Detection of Genomic Variation in *Providencia stuartii* Clinical Isolates by Analysis of DNA Restriction Fragment Length Polymorphisms Containing rRNA Cistrons

ROBERT J. OWEN, ALISTAIRE BECK, PATRICIA A. DAYAL, AND CHRISTINE DAWSON
National Collection of Type Cultures, Central Public Health Laboratory, London NW9 5HT, United Kingdom

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Chromosomal DNA from 26 strains of *Providencia stuartii* isolated mainly in hospitals in the United Kingdom and reference strains of *P. stuartii*, *P. rustigianii*, and *Proteus vulgaris* were digested with the restriction endonucleases EcoRI and HindIII. After electrophoresis in agarose gels, the fragments were subjected to Southern blot hybridization analysis with a biotin-labeled cDNA probe transcribed from a mixture of 16S and 23S rRNA from *P. stuartii* NCTC 11800T. The pattern of bands (the rDNA fingerprint), which depended on restriction fragment length polymorphisms containing rRNA genes, was used as a measure of minor genomic variation within and between strains. The *P. stuartii* clinical isolates had similar total digest patterns, but the rDNA fingerprints revealed some heterogeneity between strains, with EcoRI digests providing better strain discrimination than HindIII. Such rDNA fingerprints comprised between five and seven bands with sizes in the range of 5 to 28 kilobases. The 11 different EcoRI patterns were compared by numerical analysis, and several groups or subgroups of strains were identified. Over half (15 of 26) of the urease-negative isolates (subgroups Aa and Ab) had patterns that differed only by the presence or absence of a 25-kilobase band. Urease-negative strains from other clinical material were more homogeneous in their patterns. No correlation was apparent between strain pattern group and urease production or geographic location of isolate. The *P. stuartii* rDNA fingerprints were quite distinct from those of allied *Providencia* and *Proteus* species and provided a more sensitive measure of minor genomic differences than total DNA digests did.

*Providencia stuartii* (27) is found most often in the urine of hospitalized and catheterized patients and less frequently in wounds, burns, and bacteremias (10). Its role as a nosocomial pathogen, albeit of low virulence, is well established, although difficulties may arise in the treatment of systemic infections because of its multiple resistance to antibiotics (10). Some strains contain cryptic plasmids, which may be a factor in the acquisition of transferable resistance to some antibiotics (11-13) and of urease activity (8). DNA-DNA sequence homologies show that *P. stuartii* has only a low level of relatedness to *P. alcalifaciens* and other *Providencia* species but that strains within *P. stuartii* are highly related (2). O-antigen serotyping, plasmid profile determination, and antimicrobial susceptibility patterns, as well as, to a lesser extent, bacteriocin and bacteriophage typing, have been used in the epidemiological investigation of *P. stuartii* infections (10, 13), although little is known about the distribution of the 17 or so O antigens and only a relatively small proportion of strains (ca. 30%) contain detectable plasmids (10).

In this study we have investigated the use of rDNA fingerprints, based on restriction restriction fragment length polymorphisms in the chromosomal DNA containing rRNA genes, as a means of detecting interspecies and interstrain nucleotide differences. In eubacteria, the rRNA genes are a small (ca. 0.1%) but highly conserved part of the genome and are found in clusters (rRNA operons), within which they are linked in the order 16S-23S-5S (21). The number of rRNA gene copies per bacterial genome is 11 or fewer, varies from genus to genus (1, 7, 17), and may be linked to the rate of microbial growth (1).

It is evident from complete (16, 22) and partial (31) rRNA sequencing and from rRNA-DNA hybridization (5) that rRNA operons provide valuable taxonomic information at the genus level and above, for which some sequence variation between taxa can be detected. At the species level rRNA sequences are highly conserved; consequently, they have been selected to provide the basis for highly specific hybridization probes for *Legionella* (30) and *Mycoplasma* (15, 32) species. Recent studies have demonstrated the potential of DNA fingerprints derived from the analysis of rRNA cistron-containing restriction fragment length polymorphisms as a basis for microbial species and strain identification (6, 9, 18).

In this report we describe the use of a biotin-labeled probe, synthesized by reverse transcriptase from *P. stuartii* 16S and 23S rRNA, to identify rRNA cistrons in Southern blots of restriction enzyme digests of *Providencia* total chromosomal DNA. Our results on selected strains indicated that the pattern of hybrid bands—the rDNA fingerprint—was species specific, and with the aid of computer analysis we provided a novel basis for identifying strains or subgroups of strains within a random set of *P. stuartii* strains isolated in hospitals in the United Kingdom.

