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Restriction Endonuclease Analysis of Clinical *Pseudomonas aeruginosa* Strains: Useful Epidemiologic Data from a Simple and Rapid Method

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Newer genetic techniques have replaced phenotypic methods of subtyping *Pseudomonas aeruginosa* strains. Widespread application of newer methodologies, however, may be limited by technologic complexity and the cost of equipment. We conducted restriction endonuclease analysis (REA) of sheared genomic DNAs from 48 clinical *P. aeruginosa* strains using the enzyme *Sal*I and electrophoresis in horizontal, low-concentration (0.3 to 0.6%) agarose gels. Each REA profile consisted of a smear of lower-molecular-mass bands as well as a countable number of well-resolved bands in the 8.3- to 48.5-kbp range which could easily be compared when isolates were run side-by-side on the same gel. In general, the REA patterns of strains recovered from different patients differed by at least seven bands, and those of serial isolates from individual patients were identical or differed by, at most, two bands over this 8.3- to 48.5-kbp range. REA of strains already subtyped by field inversion gel electrophoresis revealed that the two techniques generally paralleled each other. Overall, some unrelated strains had similar REA profiles, but the relative simplicity and low cost of the approach coupled with the ability to demonstrate differences between most unrelated strains should make this type of REA an attractive first step in the investigation of institutional *P. aeruginosa* problems.

Pseudomonas aeruginosa is a significant human pathogen, usually in the context of serious underlying disease. It is also a major cause of nosocomial infections, which are characteristically prone to antimicrobial resistance (21, 26). Epidemiologic issues are often difficult to resolve because of the ubiquitous nature of *P. aeruginosa* in the environment as well as its frequency as a commensal organism in humans and animals (26). The cornerstone of epidemiologic investigations has been subtyping of *P. aeruginosa* strains. Classic methods have mostly relied upon phenotypic traits. None of these techniques is capable of typing all strains, and each technique generally yields rather broad categories (4, 14, 17, 19, 20, 24, 32).

Newer genetic methods (pulsed-field gel electrophoresis [PFGE] and field inversion gel electrophoresis [FIGE] of restriction endonuclease digests of genomic DNA as well as comparisons of specific gene polymorphisms by Southern blotting) have improved the subtyping of *P. aeruginosa* strains. Virtually all isolates are typeable, and several studies have demonstrated the superiority of genetic methods over phenotypic methods (1, 2, 5, 7, 9, 13, 15, 18, 22, 23, 25, 28). Gene probes are not commercially available, and the necessary equipment may be too expensive or too specialized for routine acquisition by clinical laboratories.

In light of these constraints, we explored, as an alternative, restriction endonuclease analysis (REA) of *P. aeruginosa* strains by simple horizontal agarose gel electrophoresis of *Sal*I digests of sheared bacterial DNA. As predicted (10, 13, 30), the REA patterns were complex and difficult to interpret, but with modification of the electrophoresis parameters, we were able to resolve a useful set of bands in the molecular mass range of 8.3 to 48.5 kbp. We report here the results of such an REA of epidemiologically related and unrelated *P. aeruginosa* strains. We were also able to examine the REA profiles of a collection of *P. aeruginosa* strains previously typed by FIGE. In general, our results obtained with REA as an initial approach to the epidemiologic typing of *P. aeruginosa* strains are encouraging.

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MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* strains were obtained from adult patients with infections at various body sites (3). A group of 14 *P. aeruginosa* strains from patients with cystic fibrosis were kindly provided by Burkhard Tümmler, Cystic Fibrosis Research Group, Medizinische Hochschule Hannover, Germany (5). Strain ATCC 27853 was obtained from the American Type Culture Collection, Rockville, Md.

