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Biotinylated DNA Probes for Exotoxin A and Pilin Genes in the Differentiation of *Pseudomonas aeruginosa* Strains

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Biotin-labeled DNA probes derived from *Pseudomonas aeruginosa* exotoxin A and pilin genes were tested for their ability to distinguish strains among a selected group of *P. aeruginosa* isolates. Probing of Southern blots of restriction digests of DNA from test strains with the exotoxin A probe demonstrated a unique hybridization pattern for each independently isolated strain containing the exotoxin A gene. Two phenotypically distinct strains isolated from the same patient were found to be identical in their DNA hybridization patterns. By using a pilin gene probe, similar distinction was made between independent strains, while strains from the same source were confirmed to be identical. Furthermore, DNA from a strain of *P. aeruginosa* lacking the exotoxin A gene yielded a unique pattern of restriction fragments which hybridized to the pilin gene probe. The exotoxin A and the pilin probes may together prove to be useful tools in epidemiological surveys during outbreaks of *P. aeruginosa* infection.

Serious infections by the opportunistic pathogen *Pseudomonas aeruginosa* are primarily restricted to groups of compromised patients. During the past decade, *P. aeruginosa* has emerged as an important nosocomial pathogen, responsible for a significant fraction of all hospital-acquired infections. Despite the high incidence of *P. aeruginosa* infections among identifiable high-risk patient groups, the epidemiology of this pathogen is not well understood. Conflicting reports on the source and spread of *P. aeruginosa* in a patient population are primarily due to inadequate strain identification techniques. The most commonly used epidemiological techniques involve the use of standard sera in serological typing schemes or determinations of patterns of sensitivity to bacteriophages or bacteriocins. The ability of *P. aeruginosa* to undergo changes in surface antigens during infection (10), or as a result of spontaneous mutation selected by exposure to bacteriophages (4), has been well documented and contributes to the inability of currently used typing schemes to reliably establish the relationships of individual *P. aeruginosa* strains.

The importance of identification of sources of nosocomial outbreaks has necessitated the identification of stable genetic markers for strain typing of *P. aeruginosa*. The discovery by Vasil and co-workers (9, 15) that the structural gene for exotoxin A is located in the bacterial chromosome in a highly variable sequence environment has led to development of specific DNA probes which can distinguish between *P. aeruginosa* strains based on the presence of unique DNA sequences adjacent to the exotoxin A gene. The location of specific restriction sites in the variable region of the exotoxin A flanking DNA yields a characteristic restriction pattern on a Southern blot, and this pattern is stable and strain specific. With DNA probes, it was possible to distinguish among over 100 different patterns in one large-scale screening study (9). Studies such as this have shown the utility of exotoxin A-derived genetic probes in epidemiological surveys of *P. aeruginosa* outbreaks. Use of the probes has overcome the inherent shortcomings of the standard strain identification techniques involving recognition of bacterial surface components by serological reagents or by bacteriophages and bacteriocins.

Approximately 5% of *P. aeruginosa* isolates lack the exotoxin A structural gene and therefore are nontypable with the exotoxin A gene probes (15). Moreover, to determine whether any two isolates represent truly independent strains, it may be necessary to carry out a number of restriction digestions of DNA, since the probe-reactive fragments generated by restriction with any one enzyme may appear identical due to limited resolving capabilities of standard agarose gels. It would be useful, therefore, to identify additional genetic markers that may distinguish between *P. aeruginosa* strains without an exotoxin A gene and those which appear identical by Southern hybridization analysis with the exotoxin A-derived probes.

Comparison of primary sequences of pilin from several unrelated *P. aeruginosa* strains revealed extensive regions of variability in the carboxy-terminal portions, while the amino-terminal 60 amino acids were virtually identical (2, 11, 12). The variability of the C-terminal region of pilin is reflected in a variable DNA sequence encoding this region. Presence or absence of specific restriction enzyme recognition sites in the vicinity of this region will result in restriction fragment heterogeneity detectable in Southern hybridization analyses with appropriate pilin gene probes. The use of a pilin gene probe can be a valuable supplement to the exotoxin A probe in epidemiological studies, since no naturally occurring strains that lack the pilin gene have been reported as yet.