**MATERIALS AND METHODS**

Bacterial strains and culture conditions. Twenty-six clinical isolates of *P. stuartii* were obtained from various hospitals in England, Northern Ireland, and the Federal Republic of Germany. Most strains were received over a 3-month period in 1985. The laboratory numbers used and strain sources are indicated in Table 1. Further details on three (A627/85, A628/85, and A629/85) strains, which were received from P. Hawkey, University of Leeds, Leeds, United Kingdom, have been published previously (11, 12). The majority of strains (17 of 26) were isolated from urine specimens, and

* Corresponding author.
their identification as *P. stuartii* was established by standard biochemical methods (3). All but two of the field strains were urease negative (Table 1). The following four reference strains were obtained from the National Collection of Type Cultures, London: *P. stuartii* NCTC 11800T, *P. alcalifaciens* NCTC 10286T, *P. rustigianii* NCTC 11801T, and *Proteus vulgaris* NCTC 4175T. All strains were cultured in Oxoid nutrient broth no. 2 (CM67) supplemented with L-cysteine hydrochloride (0.1 g/liter) or on nutrient broth solidified with 1.5% [wt/vol] Japanese agar) at 37°C for 1 to 2 days.

**DNA preparation, digestion, and separation of fragments.** Chromosomal DNA was isolated and purified as described previously (24–26). The DNA (1 to 2 µg) was digested with the restriction endonucleases *Eco*RI and *Hind*III (5 U/µg of DNA) for 3 h at 37°C (24) in the buffer recommended by the manufacturers (GIBCO Ltd., Paisley, United Kingdom; Boehringer Corporation [London] Ltd., Lewes, United Kingdom; Amersham International, Amersham, United Kingdom). The digested DNA (a 10- to 20-µl sample) was electrophoresed at 40 V for 18 h in a horizontal 0.7% (wt/vol) agarose (ultrapure, electrophoresis grade; GIBCO Ltd.) gel in a buffer containing 89 mM Tris hydrochloride, 89 mM boric acid, and 2 mM disodium EDTA (pH 8.3). After electrophoresis, the gels were stained and photographed (24).

**Probe synthesis.** The preparation of a biotin-labeled cDNA probe, transcribed by reverse transcriptase from 16S and 23S rRNA from *P. stuartii* NCTC 11800T, was described previously (28).

**Southern blot hybridization.** The DNA in the gel was nicked further after UV illumination by treatment with 0.25 N HCl for 15 to 30 min, denatured in 0.5 M NaOH–1.5 M NaCl for 1 h, and neutralized in 0.5 M Tris hydrochloride (pH 7.2)–1.5 M NaCl–1 mM disodium EDTA for 1 h. DNA was transferred to a Hybond-N membrane (pore size, 0.45 µm; Amersham International) overnight (18 to 20 h) as recommended by the manufacturer. The membranes were washed once in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate), air dried, and baked at 80°C for 2 h.

The nylon membranes were prehybridized (19) at 42°C for 3 to 4 h in a solution containing 50% (vol/vol) formamide, 5× SSC, 5× Denhardt solution (1× Denhardt solution is 0.02% polyvinylpyrrolidone, 0.02% Ficoll 400, and 0.02% bovine serum albumin), 25 mM sodium phosphate (pH 6.5), 5% (wt/vol) sodium dodecyl sulfate, and 500 µg of freshly denatured herring sperm DNA per ml. The membranes were hybridized (19) at 42°C for 18 h in a solution containing 45% (vol/vol) formamide, 5× SSC, 1× Denhardt solution, 20 mM sodium phosphate (pH 6.5), 5% (wt/vol) sodium dodecyl sulfate, 5% (vol/vol) dextran sulfate, 200 µg of freshly denatured sheared herring sperm DNA per ml, and 1 µg of heat-denatured biotinylated probe DNA. After hybridization, the nylon membranes were washed once in 0.1% (wt/vol) sodium dodecyl sulfate–2× SSC, and twice in 0.1% (wt/vol) sodium dodecyl sulfate–0.2× SSC. The hybridization reactions were visualized colorimetrically with the BluGENE nonradioactive nucleic acid detection system (GIBCO Ltd.), which contains streptavidin–alkaline phosphatase conjugate and dyes, as recommended by the manufacturer.

