CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Sept. 1995, p. 554–562 Vol. 2, No. 5
1071-412X/95/$04.00+0

Prevalence of *gca*, a Gene Involved in Synthesis of A-Band Common Antigen Polysaccharide in *Pseudomonas aeruginosa*

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Received 6 December 1994/Returned for modification 6 March 1995/Accepted 4 May 1995

Two distinct forms of lipopolysaccharide are expressed by *Pseudomonas aeruginosa*. These forms are known as the A band and the B band. In an attempt to obtain a better understanding of A-band lipopolysaccharide synthesis, a previously isolated A-band gene known as the *gca* gene (GDP–D-mannose conversion protein for A-band common antigen polysaccharide) was sequenced and analyzed. Previous protein expression data from our laboratory, along with nucleotide sequence analysis from the present study, suggest that the Gca protein is encoded by the open reading frame ORF36.5. Amino acid homology reveals that this protein may be functioning as a dehydratase or as a bifunctional enzyme, facilitating the conversion of GDP–D-mannose to GDP–D-rhamnose. The distribution of this *gca* gene among the 20 *P. aeruginosa* O serotypes, clinical isolates, and other *Pseudomonas* species was also examined. Southern hybridization results revealed that the *gca* gene is present and conserved on a 1.6-kb *Kpn*I fragment among all 20 O serotypes with the exception of serotype O12. In addition, the *gca* gene is not universally found among all pseudomonads; however, probe-reactive profiles are similar to that of *P. aeruginosa* when the *gca* gene is present. Primers were designed from the *gca* nucleotide sequence, and PCR amplification of a 700-bp product was found with each of the 20 O serotypes. Because of the conservation of this gene, *gca* may be useful as a diagnostic tool for detecting the presence of *P. aeruginosa* as well as other *Pseudomonas* species.

Pseudomonas aeruginosa is the primary pathogen associated with debilitating infections in immunocompromised hosts such as burn wound victims and patients with cystic fibrosis (CF) (15). This organism possesses numerous virulence factors including alginate (17), phospholipase C (50), exotoxin A (18), and lipopolysaccharide (LPS) (9), all of which have been implicated in establishing chronic infections within the lungs of patients with CF.

LPS plays a significant structural role in the outer membranes of gram-negative organisms (39). *P. aeruginosa* coexpresses two chemically and antigenically distinct forms of LPS known as the A band and the B band (43). The B band is the O-antigen-containing LPS which is composed of tri- to pentasaccharide repeat units of many different monosaccharides (23). It is the B-band LPS which determines the serotype specificity of the bacterium and thus differentiates *P. aeruginosa* into 17 standard serotypes which compose the International Antigenic Typing Scheme (33). Recently, three new serotypes, serotypes 18 through 20, have been added (34). In comparison, A-band common antigen LPS is a short chain and mostly neutral homopolymer of D-rhamnose arranged as trisaccharide repeat units of $\alpha1\rightarrow2$, $\alpha1\rightarrow3$, $\alpha1\rightarrow3$ linkages (2). Interestingly, this antigenically conserved form of LPS is structurally similar to the D-rhamnan LPS of other *Pseudomonas* species such as *Pseudomonas syringae* pv. morsprunorum C28 (48) and *Pseudomonas cerasi* (24).

The role of B-band LPS in virulence has been established by its contribution to lower 50% lethal doses in animal challenge studies (9), serum resistance (20, 45), and phagocytosis (13). The influence of A-band LPS on virulence has been somewhat unclear. Studies by Lam et al. (30), however, have shown that isolates from early lung infections of patients with CF coexpress both A-band and B-band LPSs, while subsequent isolates become nontypeable with O-specific polyclonal sera or monoclonal antibodies (MAbs). These subsequent isolates were deficient in B-band LPS expression and showed reactivity to A-band MAbs, denoting a cell surface change to primarily A-band LPS. In addition, a correlation between the presence of anti-A-band LPS antibodies in patients with CF and clinical observations such as a lower degree of pulmonary function has also been established (30). To further our understanding of A-band's role in host-parasite interactions in pulmonary infections of patients with CF, a recent study has provided data to show that A-band LPS could contribute to the inflammatory process of the host infection by activating complement via the alternate pathway which mediates the chemotaxis of human peripheral neutrophils (10). Thus, A-band LPS could be a contributing factor in the persistence of inflammatory processes and lung damage in the host with CF.

Because of the severity of infections associated with *P. aeruginosa* and the high level of intrinsic resistance to various antibiotics that it displays, the need for rapid identification and typing of this organism is essential. Classical typing methods such as O serotyping and bacteriophage sensitivity testing (41), as well as pyocin typing (6), tend to be unreliable and to have a low level of reproducibility for clinical isolates because of alterations in their cell surfaces. A study conducted by the International *Pseudomonas aeruginosa* Typing Group (22) has determined that phenotypic instability can be overcome through the use of restriction fragment length polymorphism typing. Since A-band LPS is the conserved, common antigen among *P. aeruginosa* strains, it was of interest in the present study to examine the use of an A-band LPS gene as a DNA probe for identification purposes.

Lightfoot and Lam (31) reported the cloning of genes in-

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volved with the expression of A-band LPS from *P. aeruginosa*. In a subsequent study, one of the cloned genes was further characterized and was found to be involved in the conversion of GDP–D-mannose to GDP–D-rhamnose, which is presumed to be part of the pathway for the synthesis of A-band polysaccharide (32). The designation proposed for this gene is *gca* (GDP–D-mannose conversion protein for A-band common antigen polysaccharide), and here we report its nucleotide sequence. The *gca* gene was also used as a probe in Southern hybridization experiments to examine the incidence and degree of conservation of the gene among *P. aeruginosa* isolates and various other *Pseudomonas* species. PCR was also performed on each of the 20 *P. aeruginosa* O serotypes by using oligonucleotide primers designed from the *gca* gene sequence.

MATERIALS AND METHODS

Strains and plasmids. The bacterial strains and plasmids used in the study are described in Table 1.