In this communication we demonstrate the utility of exotoxin A and pilin probes in studies of relatedness of different *P. aeruginosa* strains. For the studies reported here we have used nonisotopic hybridization technology, which will facilitate the use of gene hybridization technology in routine epidemiological surveys.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains of *P. aeruginosa* used in this study and plasmids used as probes are listed in Table 1. *Escherichia coli* DH5α [*endA1 hsdR17 supE44 thi-1*

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TABLE 1. *P. aeruginosa* strains used in this study

Strain	Relevant phenotype	Source or reference
PAK	Wild type, originally ATCC 25102	D. Bradley
C340	Mucoid isolate from a cystic fibrosis patient	B. Ramsey
C341	Nonmucoid, green pigment producer, from the same cystic fibrosis patient as C340	B. Ramsey
PA103	Well-characterized exotoxin A-hyperproducing strain	5
MSH7	Environmental isolate from Mount St. Helens volcanic area	B. Iglewski
CC1	Clinical isolate from a wound infection	B. Ramsey
WR5	Clinical isolate lacking exotoxin A structural gene	15

recA1 gyrA96 relA1 Δ(*lacZYA-argF*) U169 λ d*lacZ* ΔM15] (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was the host for recombinant plasmids. Plasmid pMS27 has been described previously (14). The cloning vector pUC18cm, provided by M. Yanofsky, is a modification of pUC18 in which the β-lactamase gene of pUC18 has been replaced with the gene for chloramphenicol acetyltransferase.

Reagents and enzymes. Deoxynucleotide triphosphates used in nick translation were purchased from Pharmacia P-L Biochemicals, Milwaukee, Wis. Restriction enzymes, the Klenow fragment of *E. coli* DNA polymerase, DNA ligase, biotin-7-dATP, and streptavidin-alkaline phosphatase conjugate were purchased from Bethesda Research Laboratories. Antibiotics (chloramphenicol and tetracycline), Nitro Blue Tetrazolium, 5-bromo-4-chloro-3-indolylphosphate, and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, Mo.

Growth of bacteria and DNA isolation techniques. Total DNA was isolated from a 1-ml culture of *P. aeruginosa* grown with shaking at 37°C for 12 h in Luria broth (8) by a modification of a technique described previously (14). Bacteria were pelleted in a Microfuge and washed with 50 mM Tris hydrochloride buffer, pH 8. The washed cells were suspended in 0.9 ml of 50 mM Tris hydrochloride–50 mM EDTA, pH 8, containing 2 mg of lysozyme per ml and incubated at ambient temperature for 10 min. A 10-μl amount of 20% sodium dodecyl sulfate (SDS) and 50 μl of a 1% solution of proteinase K (Calbiochem-Behring, La Jolla, Calif.) were added to the samples. Following a 30-min incubation at 37°C, phenol saturated with 50 mM Tris hydrochloride, pH 8, was added, and incubation was continued for an additional 30 min with occasional mixing. The aqueous phase was separated from phenol by centrifuging in a Microfuge for 4 min, and the top phase was transferred to a fresh tube containing an equal volume of chloroform. The two phases were mixed by vortexing for 1 min and then separated by 1-min centrifugation. The top phase was retained, and sodium acetate, pH 4.8, was added to 0.3 M. Then 2 volumes of ice-cold absolute ethanol were added. Precipitated DNA was spooled out with a glass rod, washed twice in 75% ethanol, and suspended in 400 μl of 10 mM Tris hydrochloride–1 mM EDTA, pH 8.