**Band size estimation and analysis.** The band patterns on the nylon membranes were scanned directly (Ultroscan XL laser densitometer; LKB, Bromma, Sweden) to obtain migration distances, and fragment sizes were calculated by the DNA-SIZE program as described previously (23). *Hind*III and *Eco*RI bacteriophage λ DNA digests were used to provide molecular weight markers. Patterns were screened for 16 different bands, and positive (presence) and negative (absence) results were coded as 1 and 0, respectively. Similarity among strains was estimated by means of the simple matching coefficient, and clustering was based on the unweighted pair group arithmetic average algorithm (29). Computations were carried out with an Acorn microcomputer.

**RESULTS**

cDNA probing of *Eco*RI DNA digests. Chromosomal DNA from NCTC 11800T and 26 clinical isolates of *P. stuartii* was completely digested with *Eco*RI, producing complex patterns of at least 25 fragment bands on agarose gel electrophoresis. On visual inspection, these patterns appeared to be very similar (4, 28). Southern blots of these digest were probed with biotin-labeled NCTC 11800T ribosomal cDNA, giving patterns that comprised between five and seven well-resolved bands with sizes between 5 and 28 kilobases (kb) (Fig. 1). The general features of these rRNA gene

<table>
<thead>
<tr>
<th>Group in Fig. 3</th>
<th>Strain*</th>
<th>Band sizes (kb)</th>
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<tbody>
<tr>
<td>Aa</td>
<td>NCTC 11800T, A627/85, A628/85, A631/85 (o), A632/85, A636/85, A642/85, A654/85, A658/85</td>
<td>19.5 ± 0.9, 13.9 ± 0.4, 9.4 ± 0.1, 6.2 ± 0.1, 5.8 ± 0.1</td>
</tr>
<tr>
<td>Ab</td>
<td>A633/85, A619/85, A645/85 (o), A648/85, A649/85 (o), A655/85</td>
<td>24.9 ± 1.0, 19.8 ± 0.7, 14.0 ± 0.2, 9.6 ± 0.2, 6.2 ± 0.1, 5.8 ± 0.1</td>
</tr>
<tr>
<td>Ac</td>
<td>CL142/71 (w), A657/85</td>
<td>25.4 ± 0.5, 20.4 ± 0.4, 13.6 ± 0.4, 9.5 ± 0.1, 6.1 ± 0.1</td>
</tr>
<tr>
<td>Ad</td>
<td>A624/85, A636/85</td>
<td>24.4 ± 0.6, 19.9 ± 0.3, 17.5 ± 0.5, 13.9 ± 0.3, 9.9 ± 0.4, 6.3 ± 0.1, 5.9 ± 0.1</td>
</tr>
<tr>
<td>Ae</td>
<td>A625/85 (w), A626/85 (o)</td>
<td>19.9 ± 0.5, 14.4 ± 0.2, 10.6 ± 0.1, 6.3 ± 0.1, 5.9 ± 0.1, 5.6 ± 0.1</td>
</tr>
<tr>
<td>Af</td>
<td>A630/85 (o)</td>
<td>27.9, 20.3, 14.6, 9.7, 6.1, 5.8</td>
</tr>
<tr>
<td>B</td>
<td>CL627/72 (w)</td>
<td>24.8, 19.7, 13.7, 12.0, 11.1, 6.2</td>
</tr>
<tr>
<td>C</td>
<td>A623/85 (w)</td>
<td>18.3, 17.3, 13.4, 10.2, 6.2, 6.0, 5.8</td>
</tr>
<tr>
<td>D</td>
<td>A644/85 (o)</td>
<td>25.0, 21.0, 14.2, 9.4, 8.7, 5.8</td>
</tr>
<tr>
<td>E</td>
<td>A650/85</td>
<td>27.9, 21.1, 14.6, 12.8, 6.2, 5.9</td>
</tr>
<tr>
<td>F</td>
<td>A629/85</td>
<td>21.9, 15.0, 9.8, 6.2, 6.0</td>
</tr>
</tbody>
</table>

*All strains were urine isolates except for those marked (w), which were wound swab isolates, or (o), which were isolated from other material (blood or unspecified source). |

* Urease-positive strain.
VOL. 26, NCTC groups are shown (group members of 85 or presence (group Ac); 8, A657/85 were fingerprints stuartii, but minor 10, A5.7 were discernable, and 11 different patterns were identified on the basis of the presence or absence of bands and by size difference. The fingerprints were remarkably constant among members of P. stuartii, but minor strain differences were discernable, and 11 different patterns were identified on the basis of the presence or absence of bands and by size difference. The results for the band sizes are listed in Table 1, and densitometric scans of selected patterns are shown in Fig. 2. Bands in the 5- to 6-kb, 13- to 14-kb, and 19- to 20-kb regions were distinctive common features of most of the P. stuartii fingerprints, with a band of 6.0 ± 0.2 kb present in almost all of them. The strains are listed in Table 1 according to their order of similarity in the numerical analysis, and the dendrogram obtained is illustrated in Fig. 3.