Media and culture conditions. All bacterial strains were cultured on Luria broth (Gibco/Bethesda Research Laboratories, Burlington, Ontario, Canada). For solid media, Bacto Agar (Difco Laboratories, Detroit, Mich.) was added to 1.5%. When necessary, broth and agar were supplemented with antibiotics. For culturing *Escherichia coli*, an ampicillin (Sigma Chemical Co., St. Louis, Mo.) concentration of 100 μg/ml was used.

General DNA procedures. Restriction enzymes were purchased from Gibco/Bethesda Research Laboratories and Boehringer Mannheim (Laval, Quebec, Canada). T4 DNA ligase, Klenow enzyme, and calf intestinal alkaline phosphatase were purchased from Boehringer Mannheim. All of these enzymes were used according to the supplier's specifications. Small-scale plasmid DNA from *E. coli* was prepared by the alkaline lysis method of Birnboim and Doly (5), while large-scale preparations of plasmid DNA were obtained by using the Qiagen plasmid midi kit (Qiagen Inc., Chatsworth, Calif.) according to the manufacturer's recommendations. Plasmid DNA was transformed into *E. coli* strains by the method of Chung et al. (7) and was electroporated in *P. aeruginosa* by using an electroporation unit (Bio-Rad Laboratories, Richmond, Calif.). Electrocompetent cells of *P. aeruginosa* were prepared by the method of Farinha and Kropinski (14). Genomic DNA was isolated by the hexadecyltrimethylammonium bromide method of Ausubel et al. (3), with the following minor modifications. Bacterial strains were grown in 15 ml of Luria broth overnight at 37°C in 125-ml flasks. All volumes described in their protocol were increased proportionately to accommodate 15 ml of culture.

DNA sequencing. The 1.6-kb *Kpn*I fragment containing the *gca* gene was subcloned into the vector pBluescript II KS. Both strands of DNA were sequenced by the MOBIX facility at McMaster University (Hamilton, Ontario, Canada) by using the Applied Biosystems (Foster City, Calif.) model 373A DNA sequencing system. Universal and synthetic oligonucleotide primers were used. The oligonucleotides were synthesized with an Applied Biosystems model 391 DNA synthesizer and were purified according to the manufacturer's instructions. The *Taq* DyeDeoxy Terminator Cycle Sequencing Kit from Applied Biosystems was used for all sequencing reactions. Cycle sequencing reactions were carried out in an Ericomp (San Diego, Calif.) model TCX15 thermal cycler. Excess DyeDeoxy Terminators from completed sequencing reactions were removed by passing the reaction mixture through a 1-ml Sephadex G-50 column (Pharmacia, Baie d'Urfé, Quebec, Canada). Sequence analysis was performed by using PC/GENE (IntelliGenetics, Mountain View, Calif.) in order to determine possible open reading frames (ORFs) and protein properties.

Preparation of DNA probe. The plasmid pFV39 was digested with *Kpn*I, and the 1.6-kb fragment containing the *gca* gene was gel purified from low-melting-point agarose with a Geneclean II kit (Bio/Can Scientific, Mississauga, Ontario, Canada). A DNA concentration of approximately 20 ng/ml of hybridization solution was used for labeling by the nonradioactive method of dUTP conjugated to digoxigenin (Boehringer Mannheim). A final labeling reaction volume of 100 μl was used.

Southern hybridization. Restriction enzyme-digested genomic DNA was separated on 0.7% agarose gels and was transferred to Zeta-Probe nylon membranes (Bio-Rad) by the method of Sambrook et al. (44). Hybridizations were carried out under high-stringency conditions overnight at 65°C. Subsequent washes and incubations were carried out by using the manufacturer's recommendations (Boehringer Mannheim).

Isolation of LPS. LPS was prepared by the method of Hitchcock and Brown (21), which uses sodium dodecyl sulfate (SDS) and proteinase K for whole-cell lysis and digestion of protein.

SDS-PAGE analysis and silver staining. LPS was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as described by Hancock et al. (20). The method of Dubray and Bezard (12) was used for silver staining.

Western immunoblotting. LPS samples were transferred from SDS-polyacrylamide gels to a nitrocellulose membrane, which was then reacted with the MAb N1F10 as described by Lam et al. (30).

Slide agglutination. *P. aeruginosa* transconjugates were screened for the presence of A-band LPS by the slide agglutination method of Lam et al. (29).

PCR conditions. Oligonucleotide primers (18-mer) were designed from the *gca* gene sequence for amplification of a 700-bp product from the 20 *P. aeruginosa* O serotypes: HCR1 (5′-TGGCCAAGCTGCTGCTGG-3′) and HCR2 (5′-ACGGTGGTAGTGACAACG-3′) (Fig. 1). Approximately 50 ng of genomic DNA and 100 ng of plasmid DNA were used as templates for PCR. The primers (50 pmol) and template were mixed with deoxynucleoside triphosphates, buffer, and 4 mM $MgCl_2$ from the Perkin-Elmer (Branchurg, N.J.) kit in a total volume of 100 μl. This reaction mixture was boiled for 10 min to denature the primers and the template and was immediately transferred to ice for 30 s. Amplitaq DNA polymerase (2 U) from the Perkin-Elmer kit was added to the reaction mixture, and the mixture was overlaid with mineral oil (Sigma). Thermocycling reactions were carried out in an automated Perkin-Elmer thermocycler (Applied Biosystems, Mississauga, Ontario, Canada), and the DNA was amplified through 35 cycles of 95°C (1 min), 45°C (2 min), and 72°C (2 min). The reaction products (15 μl) were then electrophoresed on a 1.1% agarose gel.

Nucleotide sequence accession number. The GenBank accession number for the *gca* nucleotide sequence is U18320.