DNA isolation. DNA was isolated from *P. aeruginosa* strains by the method of van Ketel et al. (29), with additional treatments with RNase A (200 μg/ml), RNase T_1 (200 U/ml), and proteinase K (100 μg/ml); $CHCl_3$-isoamyl alcohol (24/1) extraction; and precipitation with ethanol. DNA was quantified by measuring the A_{260} (11). Later in the study, a commercially available kit (Elu-Quick; Schleicher & Schuell, Keene, N.H.) (27) was used. The procedure for the isolation of DNA from eukaryotic cells provided with the kit was adapted to recovery of whole-cell DNA from bacteria. Approximately 10^9 bacterial cells were treated with lysozyme (3 mg/ml in 10 mM Tris–10 mM disodium EDTA [pH 8.50] at 37°C for 15 min) prior to the addition of the lysing solution (guanidine thiocyanate) provided with the kit.

Restriction digests. The restriction endonuclease *Sal*I was obtained from Bethesda Research Laboratories, Gaithersburg, Md. A typical reaction mixture consisted of 4 μg of DNA, manufacturer-provided buffer concentrate, and 50 U of *Sal*I (5 μl of 50 U of *Sal*I concentrate per μl diluted 1/5 in reaction buffer) in a total volume of 50 μl, and the reaction mixture was incubated at 37°C for 4 h. Restriction of 4 μg of

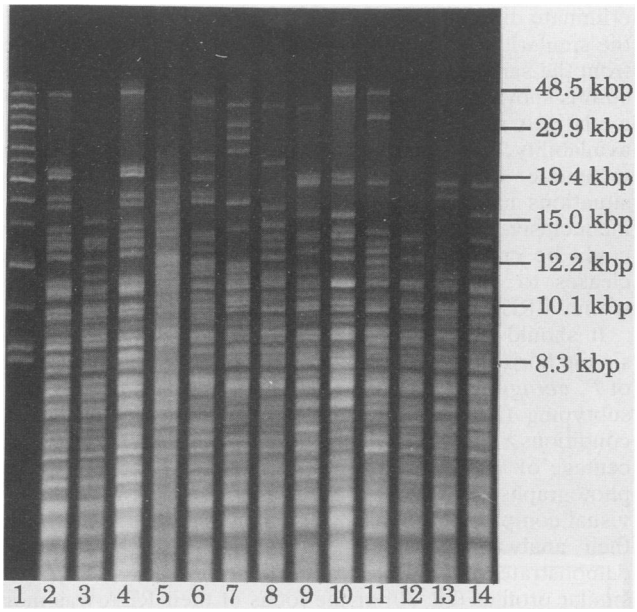

FIG. 1. Genomic DNAs from clinical *P. aeruginosa* strains were digested with the enzyme *SalI* and were subjected to electrophoresis in a 0.4% agarose gel for 33 h at 1.2 V/cm. Lane 1, molecular mass markers (8.3 to 48.5 kbp); lanes 2 to 14, strains PT-1, PT-2, PT-1A, PT-3, PT-4, PT-5, PT-6, PT-7, PT-8, PT-9, PT-10, PT-11, and PT-12, respectively. There were no obvious epidemiologic links between these patients. Strains PT-1 and PT-1A were isolated from the same patient. The cultures were obtained 76 days apart. Each of the other strains was isolated from a different patient.