The strains were all linked in the numerical analysis with similarities of 55% or greater, but the majority (22 of 26) of strains, including the type strain NCTC 11800, had similarities to each other of ≥85% (Fig. 3, group A). At the 95% S level, group A was divided into six subgroups, with most strains falling either in subgroup Aa (nine strains) or in subgroup Ab (six strains). Subgroup Aa had a fingerprint containing 5-, 6-, 9-, 13-, and 19-kb bands, and strain A631/85 was the most typical, since its fingerprint matched the average fingerprint (based on the average size of individual bands of all members in the group) most closely. The subgroup Ab strains differed from the subgroup Aa strains in possessing an additional 25-kb band, and strain A633/85 was the most typical representative. The sizes of the various bands were remarkably consistent among strains in both subgroups, with overall differences of 4% or less, although differences were typically about 2% for the 9-kb and smaller bands.

The four other subgroups (Ac to Af) linked at similarities of 86 to 91%, and each contained just one strain. The five remaining strains were linked to group A at lower similarity levels (55 to 70%) and were each designated as separate taxa (groups B to F), with A629/85 (group F) having the most different fingerprint.

There was excellent reproducibility of patterns within gels. Duplicate samples of four strains (A623/85, A636/85, A642/85, and A644/85), which were analyzed on the same gel, gave identical patterns when probed. The reproducibility of size estimation between gels was determined from the results for three strains (A633/85, A648/85, and A644/85), which were analyzed by using different DNA preparations, restriction digestions, electrophoresis runs, blots, and probes. Agreement between duplicates was excellent, and the estimated pooled error for all fragment size estimates

FIG. 1. Hybridization of biotin-labeled ribosomal cDNA from P. stuartii NCTC 11800\(^\text{\textsuperscript{T}}\) with Southern-blotted EcoRI restriction fragments of clinical isolates of P. stuartii. Lanes: 1, NCTC 11800\(^\text{\textsuperscript{T}}\) (group Aa); 2, A628/85 (group Aa); 3, A631/85 (group Aa); 4, A658/85 (group Ac); 5, A619/85 (group Ab); 6, A649/85 (group Ab); 7, A657/85 (group Ac); 8, A624/85 (group Ad); 9, A626/85 (group Ae); 10, A644/85 (group D). The percent similarities between the various groups are shown in Fig. 3.

FIG. 2. Densitometric traces of P. stuartii rDNA hybridization patterns with sizes (in kilobases) of principal bands homologous to ribosomal cDNA from P. stuartii NCTC 11800\(^\text{\textsuperscript{T}}\). (a) A658/85 (group Aa); (b) A649/85 (group Ab); (c) A626/85 (group Ae); (d) A624/85 (group Ad).

FIG. 3. Dendrogram based on rDNA hybridization fingerprints by using the simple matching coefficient and UPGMA clustering. Groups obtained are designated A through F, and individual strain numbers are given in Table 1.
was 1.5%, with a maximum observed error of 4% (generally for the 20-kb or larger fragments).

Southern blots of EcoRI digests of chromosomal DNA from P. rustigianii NCTC 11801T, P. alcalifaciens NCTC 10286T, and Proteus vulgaris NCTC 4175T were also probed with the biotin-labeled strain NCTC 11800T ribosomal cDNA, and each gave distinctive and unique banding patterns comprising 7, 5, and 6 bands, respectively (Fig. 4). The patterns were different from each other and from those of the P. stuartii strains.

cDNA probing of HindIII DNA digests. Chromosomal DNA samples from strain NCTC 11800T and 17 of the clinical isolates included in the EcoRI analysis above were completely digested with HindIII, producing a complex pattern of at least 30 fragment bands with no obvious variation between strains discernable on visual inspection (Fig. 5a). When Southern blots of these digests were probed with the NCTC 11800T ribosomal cDNA, the patterns obtained comprised six or seven well-resolved bands with sizes between 5 and 19 kb (Fig. 5b). The results showed some minor variation between strains in banding patterns, but most (15 of 18) strains had a six-band pattern, and all of these except three had a pattern comprising 5.2-, 5.5-, 6.3-, 7.7-, 12.2-, and 19.6-kb bands. Differences detected between strains were principally in the 5- to 8-kb region.