RESULTS

Sequence analysis of the *gca* gene. Our laboratory has previously reported that the plasmid pFV39 (Fig. 2), which restores A-band LPS expression in the A-band-deficient mutant rd7513, expresses a 37-kDa protein in *E. coli* maxicells (32). In the present study, the nucleotide sequence of each strand of the 1.6-kb *Kpn*I fragment in pFV39 was determined (Fig. 1), and both strands were found to contain one ORF. The first strand was found to contain an ORF encoding a protein with a predicted molecular mass of 33.4 kDa, while the ORF of the complementary strand encoded a protein with a predicted molecular mass of 36.5 kDa. Since either of these ORFs, designated ORF33.4 and ORF36.5, respectively, could encode the observed 37-kDa protein, the nucleotide sequences of each ORF were analyzed and compared with those of other *P. aeruginosa* chromosomal genes. The codon usages of the two ORFs were first examined. Of the 61 codons that can potentially encode amino acids, 26 are rarely found in *P. aeruginosa* chromosomal genes (51). Rare codons (i.e., codons which are recognized by rare tRNA species) are those which represent less than 10% of the codons from a specific synonymous group (51). Both ORF33.4 and ORF36.5 contain rare codons; however, ORF33.4 has a fourfold higher incidence of usage (16 of 26) of rare codons than does ORF36.5 (4 of 26) (data not shown). *P. aeruginosa* chromosomal genes also show a strong bias for cytosine over guanine in the third position of codons and show a relatively low level of usage of adenine and thymine in the third position. The third-position nucleotide composition of ORF36.5 was consistent with these observations; however, ORF33.4 displayed a bias for guanine over cytosine and more than twice the average incidence of adenine and thymine in the third position (data not shown).

Prior to nucleotide sequence determination of the 1.6-kb *Kpn*I fragment, studies were performed to determine if the *gca* gene was expressed from its own promoter or from the *lacZ* promoter present on pAK1900, the vector used to construct pFV39. The 1.6-kb insert from pFV39 was religated to *Kpn*I-digested pAK1900 to give rise to a recombinant plasmid containing the 1.6-kb insert in the opposite orientation. The resulting plasmid, pFVI39, was subsequently mobilized into the A-band-deficient mutant rd7513. The transconjugate, rd7513 (pFVI39), was found to react with the A-band MAb N1F10 in slide agglutination tests, indicating that the *gca* gene is transcribed from its own promoter. Nucleotide sequence analysis of ORF33.4 and ORF36.5 failed to show any evidence of a σ^{70} promoter region; however, two potential recognition se-

TABLE 1. Bacterial strains and plasmids used in the study

Strain or plasmid	Serotype, genotype, phenotype, or relevant characteristic	Reference or source[a]
Strains		
Pseudomonas aeruginosa		
IATS O1 (ATCC 33348)	O1 O antigen, A^+	33
IATS O2 (ATCC 33349)	O2 O antigen, A^+	33
IATS O3 (ATCC 33350)	O3 O antigen, A^+	33
IATS O4 (ATCC 33351)	O4 O antigen, A^+	33
IATS O5 (ATCC 33352)	O5 O antigen, A^+	33
IATS O6 (ATCC 33353)	O6 O antigen, A^+	33
IATS O7 (ATCC 33354)	O7 O antigen, A^-	33
IATS O8 (ATCC 33355)	O8 O antigen, A^+	33
IATS O9 (ATCC 33356)	O9 O antigen, A^+	33
IATS O10 (ATCC 33357)	O10 O antigen, A^+	33
IATS O11 (ATCC 33358)	O11 O antigen, A^+	33
IATS O12 (ATCC 33359)	O12 O antigen, A^-	33
IATS O13 (ATCC 33360)	O13 O antigen, A^-	33
IATS O14 (ATCC 33361)	O14 O antigen, A^-	33
IATS O15 (ATCC 33362)	O15 O antigen, A^-	33
IATS O16 (ATCC 33363)	O16 O antigen, A^-	33
IATS O17 (ATCC 33364)	O17 O antigen, A^+	33
Serotype O18	O18 O antigen, A^-	34
Serotype O19	O19 O antigen, A^+	34
Serotype O20	O20 O antigen, A^+	34
91-490 to 91-497	Serial isolates from siblings with CF; all typed as the same strain on the basis of enzyme allotype and RFLP[b] analysis with the pilin gene probe: 490-493 (patient 1), O19 O antigen[c]; 494-497 (patient 2), O1/O19 O antigen[c,d]	W. Johnson
49 to 58	Serial isolates from a single patient with CF over a 1-mo period; A^+; 49, 50, 58, O9 O antigen; 51 to 57, nontypeable	30
101	CF isolate	42
102	CF isolate	42
103	CF isolate	42
113	CF isolate	42
118	CF isolate	42
5197	CF isolate	T. Pitt
6154	CF isolate	T. Pitt
6257	CF isolate	T. Pitt
7100	CF isolate	T. Pitt
7259	CF isolate	T. Pitt
7375	CF isolate	T. Pitt
7537	CF isolate	T. Pitt
8757	CF isolate	T. Pitt
10226	CF isolate	T. Pitt
10227	CF isolate	T. Pitt
PAO1 (H103)	O5 O antigen, A^+	19
AK14O1	Mutant of OT684; lacks long-chain O antigen, A^+	4
rd7513	A^- mutant of AK14O1	31
8830	O3 O antigen, alginate producing	11, 26
FRD1	Alginate producing	40
C81M	CF isolate; O5 O antigen	28
C59M	CF isolate; O5/O16 O antigen[e]	28
C418M	CF isolate; O5/O16 O antigen[e]	28
C481M	CF isolate; O5/O16 O antigen[e]	28
Burkholderia cepacia 92-449 to 92-458	Serial clinical isolates from a single patient with CF	W. Johnson
Pseudomonas acidovorans		T. Warren
Pseudomonas stutzeri H364 (ATCC 17588)		R. Hancock
Pseudomonas fluorescens PF5		D. Cuppel
Pseudomonas putida PUG-1		D. Cuppel
Pseudomonas syringae H365 (ATCC 19310)		R. Hancock
Escherichia coli		
DH5α	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	GIBCO/Bethesda Research Laboratories
E69 O9K30H12		C. Whitfield

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Serotype, genotype, phenotype, or relevant characteristic	Reference or source[a]
Klebsiella pneumoniae CWK2	Serotype O1:K$^-$, Str^r (formerly KD2)	37
Salmonella typhimurium LT2	*metA22 metE551 trpC2* H1-b, H2-e,n,x *fla-66 rps-120*, *xyl-404*, *hsdL6*, *hsdSA29*, *hsdSB121*, *ilv-452*, *felS2*	K. Sanderson
Plasmids		
pAK1900	pGEM-3Zf(+) derivative with pRO1600 *ori*R Amp^r, 4.75 kb	A. Kropinski
pBluescript II KS	2.96 kb, Amp^r	PDI Biosciences, Aurora, Ontario, Canada
pFV39	1.6-kb *Kpn*I insert containing *gca* gene; in pAK1900	32
pFVI39	1.6-kb *Kpn*I insert containing *gca* gene in opposite orientation; in pAK1900	This study