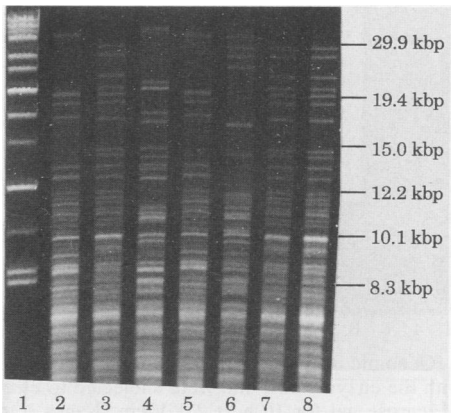

FIG. 2. Genomic DNAs from *P. aeruginosa* isolates from patient 13 were restricted with the enzyme *SalI* and were subjected to electrophoresis on a 0.4% agarose gel for 16 h at 2.4 V/cm. Lane 1, molecular mass markers (8.3 to 48.5 kbp); lanes 2 to 8, strains PT-13A, PT-13B, PT-13C, PT-13D, PT-13E, PT-13F, and PT-13G, respectively. DNAs were derived from isolates PT-13A, PT-13B, PT-13C, and PT-13D from patient sputa pretherapy by the rapid microscale method (27); each of these isolates possessed distinctive colonial morphologies. DNAs were derived from isolate PT-13E from patient sputum during antibiotic therapy. The DNAs in lanes 7 and 8 were derived from isolates PT-13F and PT-13G recovered from patient sputa after 19 days of antibiotic therapy. The DNA in lane 7 was isolated by a rapid microscale method (27), and the DNA in lane 8 was isolated by method of van Ketel et al. (29).

DNA required at least 25 U of *SalI*; 10 U of enzyme yielded a useless blur, but 25, 50, and 100 U of enzyme yielded identical REA patterns under the same conditions (data not shown).

Horizontal agarose gel electrophoresis. Restriction digests were electrophoresed in Tris-borate buffer (11) on 0.3 to 0.6% agarose (ultra PURE Agarose; Bethesda Research Laboratories) gels (0.5 by 20 by 25 cm) for 30 to 36 h at 1.2 V/cm or for 16 h at 2.4 V/cm at room temperature. Gels were fragile, but with care, they could be handled without fracturing. High Molecular Weight DNA Markers (Bethesda Research Laboratories) were used as molecular mass standards. Gels were stained with ethidium bromide (1 μg/ml) overnight, destained in distilled water for 2 to 4 h, and photographed under UV illumination through a red filter. By using the rapid microscale DNA isolation method and the shorter electrophoresis times at higher voltages, REA could be accomplished within 24 h of obtaining a pure bacterial culture on a plate.

RESULTS

Figure 1 displays the REA patterns of *P. aeruginosa* strains from 12 different patients (lanes 2, 3, and 5 to 14). The REA patterns demonstrated considerable diversity among strains (seven or more band differences by direct visual comparison over the 8.3- to 48.5-kbp range). On another, similar gel (data not shown), strains from two different patients (one a nonpersisting isolate from the sputum of patient 7; the other an isolate from the bone of a patient [patient 14] with osteomyelitis) were seen to differ by only a single band (approximately 14 kbp) among all the bands that were visible on the gel. Review of the available epidemiologic information could not establish a link between these patients.

Serial isolates were available from 14 patients: strains isolated from infected patients prior to antibiotic therapy and those cultured weeks to months after the initiation of treatment. In 11 of 14 cases, the REA patterns of all strains isolated from a single patient were either identical (eight cases) or differed at most by one to two bands (3 cases) among all visible bands on the gels (Fig. 1, lanes 2 and 4). In 2 of 14 cases, multiple colonial forms of *P. aeruginosa* were detected in cultures of sputum specimens. Some of the different colony types displayed distinctly different REA patterns, although strains with identical REA profiles were seen to persist over time (Fig. 2). In one case, pretherapy and posttherapy REA profiles differed by 14 bands over the 8.3- to 48.5-kbp range.

A group of 14 *P. aeruginosa* strains from patients with cystic fibrosis whose FIGE typing patterns have already been published (5) were examined by our REA technique (Fig. 3). Lanes 1 to 3 correspond to the isolates depicted in Figure 1 of Grothues et al. (5). These strains were isolated from the throat of the same patient with cystic fibrosis, and each strain was considered unique by FIGE typing. The REA profile showed more than 10 band differences between strain PACF11A and the other two strains, but these latter two strains, PACF11B and PACF11C, appeared to be more closely related by REA than by FIGE (>20 band differences in *DraI* FIGE patterns [5] but only 3 band differences in the visible REA profile). Strains PACF127D1 through PACF127D4, depicted as being identical in Fig. 2 of the report of Grothues et al. (5), also appeared to be closely related by our REA. Strains PACF127D2 through PACF127D4 were identical, but strain PACF127D1 differed from the others by two faint bands in the 15.0- to 17.1-kbp