DISCUSSION

Previous studies on various microbial groups, which included the eubacteria (1, 7, 9, 17), mycoplasmas (15, 32), cyanobacteria (20) and yeasts (18), demonstrated the potential of DNA fingerprints derived from RNA cistrons as a basis for species and possibly strain identification. Since P. stuartii is now recognized to be of increasing clinical importance (10), we have investigated the technique as means of characterizing hospital isolates.

Our results show that rDNA fingerprinting provides a novel method of distinguishing between strains of P. stuartii as well as for separating them from strains of allied species such as P. alcalifaciens, P. rustigianii, and Proteus vulgaris. The analysis is based on results of using EcoRI for digesting P. stuartii DNA, because previous studies (4, 28) have shown that this enzyme cuts with a relatively high frequency, giving distinctive fragment patterns. Other restriction enzymes (HindIII, PvuII, and BamHI) cut P. stuartii DNA with apparently similar frequencies to EcoRI, but the rDNA band patterns were less informative. For instance, it was found in the present study that HindIII fingerprints contained six or seven rDNA bands, but there was less variation between P. stuartii strains than was observed with the EcoRI fingerprints. Similar results to those with HindIII were obtained with BamHI. We have shown previously (A. Beck and R. J. Owen, unpublished results) that the PvuII fingerprints contained more bands (ca. 10), although there was no evidence of more strain variation. It was evident from the EcoRI analysis described here that P. stuartii strains exhibited at least 11 distinct patterns and that the majority of strains (17 of 27) had very similar patterns. The main differences between them were in the 7- to 24-kb bands. The use of computer-assisted methods to analyze data provided an objective basis for grouping strains, and our results indicate that groups formed at 85% similarity (S) or above could form the basis of a scheme for defining DNA fingerprint types and subtypes within P. stuartii.

Urine specimens were the most common source of hospital isolates in our study, and strains isolated from wound swabs were the second most common (6 of 26). The strains isolated from wound swabs were more heterogeneous with respect to their EcoRI fingerprint, although the particular types were not generally the same as those of the urine isolates. Some strains isolated in hospitals in different parts of the United Kingdom had the same fingerprint type, which suggested that individual types derived from a common clone had a wide distribution. Even so, two sets of strains isolated in hospitals in different locations (Manchester and Cambridge) contained isolates with different patterns in each. A broader survey is clearly needed to determine whether the minor genomic differences we have found when using the rRNA cistrons as markers are likely to prove of value in the study of the epidemiology of P. stuartii infections. They must be compared with other techniques such as
O-antigen serotyping and plasmid profile identification, which already have been applied to this species (13, 27).

Some 10% of \textit{P. stuartii} strains are known to produce urease (27), and urease production is one of the few conventional tests that can be used for biotyping isolates. Although it is thought that urease activity in \textit{P. stuartii} is plasmid mediated (8), the plasmid has not been identified or isolated. Our investigation of genomic DNA indicated no obvious correlation between DNA fingerprint and urease activity but showed that (i) the two urease-producing strains examined had different fingerprints from each other (type A and B), and they both differed from most of the urease-negative strains; and (ii) urease-positive strain CL672/77, isolated from urine, had a quite different fingerprint from those of the typical urease-negative urine isolates.

Since RNA sequences are highly conserved at the species level (5, 31), the DNA fingerprint differences observed in this study were attributed to the occurrence of rRNA cistrons in polymorphic restriction fragments resulting from minor base changes in the \textit{EcoRI} sites or from insertions or deletions between sites. The frequency of occurrence of \textit{EcoRI} sites within rRNA genes of \textit{P. stuartii} is not known, and so it cannot be assumed that the number of bands in the fingerprint is an exact measure of the number of rRNA operons in the genome. However, densitometric scans of the fingerprints showed that similar amounts of probe were hybridized in each fragment band. The results of the homologous hybridization (NCTC 11800) indicated that the \textit{P. stuartii} genome probably contained at least five rRNA operons, which was consistent with estimates of 11 or fewer rRNA operons in other subbacteria (1, 7, 17).

In conclusion, it seems that rDNA fingerprints generated by \textit{EcoRI} digestion of genomic DNA provide a novel means of detecting minor genomic differences. They can be used for characterizing clinical isolates of \textit{P. stuartii}, which apparently show few obvious variations in their total DNA digest fingerprints. Our results indicate that rDNA fingerprints are highly reproducible and easy to compare if they contain fewer than 10 bands. However, the assignment of strains to a particular group or subgroup is arbitrary unless computer-assisted analyses, similar to those applied to total DNA digest patterns of other bacterial species (14), are used.

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LITERATURE CITED