[a] W. Johnson, Laboratory Centre for Disease Control, Ottawa, Ontario, Canada; T. Pitt, Central Public Health Laboratory, London, United Kingdom; R. E. W. Hancock and T. Warren, University of British Columbia, Vancouver, British Columbia, Canada; D. Cuppel, Agriculture Canada, London, Ontario, Canada; K. Sanderson, University of Calgary, Calgary, Alberta, Canada; A. Kropinski, Queen's University, Kingston, Ontario, Canada.
[b] RFLP, restriction fragment length polymorphism.
[c] The O-antigen typing carried out in the present study was by slide agglutination by the method of Lam et al. (29).
[d] Agglutination with both O1 and O19 antisera.
[e] Agglutination with both O5 and O16 MAbs.

quences for the alternate σ^{54} factor were found on the strand encoding ORF36.5.

The data presented above indicate that the Gca protein is encoded on the strand containing ORF36.5. Furthermore, according to the Shine-Dalgarno consensus sequence of *P. aeruginosa* (AAGGAGAG) (47), a possible Shine-Dalgarno sequence can be identified upstream of the putative ATG start codon of ORF36.5. A hydropathy profile (25) of the predicted protein encoded by *gca* failed to show significant hydrophobic regions or membrane-spanning segments, suggesting that the Gca protein is not intimately associated with the cytoplasmic membrane (data not shown). This observation is consistent with previous studies which have demonstrated that Gca activity is localized in the cytoplasm of *P. aeruginosa* (32).

Sequence homology. A search for the amino acid similarity of the Gca protein to the amino acids of other known proteins was performed by using protein sequence databases of GenBank through the NCBI Blast network server (1, 16). Two proteins, YefA (GDP–D-mannose dehydratase) (accession number P32054) from *E. coli* and RfbD (dehydratase, oxidoreductase, or epimerase) (accession number S28470) (35) from *Vibrio cholerae*, showed the most significant homology to the Gca protein encoded by ORF36.5. Both proteins, YefA and RfbD, showed homology to four specific regions of the Gca protein, while RfbD was homologous to one additional region (Fig. 3).

Conservation of the *gca* gene and expression of A-band LPS in *P. aeruginosa*. Results from Southern hybridization studies by using the *gca* gene as a DNA probe are presented in Tables 2 and 3. All *P. aeruginosa* serotypes except serotype O12 showed extensive conservation of the *gca* gene on a 1.6-kb *Kpn*I fragment. In the case of serotype O12, the *gca* gene probe hybridized to a 7.0-kb *Kpn*I fragment. Some variability among the serotypes was also observed when *Bam*HI or *Eco*RI was used to digest the chromosomal DNA; however, the majority hybridized to a 9.4-kb *Bam*HI fragment and a 9.4-kb *Eco*RI fragment.

A number of *P. aeruginosa* clinical isolates from patients with CF (CF isolates) were also used for hybridization studies with the *gca* gene probe. *P. aeruginosa* CF isolates 91-490 to 91-497 hybridized to the 1.6-kb *Kpn*I, 9.4-kb *Bam*HI, and 9.4-kb *Eco*RI probe-reactive fragments, consistent with the results observed for IATS O1 and O19 (isolates 91-490 to 91-493 are of serotype O1, and isolates 91-494 to 91-497 belong to the O1/O19 cross-reactive serogroup) (Tables 1 and 2). In a second group of *P. aeruginosa* CF isolates, CF isolates 49 to 58, the *gca* gene probe also hybridized to 1.6-kb *Kpn*I and 9.4-kb *Eco*RI fragments, consistent with that observed for IATS O9 (isolates 49, 50, and 58 are of serotype O9, and isolates 51 to 57 are nontypeable) (Tables 1 and 2). Both of the sets of *P. aeruginosa* CF isolates described above expressed A-band LPS on Western immunoblots.

Since some IATS serotypes such as O5 and O12 displayed differences in their probe-reactive fragments compared with the reactivities of other serotypes (Table 2), we decided to examine the use of this probe with both CF clinical isolates of serotype O5 and CF clinical isolates of serotype O12 (Table 3). The group of O5 isolates C81M, C59M, C418M, and C481M expressed A-band LPS and hybridized to 1.6-kb *Kpn*I, 20.0-kb *Bam*HI, and 9.4-kb *Eco*RI probe-reactive fragments which are genotypically characteristic of IATS O5. In the case of the O12 isolates, 3 hybridized to a 7.0-kb *Kpn*I fragment and failed to express A-band LPS, resembling the hybridization pattern of IATS O12, while the remaining 12 hybridized to a 1.6-kb *Kpn*I fragment.

Presence of the *gca* gene and A-band LPS expression in *Burkholderia cepacia* and other pseudomonads. *B. cepacia* has raised serious concerns recently because of its ability to cause rapid and severely debilitating chronic lung infections in patients with CF. Since this organism had previously been classified under the family *Pseudomonadaceae*, it was of interest to determine if the *gca* gene was present in this organism and if A-band LPS was expressed. Southern hybridization was carried out on 10 *B. cepacia* CF isolates. The *gca* gene probe hybridized to 1.6-kb *Kpn*I, 20.0-kb *Bam*HI, and 9.4-kb *Eco*RI probe-reactive fragments in only 6 of the 10 isolates (Table 2). These six isolates showed no expression of A-band LPS. Various other pseudomonads were also analyzed to determine if other members of the family *Pseudomonadaceae* contain the *gca* gene and express A-band LPS. *Pseudomonas acidovorans*, *Pseudomonas putida*, and *P. syringae* were found to hybridize to the 1.6-kb *Kpn*I, 9.4-kb *Bam*HI, and 9.4-kb *Eco*RI conserved probe-reactive fragments (Table 2), similar to most *P. aeruginosa* serotypes. *P. acidovorans* and *P. putida* were also found to express A-band LPS, while *P. syringae* lacked A-band LPS. Two other pseudomonads, *Pseudomonas stutzeri* and *Pseudomonas fluorescens*, did not hybridize to the *gca* gene probe and showed no A-band LPS expression, indicating that this