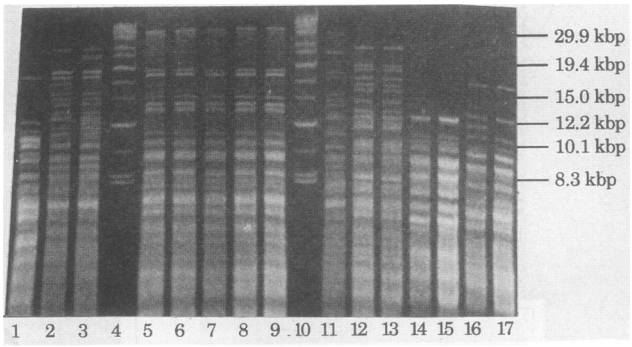

FIG. 3. Genomic DNAs from *P. aeruginosa* strains were restricted with the enzyme *SalI* and were subjected to electrophoresis in a 0.6% agarose gel for 16 h at 2.4 V/cm. Lanes 1 to 3, strains PACF11A, PACF11B, and PACF11C, respectively; lane 4, molecular weight markers (8.3 to 48.5 kbp); lanes 5 to 9, strains PACF127D1, PACF127D2, PACF127D3, PACF127D3, and PACF127D4, respectively; lane 10, molecular weight markers (8.3 to 48.5 kbp); lanes 11 to 17, strains ATCC 27853, BTCFPA-1a, BTCFPA-1b, BTCFPA-2a, BTCFPA-2b, BTCFPA-3a, and BTCFPA-3b, respectively. All strains except ATCC 27853 were cultured from patients with cystic fibrosis. FIGE typing of cystic fibrosis strains has been reported previously by Grothues et al. (5). Strains PACF11A, PACF11B, and PACF11C were all isolated from the throat of the same patient. Strains PACF127D1, PACF127D2, PACF127D3a, PACF127D3b, and PACF127D4 were serial isolates from another patient obtained 0, 6, 12, 12, and 17 months, respectively, from the onset of *P. aeruginosa* throat colonization. Strains BTCFPA-1 through BTCFPA-6 were not given designations by Grothues et al. (5). Those strains are the *P. aeruginosa* isolates from three sibling pairs with cystic fibrosis shown in Fig. 3 of their report (5).

range. These subtle differences were reproducible (three gels) and most likely were not an artifact. Similarly, strains from sibling pairs with cystic fibrosis showed REA differences between isolates from sibling pairs, and REA similarities between isolates from each pair (lanes 12 to 17 in Fig. 4 in the report of Grothues et al. [5]) (Fig. 3).

Comparison of REA profiles was straightforward when all isolates for comparison could be accommodated on the same gel. Direct comparison of strains run on different gels, however, was not attempted because of the relatively large number of bands, especially in the 10- to 20-kbp range. Qualitatively, however, the patterns seemed quite reproducible. Strains 6G and ATCC 27853 were analyzed on four different gels, and serial isolates that were run on other gels could be compared with same strains on the gel shown in Fig. 1. In each case, there was no variation in the number of bands or their positions (qualitative visual determination) relative to the positions of the molecular mass standards. It seems unlikely that diverse banding patterns are artifacts of inconsistent or incomplete endonuclease restriction.

DISCUSSION

Typing of *P. aeruginosa* strains by genomic fingerprinting with PFGE, FIGE, or demonstration of gene polymorphisms has been shown to be reproducible and accurate on the basis of studies with large numbers of strains (2, 15, 16, 25), and appears to be the state of the art. Nevertheless, *P. aeruginosa* infections are common problems (1, 9), and laboratories other than those devoted to the study of *P. aeruginosa* infections may need access to epidemiologic typing.

Our data demonstrated a diversity of REA patterns among isolates from different patients, the ability of REA to discriminate different colony types from the same patient, and the similarity and stability of REA profiles of serial isolates from the same patient. Although some apparently unrelated strains showed great similarity by REA, this appeared to be a relatively infrequent event and may be offset by the speed, availability, and other useful discriminatory powers of this technique. FIGE or PFGE analysis may be reserved for situations in which clinical and REA data conflict or are inconclusive. Alternatively, the type of REA described here could be enhanced by using different restriction endonucleases to confirm the identities or uncover differences within a REA group.

