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# Ribotyping for Use in Studying Molecular Epidemiology of *Serratia marcescens*: Comparison with Biotyping

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**Ribotyping carried out with a nonradioactive probe (acetylaminofluorene ribosomal RNA kit I from Eurogentec, Seraing, Belgium) was performed for the characterization of 139 hospital strains of *Serratia marcescens*. These strains, which belonged to 11 biotypes and 1 nontypeable group, were isolated in seven hospitals in Belgium between 1986 and 1992. *Eco*RI and *Hin*dIII were used to obtain cleavage patterns. Analysis of the results produced 27 different patterns with *Eco*RI and 23 patterns with *Hin*dIII. Typeability reached 100%. Combination of the patterns obtained with each enzyme produced 38 distinct ribotypes. Percent similarity values, calculated by using the Dice coefficient and unweighted-pair group average linkage clustering, showed four main clusters and nine subclusters of ribopatterns at a similarity rate of approximately 80% or less. These groups did not coincide with those delimited by biotyping, although a rather good correlation was observed. The simultaneous use of the two methods has potential value in epidemiological studies.**

During the last three decades, *Serratia marcescens* has emerged as an opportunistic pathogen and nosocomially significant microorganism (17). Outbreaks of infections due to this bacterium in hospital wards have been well documented (10, 17). The availability of simple, unambiguous, and reproducible typing methods is essential to trace the source of contamination or to prevent patient-to-patient transmission.

In 1986, Grimont and Grimont (12) proposed ribotyping as a general method for molecular bacterial identification. This method can also discriminate between isolates of the same species (24), and it has proven to be a useful epidemiological tool in the study of various bacterial pathogens (1, 4–6, 21, 22). To our knowledge, ribotyping has been applied only once for the epidemiological evaluation of 15 *S. marcescens* strains in a pediatric hospital. The results were found to be more easily interpreted than those of total DNA analysis, with an equivalent degree of discrimination, and it was concluded that the method is particularly well suited for differentiation of nosocomial strains (3).

The purpose of our study was to establish a ribotyping scheme for *S. marcescens* and to compare this genotypic technique with biotyping in order to examine the potential application of these approaches in epidemiological investigations.

## MATERIALS AND METHODS

**Bacterial strains.** A series of 139 isolates recovered from 120 patients and identified as *S. marcescens* with the API 20E system (Analytal. Products, Marcy l'Etoile, France) was included in this study. Strains were selected from a collection of 772, isolated between 1986 and 1992 in seven Belgian hospitals, in such a way that all the biotypes were represented with respect to their frequency. Characteristics and ribopatterns of these isolates are listed in Table 1.

**Biotyping.** Biotyping was performed as described by Grimont and Grimont (13), using eight carbon sources in assimilation tests (benzoate, DL-carnitine, *m*-tartrate, *m*-erythritol, 3-hydroxybenzoate, 4-hydroxybenzoate, D-quinate, and trigonelline) and a tetrathionate reduction test.

**Ribotyping. (i) Extraction of bacterial DNA.** Total *S. marcescens* DNA was prepared by the method of Grimont et al. (11). Briefly, overnight cultures in 50 ml of Columbia broth shaken at 30°C were centrifuged at 10,000 × *g* for 30 min, and each pellet was suspended in 10 ml of a lysis solution (0.05 M Tris-HCl, 0.5 M EDTA, 0.1 M NaCl; pH 8.0). Then sodium dodecyl sulfate and pronase were added (1% and 50 μg/ml, respectively). The reaction mixture was incubated for 60 min at 37°C. An equal volume of phenol-chloroform-isoamyl alcohol mixture (25:24:1) was added. After shaking and centrifugation at 4,000 × *g* for 10 min, RNase A was added to the aqueous phase at a final concentration of 50 μg/ml, and the mixture was incubated at 60°C for 1 h. Extraction of DNA by phenol-chloroform-isoamyl alcohol was repeated until there was no longer a white interface. Finally, the DNA was cold ethanol precipitated, washed with 70% ethanol, and resuspended in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0).

**(ii) Gel electrophoresis of endonuclease-cleaved DNA.** Purified DNA samples (3 μg) were cleaved by restriction enzymes *Eco*RI and *Hin*dIII (10 U) according to the manufacturer's instructions (Eurogentec, Seraing, Belgium). The digestion was done at 37°C for 4 h. The optimal conditions for horizontal electrophoresis were obtained with a 0.8% (wt/vol) agarose gel in Tris-borate buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA; pH 8.0), an agarose gel length of 20 cm, and a voltage of 65 V. Raoul I (Appligene, Illkirch, France), consisting of linear DNA fragments from 230 to 48,500 bp, was used as a size marker.