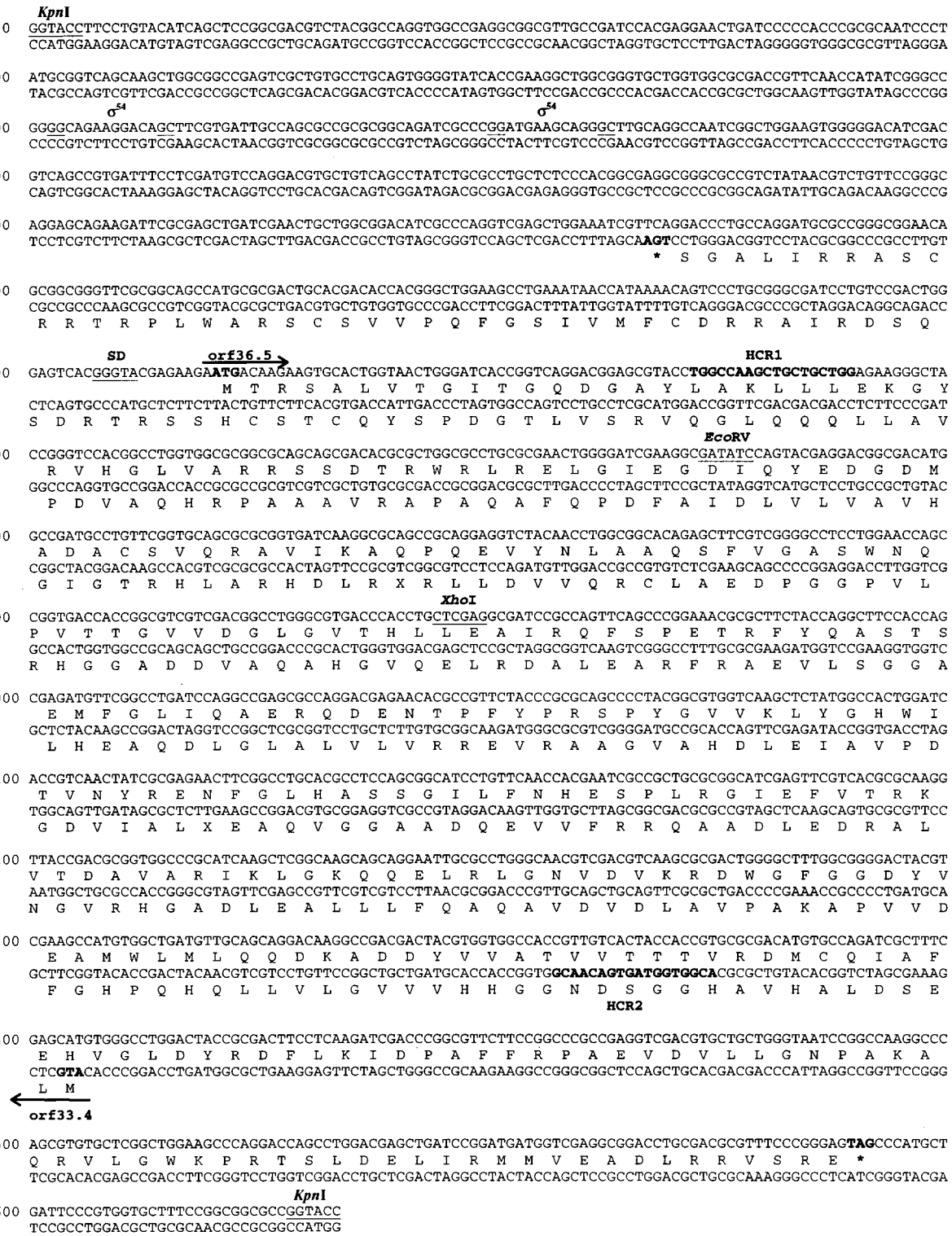

FIG. 1. Nucleotide sequence of the 1.6-kb *Kpn*I insert containing the *gca* gene from pFV39 and the corresponding amino acid sequences for both ORF36.5 and ORF33.4. Both ORFs are indicated on their respective strands, as are the putative start (ATG) and stop (*) codons for each. Two possible sites containing consensus recognition sequences (GG-N_{10}-GC) for σ^{54} are shown on the strand containing ORF36.5. In addition, a potential Shine-Dalgarno sequence (S-D) is indicated upstream of the putative ATG start codon of ORF36.5. The sequences of two PCR oligonucleotide primers, HCR1 and HCR2, are shown on their respective strands.

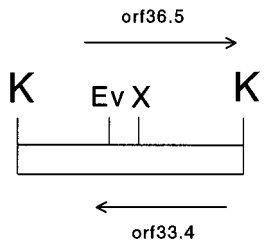

FIG. 2. Plasmid pFV39 containing the 1.6-kb *Kpn*I fragment on which the *gca* gene resides. The arrows indicate the direction of transcription for both ORF36.5 and ORF33.4 on their respective strands.

gene is not universally present among all *Pseudomonas* species.

PCR amplification of the *gca* gene from *P. aeruginosa*. PCR amplification was carried out on each of the 20 O serotypes of *P. aeruginosa* with primers specific for the *gca* gene. Amplification of a 700-bp product from all 20 O serotypes was observed (Fig. 4). The plasmid containing the *gca* gene, pFV39, and IATS O5 were also amplified by PCR, and the products were subjected to Southern hybridization by using the *gca* gene as a DNA probe. Both products hybridized to the probe, indicating that the amplified product was derived from the *gca* gene (data not shown). No amplification was seen with the

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1
RfbD   6 ALITGITGQDGSYLAEFLLEKGYEVHGIKRRSS 38
         **|********|***| ****** ***|  * *
Gca    5 ALVTGITGQDGAYLAKLLLEKGYRVHGLVARRS 37