Preparation of DNA probes and Southern blot analysis. Probes were prepared from recombinant plasmids containing the gene sequences of interest (see Fig. 1). The appropriate fragments were labeled by nick translation as described previously, substituting biotin-7-dATP for [^{32}P]dATP (3, 7). Following a 2-h labeling, the DNA was precipitated with ammonium acetate and ethanol.

Total DNA from *P. aeruginosa* was digested with appropriate restriction enzymes, and the resulting fragments were separated by electrophoresis on 0.8% agarose gels. The fragments were transferred onto nylon membranes (Nytran; Schleicher & Schuell, Keene, N.H.) by the method of Southern (13) as modified by the manufacturer. Prior to hybridization, filters were incubated for 30 min in a solution consisting of 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7), 5× Denhardt solution (7), 25 mM sodium phosphate buffer (pH 7), 5% dextran sulfate, 0.5% SDS, and 50 μg of sheared and boiled calf thymus DNA per ml. The nylon membranes were then hybridized with probe DNA for 18 h at 37°C. The hybridization solution was similar to the prehybridization solution, except the Denhardt solution was lowered to 1× and the heat-denatured, biotin-labeled probe (100 ng/ml) was included. After hybridization, the filters were washed at room temperature for 5 min in 2× SSC–0.1% SDS, followed by a wash in 0.2× SSC–0.1% SDS for an additional 5 min. The final wash was in 0.2× SSC–0.1% SDS for 30 min at 55°C. Filters were then blocked by incubation for 30 min in 3% (wt/vol) bovine serum albumin–0.1 M Tris hydrochloride buffer (pH 7.5)–0.15 M NaCl at 65°C. The filters were then immersed in a minimal volume of a solution of streptavidin-alkaline phosphatase conjugate diluted to 1 μg/ml in 0.1 M Tris hydrochloride (pH 7.5)–0.15 M NaCl. Following incubation for 15 min at room temperature with gentle agitation, the membranes were washed twice for 10 min each in 500 ml of 0.1 M Tris hydrochloride (pH 7.5)–0.15 M NaCl, followed by a single wash with 250 ml of 0.1 M Tris hydrochloride (pH 9.5)–0.1 M NaCl–50 mM $MgCl_2$.

The alkaline phosphatase bound to DNA was visualized following treatment of membranes with Nitro Blue Tetrazolium dye. This dye solution was prepared just prior to use by mixing, in 10 ml of 0.1 M Tris hydrochloride (pH 9.5)–0.1 M NaCl–50 mM $MgCl_2$, 50 μl of freshly prepared 7.5% (wt/vol) solution of Nitro Blue Tetrazolium (in 70% dimethylformamide) with 35 μl of a 5% solution (wt/vol) of 5-bromo-4-chloro-3-indolylphosphate (in dimethylformamide). The nylon membranes were incubated in the dye solution in the dark at room temperature for 30 min to 2 h until bands became visible. When maximal color development was achieved, the reaction was stopped by rinsing the filters in 20 mM Tris hydrochloride (pH 7)–0.5 mM EDTA. The membranes were blotted dry with paper towels, photographed immediately, and stored in the dark. Color was retained on membranes for at least 6 months under these conditions.

RESULTS

The pilin gene probe used in Southern blot analysis was a 1.2-kilobase (kb) *Hin*dIII fragment from pMS27 (14) (Fig. 1A), encoding the entire pilin polypeptide of *P. aeruginosa* PAK as well as additional sequences flanking the pilin structural gene. Comparison of DNA sequences of pilin genes from three independently isolated strains of *P. aeruginosa*, PAK, PAO, and PA103 (2, 12), showed the presence of unique restriction sites in the region of the gene that encodes the hypervariable domain of the pilin polypeptide. For example, the pilin gene from PAK contains unique *Cla*I and *Pvu*II sites near the 3′ end of the gene, while these sites are absent from the other two genes. Similarly, the PA103 pilin gene contains a unique *Kpn*I site not found in PAK or PAO pilin genes, while the PAO pilin gene contains a unique *Bam*HI site absent from the corresponding position in the other two strains.