**(iii) Southern transfer and hybridization.** The transfer of DNA fragments to Hybond-N nylon membranes (Amersham International, Amersham, United Kingdom) was realized by the method of Southern (23). On completion of the DNA transfer, the nylon membranes were dried for 10 min at 37°C, wrapped in aluminum paper, and then stored at room temperature until use. Ribosomal 16 and 23S RNA from *Escherichia coli* labeled with acetylaminofluorene was used as a probe (11). The immunodetection of rRNA and genes coding for rRNA was done with monoclonal anti-acetylaminofluorene mouse antibody and sheep anti-mouse alkaline phosphatase-conjugated immunoglobulin G antibody (acetylaminofluorene ribosomal RNA kit I from Eurogentec).

**(iv) DNA fragment size determination.** The 15 largest fragments of DNA fragment size standard Raoul I hybridizing with labeled pBR322 DNA were used to visually determine DNA sizes and migration distances.

**(v) Analysis of DNA relatedness.** Dice coefficients of similarity (number of shared fragments × 2 × 100/total number of fragments in the two samples) were determined for each pair of isolates by visual comparison of ribotype patterns (14). Strains were clustered by the unweighted-pair group method of average linkage (UPGMA) (18).

## RESULTS

**Typing ability and reproducibility.** Different restriction endonuclease patterns were established after digestion with *Eco*RI or *Hin*dIII, electrophoresis, and ribotyping using the *E. coli* rRNA probe. rDNA profiles were considered distinct when there was a single band difference and were designated according to the restriction enzyme used (E for *Eco*RI and H for *Hin*dIII). Figure 1 shows rRNA gene *Hin*dIII restriction patterns for a set of isolates, and Fig. 2 and 3 illustrate the patterns obtained. All 139 isolates studied were typeable. Twenty-seven *Eco*RI rDNA profiles (E1 to E27) were identified. They had 11 to 14 rDNA bands clearly focused from 1.4

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TABLE 1. Origins, biotypes, and ribotypes of *S. marcescens* strains