2
RfbD  64 GDLTDSSNLTRILAEVQPDEVYNLGAQSHVAVSFQSPEYTADVDAIGTLRLLEAIR 119
         **| *| || * | | ** ***** *** *  *|  *  *  ** |*   ******
Gca   58 GDMADACSVQRAVIKAQPQEVYNLAAQSFVGASWNQPVTTGVVDGLGVTHLLEAIR 113
         **||*  || * | | ** ***** * * *  *|  *  *  ** |*   ******
YefA  14 GDLSDTSNLTRILREVQPDEVYNLGAMSHVAVSFESPEYTADVDAMGTLRLLEAIR  69

3
RfbD 126 KTKFYQASTSELYGLVQEIPQKETTPFYPRSPYAVAKMYAYWITINYRESYGIYAC 181
         |*|********||**|*   * * ********* * *|* |***|****||*||*
Gca  118 ETRFYQASTSEMFGLIQAERQDENTPFYPRSPYGVVKLYGHWITVNYRENFGLHAS 173
         |**********||**|*   * * ********** * *** |********||*||*
YefA  76 KTRFYQASTSELYGLVQEIPQKETTPFYPRSPYAVAKLYAYWITVNYRESYGMYAC 131

RfbD 182 NGILFNHESPRRGETFVTRKITRGMANIAQGLEKCLFMGNLDALRDWGHAKDYVKM 237
         |********* **  *****|*  |* *  * || * |**|*  ****   ***|
Gca  174 SGILFNHESPLRGIEFVTRKVTDAVARIKLGKQQELRLGNVDVKRDWGFGGDYVEA 229
         |********* **  *****|* *|* *  * |  * ***|*  ****   ***|
YefA 132 NGILFNHESPRRGETFVTRKITRAIANIAQGLESCLYLGNMDSLRDWGHAKDYVKM 187

RfbD 238 QWMMLQQDEPRDFVIATGV 256
          *|** * |  *  |   *
Gca  230 MWLMLAQQDKADDYVVATV 248
          *|** *|   *  |   *
YefA 188 QWMMLQQEQPEDFVIATGV 206

4
RfbD 237 MQWMMLQQDEPRDFVIATGVQYSVREFIDMSARELGIE 274
         *  *| ***|  *|*|** *  |**|   ||   |*||
Gca  230 MWLMLAQQDKADDYVVATVVTTTVRDMCQIAFEHVGLDYR 269
         *  *| **|| |*|*|** *  |**   ||*   |*|  *
YefA 187 MQWMMLQQEQPEDFVIATGVQYSVRQFVEMAAAQLGIKLR 226