It should be noted that other investigators have found simple horizontal agarose gel electrophoresis of *SalI* digests of *P. aeruginosa* chromosomal DNA to be useful for strain subtyping (10, 13, 30). In general, their electrophoretic conditions were somewhat different from ours (higher percentage of agarose gels) or were not clearly specified, and photographs of their REA patterns did not allow such easy visual comparison of strains as our REA did. Furthermore, their analyses either did not include enough strains to demonstrate that some unrelated strains may have very similar profiles (10, 30) or the focus of their REA was more to confirm the results of other subtyping methods than to survey a population of strains (13).

The rational basis for selection of the best restriction enzyme for such an REA, however, is not clear-cut. The enzyme *SalI* was chosen after reviewing other investigators' experience (13) with REA and *P. aeruginosa*, although their selection of *SalI* may have been influenced more by the restriction map of the region encompassed by their *PstI-NruI* probe and their desire to exploit the inherent polymorphism of this specific region than the anticipated effect of *SalI* on the whole bacterial genome.

When PFGE and FIGE systems are used, DNA restriction patterns may be qualitatively predicted when the G+C content and restriction site recognition sequences are considered (12). Enzymes such as *XbaI*, *SspI*, *SpeI*, and *DraI* (12) are recommended for PFGE and FIGE analysis of *P. aeruginosa*, because the resultant DNA restriction fragments are quite large (100 to 500 kbp), few in number, and amenable to resolution by these electrophoretic techniques (6).

Standard methods yield sheared DNA with an average size of 50 kbp. Digestion of this DNA with infrequently cutting enzymes (*XbaI*, *SspI*, *SpeI*, and *DraI*) would likely yield no resolvable fragments (12). Given the G+C content of *P. aeruginosa* (67%) (22) and the relatively G+C-rich recognition sequence of *SalI*, one would expect a fairly large number of bands from *SalI* digestion of *P. aeruginosa* DNA, and the distribution of fragments of the expected size would be unpredictable. Fortuitously, we obtained our typing information from a relatively small number of larger but polymorphic DNA fragments with G+C contents that were lower than that of the *P. aeruginosa* genome as a whole. Admittedly, this technique examines a limited portion of the bacterial genome, and it should not be surprising that our REA results sometimes differed from FIGE typing results.

A similar analysis could be extended to other bacterial species, but a fair amount of trial and error would be anticipated in the selection of a restriction endonuclease. Our best advice is a variation of that given by McClelland et al. (12). Restriction enzymes that would predictably yield a large number of restriction fragments should be chosen (on the basis of the enzyme recognition sequence and percent G+C content) with the hope that strain discrimination can be

achieved by the presence of genomic segments with anomalous G+C contents that would yield larger, resolvable, and polymorphic fragments. For example, enzymes with 6-base recognition sequences rich in G and C would be chosen for species with a relatively high G+C content (≥60%), 6-base recognition sequence enzymes with AT-rich sites would be chosen for species with a low G+C content (≤40%), and 4-base recognition sequence enzymes would be chosen for species with G+C contents in the middle range. The best resolution of fragments would require adjustment of the electrophoresis parameters to fit the resulting fragment sizes. These principles are supported by our data as well as a recent report of REA of *Pasteurella multocida* (G+C content, 40.8 to 43.2%) with the enzyme *Hha*I (31), a 4-bp restriction endonuclease, which yielded well-resolved REA patterns in a somewhat lower-molecular-mass range.

In conclusion, we described methods of DNA isolation and restriction endonuclease digestion and electrophoresis conditions which enabled us to quickly and easily subtype clinical *P. aeruginosa* strains. A small group of isolates was examined, but the results were encouraging. Despite some limitations, the method described here may be useful, especially to clinical investigators, clinical microbiologists, or epidemiologists interested in *P. aeruginosa* infections but not fully committed to sophisticated molecular biologic techniques.

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