| Strain no. | Source[a] | Date(s) of isolation (mo and yr) | Biotype | rRNA RFLP pattern[c] | | Ribotype | No. of patients |
|---|---|---|---|---|---|---|---|
| | | | | *Eco*RI | *Hin*dIII | | |
| 1–27 | CHU | Dec. 87 to Sept. 89 | A5 | E14 | H16 | 1 | 19 |
| 28–31 | CHU | Feb. 89 to May 89 | A5 | E25 | H16 | 2 | 4 |
| 32–34 | CHU | Nov. 89 | A5 | E14 | H12 | 3 | 2 |
| 35 | CIT | ? 87 | A5 | E14 | H12 | 3 | 1 |
| 36–37 | CIT | ? 88 | A5 | E14 | H16 | 1 | 2 |
| 38 | STJ | ? 87 | A5 | E14 | H12 | 3 | 1 |
| 39 | STJ | ? 88 | A5 | E14 | H16 | 1 | 1 |
| 40–42 | BRU | ? 88 | A5 | E14 | H16 | 1 | 3 |
| 43 | ESN | ? 87 | A5 | E14 | H16 | 1 | 1 |
| 44 | BXL | Dec. 89 | A5 | E14 | H12 | 3 | 1 |
| 45–61 | CHU | Dec. 87 to Mar. 91 | A8a | E14 | H16 | 1 | 13 |
| 62 | CHU | Sept. 89 | A8a | E14 | H13 | 4 | 1 |
| 63 | CIT | ? 88 | A8a | E14 | H13 | 4 | 1 |
| 64 | CIT | Jan. 88 | A8a | E14 | H12 | 3 | 1 |
| 65–66 | STJ | ? 88 | A8a | E14 | H13 | 4 | 2 |
| 67 | BXL | Dec. 90 | A8a | E14 | H12 | 3 | 1 |
| 68 | BXL | Dec. 90 | A8a | E14 | H13 | 4 | 1 |
| 69 | BXL | June 86 | A8a | E14 | H16 | 1 | 1 |
| 70–72 | CHU | Oct. 89 | A8b | E14 | H15 | 5 | 2 |
| 73–75 | CHU | Oct. 88 | A8b | E15 | H13 | 6 | 3 |
| 76–77 | CHU | Feb. 88 | A8b | E17 | H13 | 7 | 1 |
| 78–79 | CHU | Nov. 87 | A8b | E14 | H13 | 4 | 2 |
| 80 | CHU | Nov. 89 | A8b | E14 | H16 | 1 | 1 |
| 81 | CHU | Oct. 88 | A8b | E15 | H14 | 8 | 1 |
| 82 | CHU | Oct. 89 | A8b | E15 | H17 | 9 | 1 |
| 83 | CHU | Mar. 92 | A8b | E19 | H15 | 11 | 1 |
| 84 | CHU | Dec. 87 | A8b | E18 | H13 | 10 | 1 |
| 85 | CIT | Dec. 87 | A8b | E18 | H13 | 10 | 1 |
| 86 | STJ | Aug. 88 | A8b | E14 | H16 | 1 | 1 |
| 87–88 | SER | Jan. 88 | A8b | E16 | H13 | 12 | 1 |
| 89 | SER | Jan. 88 | A8b | E20 | H13 | 13 | 1 |
| 90–93 | BXL | July 86 to Dec. 90 | A8b | E15 | H13 | 6 | 4 |
| 94 | BXL | July 86 | A8b | E14 | H15 | 5 | 1 |
| 95–99 | CHU | Mar. 88 to Oct. 91 | TCT | E21 | H20 | 14 | 5 |
| 100–102 | CHU | Oct. 88 to Sept. 89 | TCT | E21 | H18 | 15 | 3 |
| 103–104 | CHU | May 89 | TCT | E22 | H19 | 16 | 1 |
| 105 | CHU | June 89 | TCT | E19 | H12 | 17 | 1 |
| 106 | CHU | Aug. 89 | TCT | E21 | H22 | 18 | 1 |
| 107 | CHU | Aug. 88 | TCT | E23 | H21 | 19 | 1 |
| 108 | CHU | May 89 | TCT | E24 | H20 | 20 | 1 |
| 109–118 | CHU | Aug. 88 to Nov. 89 | TT | E14 | H13 | 4 | 9 |
| 119 | STJ | ? 87 | TT | E26 | H18 | 21 | 1 |
| 120–121 | BRU | Dec. 89 | TT | E27 | H23 | 22 | 1 |
| 122 | CHU | ? 87 | A4a | E5 | H4 | 23 | 1 |
| 123 | CHU | ? 87 | A4a | E7 | H6 | 24 | 1 |
| 124 | CIT | Feb. 88 | A4a | E6 | H5 | 25 | 1 |
| 125 | BRU | ? 89 | A4a | E6 | H7 | 26 | 1 |
| 126 | STJ | Aug. 88 | A4a | E8 | H7 | 27 | 1 |
| 127 | CHU | July 88 | A3a | E3 | H2 | 28 | 1 |
| 128 | CIT | Mar. 88 | A3a | E1 | H1 | 29 | 1 |
| 129 | BRU | ? 88 | A3a | E2 | H1 | 30 | 1 |
| 130 | BXL | July 86 | A3a | E4 | H3 | 31 | 1 |
| 131 | CHU | Nov. 89 | A3b | E10 | H1 | 32 | 1 |
| 132 | CHU | Aug. 89 | A3b | E10 | H9 | 33 | 1 |
| 133 | CHU | July 89 | A3b | E11 | H1 | 34 | 1 |
| 134 | CHU | July 89 | A3b | E11 | H9 | 35 | 1 |
| 135–136 | CHU | Apr. 89 to Mar. 91 | A2a | E9 | H8 | 36 | 2 |
| 137 | BXL | Feb. 91 | Aux | E14 | H12 | 3 | 1 |
| 138 | CHU | Aug. 89 | A6a | E13 | H11 | 37 | 1 |
| 139 | CHU | Oct. 89 | A1b | E12 | H10 | 38 | 1 |

[a] CHU, Centre Hospitalier Universitaire, Liège, Belgium; CIT, STJ, BRU, SER, and ESN, five other hospitals in the Liège area; BXL, Hôpital Universitaire Brugmann, Brussels, Belgium.
[b] ?, month unknown.
[c] RFLP, restriction fragment length morphism.