5
RfbD 308   VDPAYFRPAEVETLLGDPSLAKKELGWVPEITLQQMVSEMVASDLEQ 354
           |***|******| ***|*| *|| *** *  |* ||||  ** |** |
Gca  272 LKIDPAFFRPAEVDVLLGNPAKAQRVLGWKPRTSLDELIRMMVEADLRRVSR 323
         | |** |******| ***|* **   *****  |* *||  **  **    |
YefA 256 IAVDPRYFRPAEVETLLGDPTKAHEKLGWKPEITLREMVSEMVANDLEAAKK 307
```

FIG. 3. Alignment of homologous regions of the Gca protein with YefA of *E. coli* (accession no. P32054) and RfbD of *V. cholerae* (accession no. S28470) (35). Identical amino acids are represented by asterisks, while similar amino acids are represented by vertical lines. The YefA protein shares four regions of homology: region 2, 46% identity and 60% homology; region 3, 58% identity and 72% homology; region 4, 35% identity and 62% homology; region 5, 48% identity and 65% homology. The RfbD protein shares five regions of homology: region 1, 72% identity and 84% homology; region 2, 48% identity and 62% homology; region 3, 54% identity and 72% homology; region 4, 34% identity and 63% homology; region 5, 48% identity and 76% homology.

TABLE 2. Southern hybridization results for various pseudomonads obtained by using the *gca* gene as a DNA probe and Western immunoblot results obtained by using A-band LPS-specific MAb N1F10

Strain	Size of Southern hybridization probe-reactive fragment (kb)			Western immunoblot reactivity with A-band MAb N1F10 (reference)
	*Kpn*I	*Bam*HI	*Eco*RI	
Pseudomonas aeruginosa				
IATS O1	1.6	9.4	9.4	A^+ (30)
IATS O2	1.6	9.4	9.4	A^+ (30)
IATS O3	1.6	9.4	9.4	A^+ (30)
IATS O4	1.6	9.4	9.4	A^+ (30)
IATS O5	1.6	20.0	9.4	A^+ (30)
IATS O6	1.6	9.4	9.4	A^+ (30)
IATS O7	1.6	9.4	9.4	A^- (30)
IATS O8	1.6	9.4	5.0	A^+ (30)
IATS O9	1.6	9.4	9.4	A^+ (30)
IATS O10	1.6	9.4	5.0	A^+ (30)
IATS O11	1.6	9.4	9.4	A^+ (30)
IATS O12	7.0	3.5	2.5	A^- (30)
IATS O13	1.6	9.0	5.0	A^- (30)
IATS O14	1.6	9.4	9.4	A^- (30)
IATS O15	1.6	9.4	4.8	A^- (30)
IATS O16	1.6	9.4	17.0	A^- (30)
IATS O17	1.6	9.4	5.0	A^+ (30)
Serotype O18	1.6	9.4	9.4	A^- (30)
Serotype O19	1.6	9.4	9.4	A^+ (30)
Serotype O20	1.6	20.0	5.0	A^+ (30)
91-490 to 91-497	1.6	9.4	9.4	A^+ (30)
49 to 58	1.6	ND[a]	9.4	A^+ (30)
PAO1 (H103)	1.6	ND	ND	A^+ (30)
AK14O1	1.6	ND	ND	A^+ (30)
rd7513	1.6	ND	ND	A^- (31)
8830	1.6	ND	ND	A^+
FRD1	1.6	ND	ND	A^+
Burkholderia cepacia				
92-449	1.6	20.0	9.4	A^-
92-450	—[b]	—	—	A^-
92-451	—	—	—	A^-
92-452	1.6	20.0	9.4	A^-
92-453	—	—	—	A^-
92-454	1.6	20.0	9.4	A^-
92-455	1.6	20.0	9.4	A^-
92-456	1.6	20.0	9.4	A^-
92-457				A^-
92-458	1.6	20.0	9.4	A^-
Pseudomonas acidovorans	1.6	9.4	9.4	A^+
Pseudomonas stutzeri H364	—	—	—	A^-
Pseudomonas fluorescens PF5	—	—	—	A^-
Pseudomonas putida PUG-1	1.6	9.4	9.4	A^+
Pseudomonas syringae H365	1.6	9.4	9.4	A^-
Klebsiella pneumoniae CWK2	—	ND	ND	A^-
Salmonella typhimurium LT2	—	ND	ND	A^-

[a] ND, not determined.
[b] —, no hybridization.

TABLE 3. Southern hybridization results for *P. aeruginosa* O5 CF isolates and O12 clinical isolates obtained by using the *gca* gene as a DNA probe and Western immunoblot results obtained by using A-band LPS-specific MAb N1F10

Serotype and strain	Size of Southern hybridization probe-reactive fragments (kb)			Western immunoblot reactivity with A-band MAb N1F10 (reference)
	*Kpn*I	*Bam*HI	*Eco*RI	
Serotype O5				
IATS O5	1.6	20.0	9.4	A^+ (30)
C81M	1.6	20.0	9.4	A^+
C59M	1.6	20.0	9.4	A^+
C418M	1.6	20.0	9.4	A^+
C481M	1.6	20.0	9.4	A^+
Serotype O12[a]				
IATS O12	7.0	3.5	2.5	A^- (30)
101	1.6	ND[b]	ND	A^+
102	1.6	ND	ND	A^+
103	1.6	ND	ND	A^+
113	1.6	ND	ND	A^+
118	7.0	ND	ND	A^-
5197	1.6	ND	ND	A^+
6154	1.6	ND	ND	A^-
6257	1.6	ND	ND	A^+
7100	7.0	ND	ND	A^-
7259	1.6	ND	ND	A^+
7375	7.0	ND	ND	A^-
7537	1.6	ND	ND	A^+
8757	1.6	ND	ND	A^-
10226	1.6	ND	ND	A^+
10227	1.6	ND	ND	A^+

[a] Strains 101 to 7537 are multidrug resistant (42).
[b] ND, not determined.

negative controls, *E. coli* E69 and *Salmonella typhimurium* LT2, or in the blank containing all reaction components except template DNA (data not shown).

DISCUSSION

In an attempt to obtain a better understanding of the role of the *gca* gene in A-band LPS synthesis and to assess its applicability as a diagnostic tool, sequence analysis, Southern hybridization, and PCR amplification were carried out in the present study. Protein expression data from Lightfoot and Lam (32) revealing a 37-kDa protein, along with nucleotide sequence analysis, suggest that the Gca protein is encoded by ORF36.5 rather than ORF33.4. ORF36.5 may contain its own promoter region upstream because of the presence of two possible σ^{54} recognition sequences.

Sequence homology often provides information that can lead to identification of the definitive function of a protein. In this case, the Gca amino acid sequence was found to be homologous to specific regions within two proteins (Fig. 3), YefA of *E. coli*, which encodes the GDP–D-mannose dehydratase involved in fucose biosynthesis, and RfbD of *V. cholerae*, which is thought to function as either a dehydratase, an oxidoreductase, or an epimerase in the production of the O-antigen sugar perosamine from GDP–D-mannose (35). Previously, Lightfoot and Lam (32) demonstrated that the Gca protein is involved in the conversion of GDP–D-mannose to GDP–D-rhamnose. The enzymatic steps required for this conversion are thought to involve a GDP–D-mannose dehydrase (functionally homologous to a dehydratase) and a GDP–4-keto-D-rhamnose reductase (36). Thus, the homology of the Gca protein to enzymes that function as dehydratases or reductases correlates with those enzymes required for the biosynthesis of GDP–D-rhamnose. On the basis of the sequence homology to YefA and RfbD and taking into consideration the steps required to convert GDP–D-mannose to GDP–D-rhamnose, we propose that the Gca protein functions either as a dehydratase or as a bifunctional enzyme which facilitates a simple conversion of GDP–D-mannose to GDP–D-rhamnose. The ability of the Gca protein to function as a bifunctional enzyme may be compared with those of *algA* (phosphomannose isomerase; GDP-mannose pyrophosphorylase) (46) and *algC* (phosphomannomutase; phosphoglucomutase) (8, 52) enzymes of the *P. aeruginosa* alginate pathway, both of which have dual functions.

In order to determine if this *gca* gene was present and conserved in strains of all 20 O serotypes, Southern hybridizations were performed. The Southern hybridizations revealed that the *gca* gene was conserved in each of all 20 serotypes on a 1.6-kb *Kpn*I fragment with the exception of strains of serotype O12, in which it hybridized to a 7.0-kb fragment (Table 2).

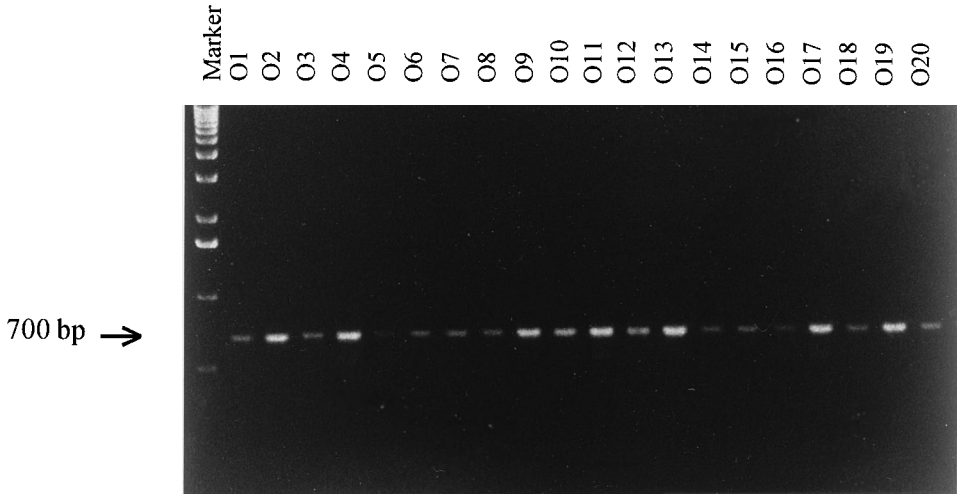

FIG. 4. PCR amplification of serotypes O1 to O20 with primers specific for the *gca* gene. Amplification of a 700-bp product can be seen in strains of all 20 O serotypes compared with the marker (1-kb ladder).