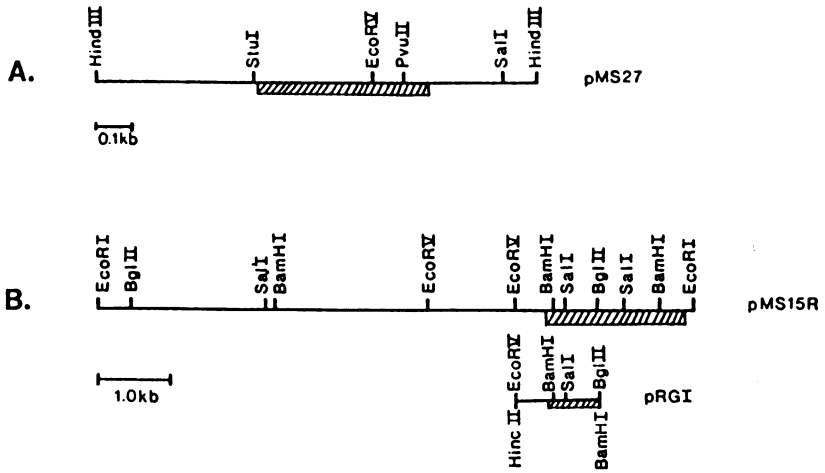

FIG. 1. Restriction map of DNA probes used in this study. (A) Insert of plasmid pMS27 (14) containing the entire cloned pilin gene of *P. aeruginosa* PAK. (B) Insert of plasmid pRGI, prepared by cloning the *Bgl*II-*Eco*RV fragment of pMS15R into the *Bam*HI-*Hinc*II sites of plasmid pUC18cm. Hatched region denotes pilin and the exotoxin A-coding sequence.

The exotoxin A gene probe was plasmid pRGI (Fig. 1B), which is an *Eco*RV-*Bgl*II fragment of pMS15R (6), cloned in pUC18cm. Plasmid pRGI contains approximately 0.7 kb of coding sequence from the N terminus of exotoxin A in addition to 0.4 kb of DNA from the 5′ flanking region. This probe allows detection of unique fragments from a variable portion of the *P. aeruginosa* chromosome, since extensive divergence of DNA sequence has been identified in the regions upstream from the exotoxin A gene (15).

DNA from the seven test strains was extracted, digested with restriction enzymes, blotted onto nylon membranes following agarose gel electrophoresis, and probed with exotoxin A and pilin gene probes. Digests of DNA with *Bam*HI, *Sal*I, and *Bgl*II were probed with pRGI to determine the variation of fragment sizes that contain a portion of the exotoxin A-coding sequence. Six strains showed hybridization to specific fragments (Fig. 2); no hybridization of the probe was detected to DNA fragments from *P. aeruginosa* WR5, a strain lacking the exotoxin A structural gene (15). *Bam*HI digests of the other six strains yielded two fragments that hybridized with the probe: a 1.5-kb fragment and a larger fragment that varied in length. This fragment was 3.4 kb from genomic digests of strains PAK, C340, C341, and PA103, 3.2 kb from MSH7, and 3.9 kb from CC1. The 1.5-kb fragments are due to the presence of two *Bam*HI recognition sites within the exotoxin A-coding sequence, while the longer variable fragments are generated by cleavage at restriction sites within the 5′ flanking regions. Similarly, hybridization of the *Sal*I digest revealed the presence of unique as well as conserved fragments, with strain PAK containing a 4.1-kb probe-reactive fragment, strains C340, C341, and PA103 hybridizing with identical fragments of 4.2 kb, and MSH7 and CC1 containing a 8.4-kb fragment. Interpretation of hybridization patterns of digests with *Bam*HI and *Sal*I restriction enzymes suggested that strains C340 and C341 are related to each other and to PA103. Southern analysis of DNAs digested with the restriction enzyme *Bgl*II revealed a 7.5-kb restriction fragment hybridizing to the exotoxin A probe from digests of strains PAK, C340, and C341, a 10.0-kb fragment from PA103, a 4.8-kb