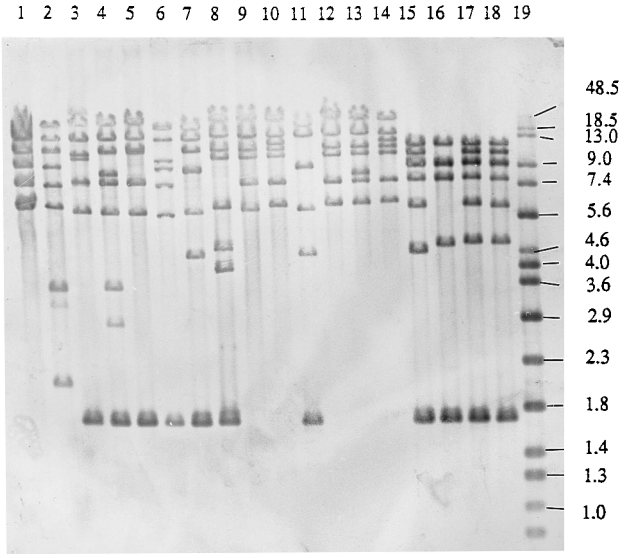


FIG. 1. Ribopatterns obtained by Southern blot analysis of *Hin*dIII digests. Lanes: 1, H18; 2, H21; 3, H16; 4, H14; 5, H13; 6, H12; 7, H11; 8, H10; 9, H9; 10 and 14, H1; 11, H8; 12, H3; 13, H2; 15, H7; 16, H6; 17, H5; 18, H4; and 19, molecular weight standard Raoul 1. Sizes (in kilobase pairs) are indicated on the right.

to 18.5 kb. Certain bands were common: fragments of 1.4, 2.2, and 8.5 kb were found in all profiles (Fig. 2). Twenty-three *Hin*dIII rDNA profiles (H1 to H23) were observed, including five to nine bands from 1.8 to 48.5 kb (Fig. 3). The combined results of the two enzymes produced maximum discrimination between isolates and identified 38 ribopatterns (Table 1).

Reproducibility was not statistically studied, but it appeared quite high. Indeed, 20 strains ribotyped twice yielded the same results in all instances. Moreover, stability of the profiles was noted for strains isolated from the same patient over a period ranging from a few days to several months.

Percent similarities based on the Dice coefficient and UPGMA clustering are presented in Fig. 4. The dendrogram shows four major clusters of ribopatterns (A through D) with more than 65% similarity. Nine subclusters with 70.5 to 81.6% similarity can also be identified. Correlation between results of ribotyping and those of biotyping has been established. Within some subclusters, discrimination between strains within one biotype can be observed: biotype A3a in subcluster A1, biotype A3b in subcluster A2, biotype A4a in subcluster C1, and biotype A1b in subcluster D1. The other subclusters did not include all the strains from one biotype or consisted of isolates belonging to several biotypes. However, most strains of the major biotypes were distributed in one subcluster: biotype TCT in subcluster $B_2$, biotypes A5 and A8a in subcluster $D_2$, and biotypes A8b and TT in subcluster $D_3$. Moreover, subcluster $D_2$ suffered from poor discrimination, particularly in ribotype E14 H16, which characterized 55 of 139 strains studied.

**Subdivision of biotypes by ribotyping for epidemiological studies.** Ribotyping is more discriminatory than biotyping: ribotyping revealed 38 patterns, while biotyping produced 12 types (11 biotypes and 1 nontypeable group). To increase discrimination between isolates, the results of the two methods have been combined. Indeed, some biotypes exhibited a great deal of heterogeneity in ribotypes, and the same ribotype could be noted for isolates belonging to distinct biotypes.

In this analysis, only one isolate per patient was retained, since all isolates from the same origin were identical, reducing the number of strains to 120 (Table 1). According to the distribution of ribotypes within biotypes, 44 groups could be defined as follows.