Substantial differences among the 20 serotypes were observed when the restriction enzymes *Bam*HI and *Eco*RI were used. These variations in probe-reactive fragment profiles confer certain hybridization patterns on specific serotypes, such as those seen with serotypes O5 and O12. On the basis of these unique genotypic characteristics, it appears that the pattern of probe-reactive fragments exhibited by a strain of a certain serotype is diagnostic of that serotype and reproducible when other strains of that same serotype are probed with the *gca* gene.

P. aeruginosa CF isolates were also used for hybridization studies to determine if these isolates displayed hybridization patterns similar to those of the IATS strains. Two sets of isolates, 91-490 to 91-497 and 49 to 58, hybridized to the predicted probe-reactive fragments, which correlates with the results obtained for their respective serotypes. A third set of CF isolates had previously been typed by Lam et al. (28), with C81M typing as O5 and C59M, C418M, and C481M typing as O5/O16. Typing of these three strains as more than one serotype is indicative of the presence of cross-reactive epitopes which O5 and O16 share in their LPSs (27). When the probe-reactive fragment profiles of these isolates were examined, it was evident that these three isolates genotypically typed as serotype O5 on the basis of their unique *Bam*HI hybridization patterns which correspond to that of IATS O5. This *gca* probe may be useful as a means of determining or confirming a particular clinical isolate's serotype rather than relying solely on phenotype-based typing methods.

As previously mentioned, IATS O12 was the only serotype in which the *gca* gene probe hybridized to a fragment other than that of the common 1.6-kb *Kpn*I fragment. It is of interest that IATS O12 is one of six IATS strains (O7, O12, O13, O14, O15, and O16) which do not express A-band LPS (30). A correlation can be drawn between IATS O12 and the three clinical O12 isolates in the present study which also hybridized to a 7.0-kb *Kpn*I fragment and failed to express A-band LPS. From these results it can be speculated that some strains of serotype O12 have an altered *Kpn*I restriction map which is associated with a lack of A-band LPS expression. Additional support for a different restriction map comes from a study by Ullstrom et al. (49) in which mapping of an outer membrane protein gene by Southern hybridization revealed that serotype O12 is the only serotype with an altered *Kpn*I restriction map.

Various pseudomonads were also examined in the present study to ascertain the presence of the *gca* gene among different species. Results indicate that this gene is not found in all pseudomonads; however, when it is present it is well conserved with respect to probe-reactive profiles observed by Southern hybridization. Among those pseudomonads containing *gca*, only *P. acidovorans* and *P. putida* showed reactivity to the A-band MAb. This indicates that these two organisms possess either a different LPS molecule with similar antigenic epitopes or express A-band common antigen. Interestingly, Western immunoblots showed that *P. syringae* failed to react with the A-band MAb. Since previous studies by Smith et al. (48) had demonstrated that *P. syringae* pv. morsprunorum C28 has an O repeat unit composed of D-rhamnose, we thought that perhaps other *P. syringae* strains may also express such an LPS molecule and that it may react with our A-band LPS-specific MAb. However, the *P. syringae* isolate in our study, as well as all of the *B. cepacia* isolates from patients with CF, displayed rough LPS phenotypes (no O repeat units) on SDS-polyacrylamide gels (data not shown). This accounts for their lack of reactivity with the A-band MAb; however, it is not clear whether A-band LPS is present in other isolates of the same species.

From the Southern hybridization results for the 20 *P. aeruginosa* O serotypes, it is evident that the *gca* gene is well conserved. On this basis, PCR primers designed from the *gca* gene sequence were used to amplify a 700-bp product from each of the 20 O serotypes (Fig. 4). It is tempting to consider the possibility of using PCR amplification of the *gca* gene as a means of detecting the presence of *P. aeruginosa*. The drawback of such a diagnostic tool is that the *gca* gene is not unique to *P. aeruginosa* and is, instead, present in other pseudomonads that may also be found in clinical isolates. McIntosh et al. (38) demonstrate the application of PCR with primers specific for the *algD* gene (GDP–D-mannose dehydrogenase) of *P. aeruginosa* to identify this organism from clinical sputum samples from patients with CF. The *algD* gene, like the *gca* gene, is present and well conserved in strains of all 20 O serotypes and was found in the study by McIntosh et al. (38) to be unique to *P. aeruginosa*. However, the study failed to examine amplification of *algD* in *B. cepacia*, which may also be present in the sputa of patients with CF. In the future, a combination of both *algD* and *gca* gene primers may be useful in PCR amplification to increase the accuracy of *P. aeruginosa* identification. Such rapid detection, particularly in early lung infections of patients with CF, is essential for preventing chronic states of infection.

Results from the sequence analysis indicate that the Gca protein may be functioning either as a dehydratase or as a bifunctional enzyme, converting GDP–D-mannose to GDP–D-rhamnose. The *gca* gene, which codes for this protein, is present and conserved throughout the 20 *P. aeruginosa* O serotypes; however, variability is seen in the *Bam*HI and *Eco*RI restriction maps of some serotypes, which may prove to be useful in determining or verifying the serotypes of clinical isolates. This gene is not universally distributed among all pseudomonads, but it is well conserved when it is present. In conclusion, PCR amplification with primers specific for the *gca* gene sequence may prove to be useful in the identification of *P. aeruginosa* among clinical isolates.

ACKNOWLEDGMENTS

This work was supported by grants (to J.S.L.) from the Canadian Cystic Fibrosis Foundation and the Canadian Bacterial Diseases Network. H.L.C. is a recipient of a Canadian Cystic Fibrosis studentship, and J.L. was a recipient of a Medical Research Council studentship and a Natural Sciences and Engineering Research Council postgraduate scholarship.

We thank J. Foster for technical assistance and the numerous colleagues (listed in Table 1) who kindly provided us with many strains and clinical isolates.

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