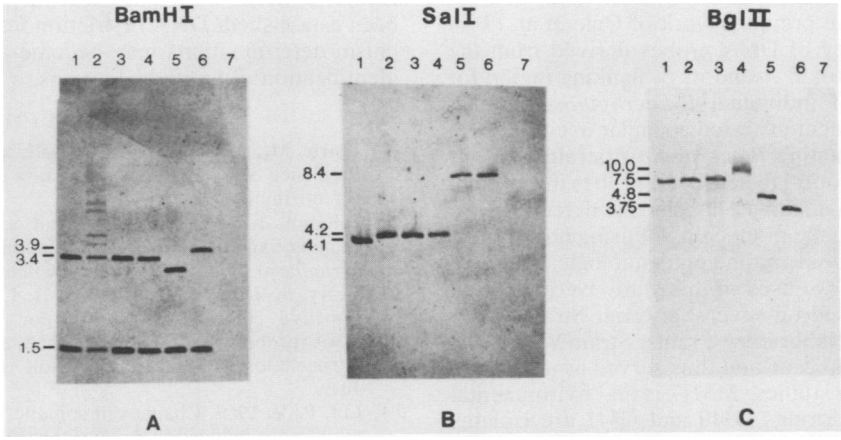

FIG. 2. Southern blot analysis of restriction digests of various *P. aeruginosa* strains with the exotoxin A gene probe pRGI. DNA was extracted from individual strains and digested with restriction enzymes, and following agarose gel electrophoresis fragments were blotted onto nylon membranes. Membranes were probed as described in Materials and Methods. Digests with *Bam*HI (A), *Sal*I (B), and *Bgl*II (C) are shown. Strains of *P. aeruginosa* analyzed are: 1, PAK; 2, C340; 3, C341; 4, PA103; 5, MSH7; 6, CC1; 7, WR5. Hybridization of additional DNA fragments with the probe in lane 2 is probably due to incomplete digestion of DNA with *Bam*HI.

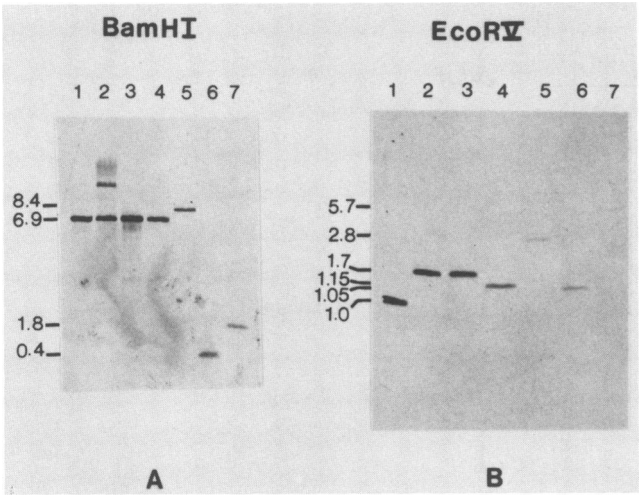

FIG. 3. Southern blot analysis of *P. aeruginosa* strains with pilin probe pMS27. DNA was digested with *Bam*HI (A) and *Eco*RV (B). Lanes 1 to 7 contain DNA from the same strains as in the legend to Fig. 2.

fragment from MSH7, and a 3.75-kb fragment from CC1. Therefore, in the analysis with the exotoxin A gene probe, digestion with three restriction enzymes yielded unique patterns for unrelated strains, while strains C340 and C341 appeared to be related, a finding consistent with the two strains having been isolated from the same patient.