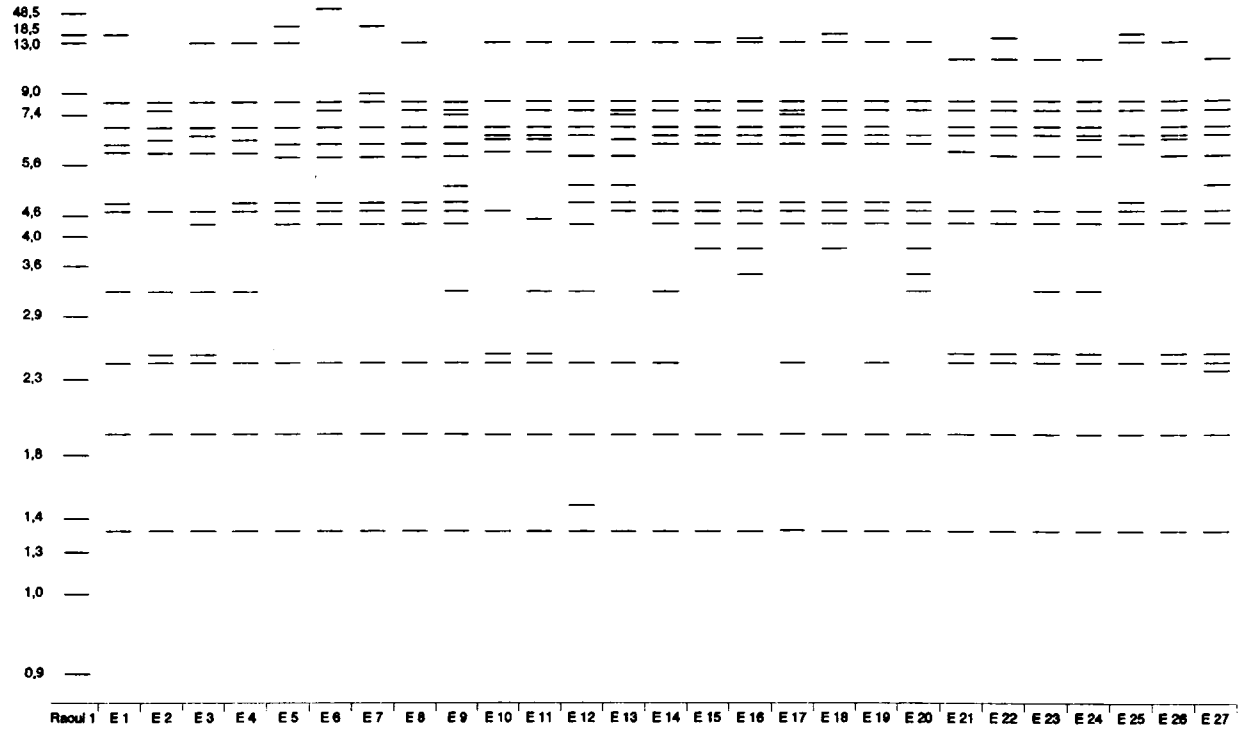


FIG. 2. Schematic representation of the *Eco*RI restriction enzyme rRNA patterns. Fragment sizes (in kilobase pairs) are shown on the left.

