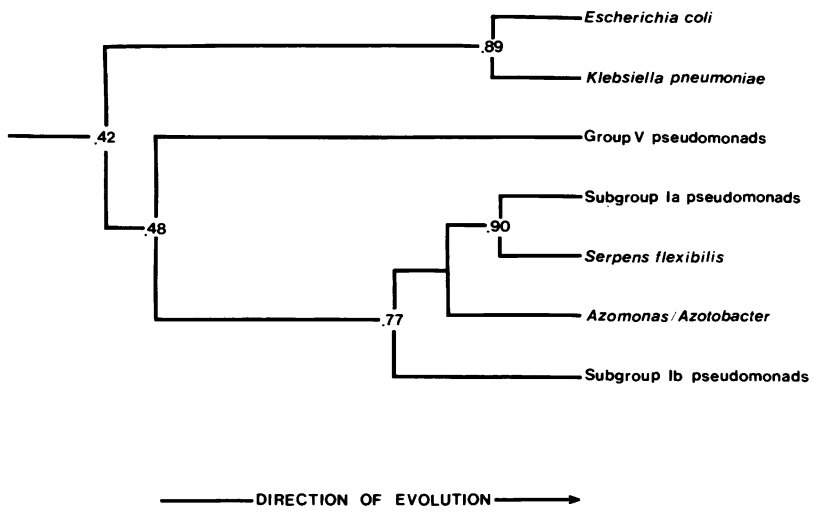

FIG. 6. Dendrogram positions of superfamily B procaryotes used in this study. Numbers at branch points are similarity coefficients (S_{AB} values) (22) determined by oligonucleotide cataloging of 16s rRNA (39). S_{AB} values range from 0 to 1.0, with S_{AB} = 1.0 representing perfect identity between two organisms. Superfamily B diverged from superfamilies A and C at an S_{AB} value of 0.3 (4). Branch points lacking numbers have not yet been assigned S_{AB} values and were established by using methods other than oligonucleotide cataloging of 16s rRNA (4, 5, 14).

show that this is indeed the case. *Azotobacter vinelandii* possessed sequences homologous to all the *P. aeruginosa alg* genes tested, while *Azomonas macrocytogenes* lacked only *algD*. It is not known whether *Azomonas* species produce alginate. One would expect not, since the *algD* gene is lacking, and GMD (the *algD* gene product) is believed to be the committing step in alginate synthesis (11). However, *Azomonas* species could possess a gene that encodes GMD but is not homologous to *algD*.

Govan et al. (26) reported that no alginate-producing mutants of *P. stutzeri* could be obtained by selection for carbenicillin resistance, which allows isolation of alginate-producing variants of other *Pseudomonas* species. A priori this would appear to be due to the lack of the *algD* gene in *P. stutzeri* (Table 2). However, *P. stutzeri* could have a gene encoding GMD that is not homologous to *algD* and thus be capable of alginate synthesis even though carbenicillin selection failed to yield alginate-producing variants. Recent findings by Goldberg and Ohman (personal communication) support the latter possibility. These workers found that another *P. aeruginosa* alginate gene, *algT* (20), hybridized with DNA from *P. fluorescens*, *P. putida*, and *P. stutzeri* but not with DNA from *P. acidovorans*, *P. diminuta*, or *P. maltophilia* (*X. maltophilia*). Furthermore, when the cloned *algS(on)T* (21) region was introduced into the above *Pseudomonas* species, *P. fluorescens*, *P. putida*, and *P. stutzeri* (as well as alginate-negative *P. aeruginosa* strains) produced alginate.

Woese et al. (41) demonstrated a close phylogenetic relationship between the helical bacterium *Serpens flexibilis* and *P. pseudoalcaligenes*, a member of *Pseudomonas* subgroup Ia (35). This was supported by the work of Ahmad and Jensen (1), who found *Serpens flexibilis* to be identical to subgroup Ia pseudomonads based on enzymological patterning of aromatic amino acid biosynthesis. Although the *P. aeruginosa algA*, *pmm*, and *algD* genes failed to hybridize with *Serpens flexibilis* DNA, the regulatory gene *algR1* did hybridize. It is possible that *Serpens flexibilis* in nature never encounters environmental conditions that promote alginate production and as a result has lost the alginate biosynthetic genes during its evolution. The *algR1* homolog in *Serpens flexibilis* may play a critical role in regulating some other gene(s) in this organism and consequently has been evolutionarily conserved.

Because *X. campestris* (a group V pseudomonad) is more closely related to *Pseudomonas* group I than it is to *Pseudomonas* group II, III, or IV (35) and since PMM is an essential step in xanthan synthesis in *X. campestris*, we expected to see homology between the *P. aeruginosa pmm* gene and *X. campestris* DNA. We observed hybridization not only with *pmm* but also with *algR1*. It is not known whether the *X. campestris* gene homologous to *algR1* is involved in the regulation of xanthan biosynthesis. It would be interesting to determine whether the *P. aeruginosa algR1* or *pmm* gene or both are able to complement certain xanthan-negative mutants of *X. campestris*.

We predicted that because PMI and PMM are involved in general carbohydrate metabolism, the genes encoding these enzymes in *P. aeruginosa* (*algA* and *pmm*, respectively) would be found in many bacteria, while the alginate-specific genes (*algD* and *algR1*) would be restricted to group I *Pseudomonas* species or other organisms known to produce alginate. On the contrary, *E. coli* showed no sequences homologous to either *algA* or *pmm* (Table 2). Darzins et al. (8) sequenced the *P. aeruginosa algA* gene and showed that *algA* had no homology with the *E. coli manA* gene, which encodes PMI in *E. coli*. Furthermore, the *algA* gene failed to hybridize with DNA from *E. coli*, *K. pneumoniae*, *Enterobacter aerogenes*, *Arthrobacter viscosus*, and *Agrobacterium tumefaciens* (8). The hybridization of the *algD* gene to *E. coli* DNA (Table 2) may be a result of the homology between *algD* and the *E. coli* gene encoding histidinol dehydrogenase (12), while the hybridization of the *algR1* gene to *E. coli* DNA may be due to the homology between *algR1* and the *E. coli ompR* gene (10).

The virtual absence of sequences homologous to the *P. aeruginosa* alginate genes in the enteric bacteria (*E. coli*, *Salmonella typhimurium*, *K. pneumoniae*) suggests that the alginate genes were either (i) lost in the enteric lineage or (ii) gained in the *Pseudomonas* group I-group V lineage, after the evolutionary divergence of the enterics from *Pseudomonas* groups I and V (Fig. 6). This may reflect the differences

in the natural environments of the enterics versus pseudomonads; the enterics may never encounter conditions that promote alginate production and thus lack some of the genes for its synthesis. On the other hand, the enteric bacteria may have evolved to produce a different EPS (e.g., colanic acid in *E. coli*) that is more beneficial in their given ecological niche. Since PMI and PMM activities both exist in *E. coli*, the lack of homology between the genes encoding these enzymes in *E. coli* and *P. aeruginosa* is apparently a reflection of the considerable evolutionary distance between these organisms (Fig. 6).

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