The pilin gene probe was used to examine *Bam*HI and *Eco*RV digests of DNA from the seven test strains (Fig. 3). The pilin probe hybridizes to a 6.9-kb fragment in *Bam*HI digests of DNA from PAK, C340, C341, PA103, and different size fragments from strains MSH8, CC1, and WR5 (8.4, 0.4, and 1.8 kb, respectively). When the same probe was used in a hybridization analysis of *Eco*RV digests, 1.7-kb probe-reactive fragments were observed for C340 and CC341 and 1.05-kb fragments were detected in strains PA103 and CC1. Unique *Eco*RV fragments were present in blots of DNA from PAK (1.0 kb), MSH7 (2.8 kb), and WR5 (5.7 kb). Thus, pilin probe analysis confirmed that strains C340 and C341 are indeed related.

DISCUSSION

We have extended the previous work of Ogle et al. (9) in demonstrating the utility of DNA probes derived from the structural gene for exotoxin A and its 5′ flanking region for use in identification of individual *P. aeruginosa* strains. Furthermore, we have demonstrated a similar use for a pilin gene probe in discriminating *P. aeruginosa* strains. In our studies we have used biotin-labeled DNA probes in conjunction with a streptavidin-alkaline phosphatase detection system, thus demonstrating the practicality of using nonisotopic gene-probing technology in routine epidemiological studies.

Strains of *P. aeruginosa* used in this study were selected from our collection based on several criteria. Strains PAK and PA103 are standard laboratory strains. Strain WR5 lacks the exotoxin A structural gene and thus served as a negative control in hybridization studies. MSH7 is an environmental *P. aeruginosa* isolate. Strains C340 and C341 are isolates from a single patient suffering from cystic fibrosis. Strain CC1 is a clinical isolate cultured from a wound sample. Thus, all test strains with the exceptions of C340 and C341 are independent isolates of *P. aeruginosa* from unrelated sources.

Results of a Southern blot analysis of genomic digests of the various *P. aeruginosa* strains, using the exotoxin A gene probe (Fig. 2), show patterns obtainable with different enzymes. The results show that a number of strains yielded the same size fragments with one enzyme, but not with all three. Two different isolates (C340 and C341) of *P. aeruginosa* from the same patient showed identical hybridization patterns in each of the enzyme digests. Since these strains show strikingly different growth properties in both solid and liquid media, they therefore represent phenotypic variants of the same strain. Some of the strains showed identical hybridization patterns with two enzymes, but not with all three used in this study.

A small but significant fraction of *P. aeruginosa* isolates lacks the structural gene for exotoxin A, and identification of this subpopulation of *P. aeruginosa* isolates presents a limitation in using the exotoxin A-derived DNA probe in epidemiological studies. We have therefore identified a potentially useful alternate DNA probe, derived from the gene encoding the antigenically variable *P. aeruginosa* pilin polypeptide. The variability in the coding region results in the presence or absence of unique restriction sites yielding unique hybridization patterns following Southern blot analysis of digested DNA with appropriate enzymes and probed with the pilin gene probe.

Comparison of blots presented in Fig. 3 shows that Southern blot analysis of DNA digested with *Bam*HI and *Eco*RV yields sufficient information to discriminate all strains except C340 and C341, further demonstrating that these two strains from the same patient are closely related or identical. Furthermore, strain WR5 contains a hybridizing fragment and can be included in a classification based on the pilin gene probe. Both exotoxin A and pilin gene probes are therefore useful in discriminating *P. aeruginosa* strains and can provide a basis for future epidemiological studies based on restriction fragment length polymorphisms.

We have utilized nonisotopic detection techniques for the identification of DNA fragments containing a hybridization target present in a single copy on the bacterial chromosome. The sensitivity of this technique was comparable to use of identical DNA probes labeled by nick translation with ^{32}P (data not shown). This technology is thus accessible to most clinical or epidemiological laboratories for use in strain identification. Once a proper reference collection of hybridization patterns with the exotoxin A and pilin probes has been established, DNA restriction fragment length polymorphism determinations may become a method of choice for identification of individual strains.

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