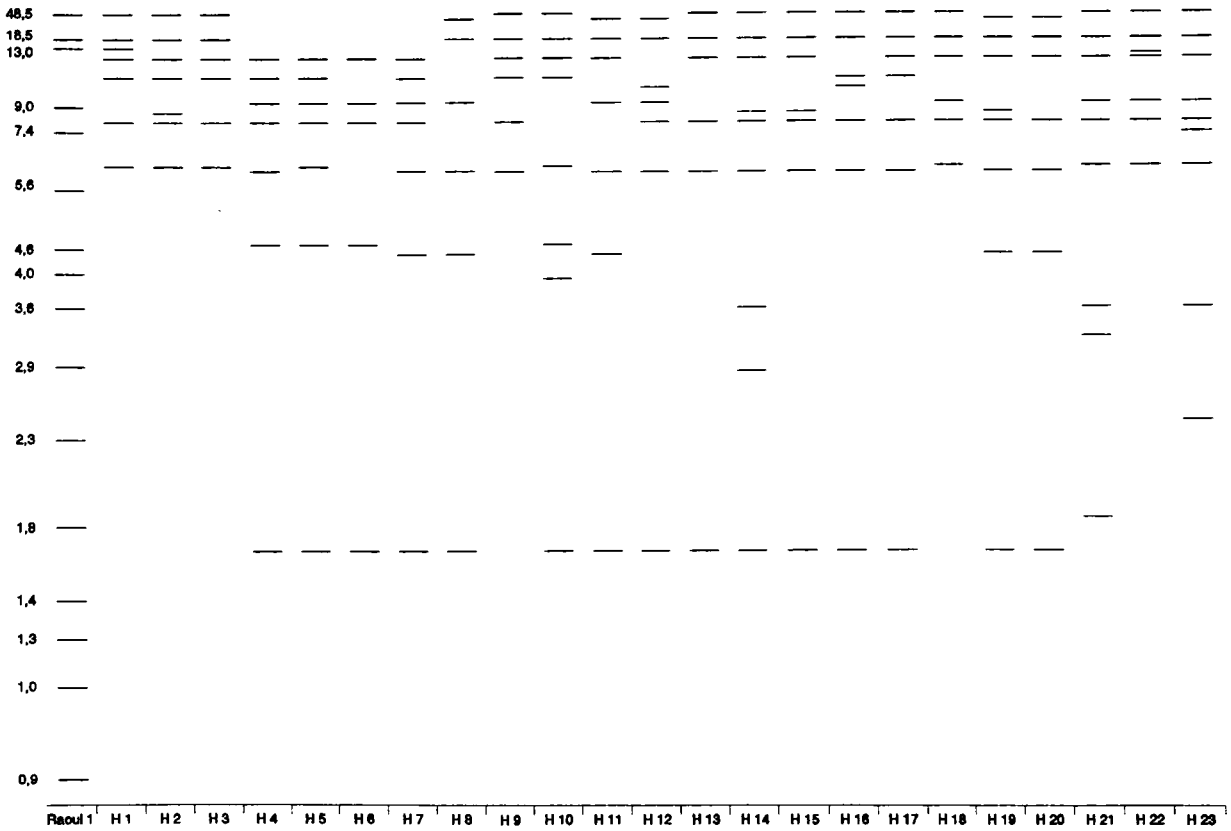


FIG. 3. Schematic representation of the *Hin*dIII restriction enzyme rRNA patterns. Fragment sizes (in kilobase pairs) are shown on the left.

**Biotype A5.** With biotype A5, we observed three different ribopatterns (ribotypes 1 through 3) among 35 strains studied. The majority of the isolates (26 strains), which came from five hospitals in the area of Liège, showed the same ribotype (ribotype 1). Ribotype 2 characterized four isolates from one hospital, and ribotype 3 was common to five strains originating from three hospitals in Liège and one hospital in Brussels over a 3-year period.

**Biotype A8a.** Among 21 isolates of biotype A8a, we also found three ribopatterns (ribotypes 1, 3, and 4). Ribotype 1 characterized most of the isolates (14 strains). The three ribotypes included strains isolated from various hospitals in Liège or Brussels over 6 years (1986 to 1991).

**Biotype A8b.** Among 22 biotype A8b isolates, ribotyping revealed 11 distinct ribopatterns (ribotypes 1 and 4 through 13), demonstrating the heterogeneity of the isolates belonging to this biotype. Ribotypes 5 and 6 did not discriminate between strains from different origins. Thus, ribotype 5 was yielded by one strain from Brussels isolated in 1986 and by two strains originating in Liège 3 years later.

**Biotype TCT.** Like biotype A8b, biotype TCT was heterogeneous, because seven ribopatterns (ribotypes 14 to 20) were identified among 13 strains from one hospital. Ribotypes 14 and 15 were the most frequently encountered, including five and three strains, respectively.

**Biotype TT.** The same ribopattern (ribotype 4) was yielded by 9 of 11 strains studied. All these strains were isolated in the same hospital over a 1-year period. The last two strains had different origins and showed two other ribopatterns (ribotypes 21 and 22).

**Biotype A4a.** A distinct ribopattern (ribotypes 23 to 27) characterized each of the five strains of biotype A4a, which came from four hospitals.

**Biotype A3a.** The four biotype A3a strains, which originated from four hospitals, yielded four ribopatterns (ribotypes 28 to 31).

**Biotype A3b.** Ribotyping allowed differentiation between the four isolates of biotype A3b (ribotypes 32 to 35). All the strains were isolated in one hospital during the same period.

**Biotype A2a.** The two biotype A2a strains had the same ribotype (ribotype 36). They were isolated from one hospital over a 2-year period.

**Biotypes A1b and A6a and a nontypeable strain.** The prototype strains of biotypes A1b and A6a and the nontypeable group yielded ribotypes 38, 37, and 3, respectively.

**Distribution and spread of epidemic clones in the University Hospital in Liège.** By retaining only one isolate per patient, the distribution of epidemic clones in the University Hospital in Liège (Centre Hospitalier Universitaire) from 1987 to 1991 was examined (Table 1). Of 32 distinct biotype-ribotype combinations, 11 types accounted for 75.3% of the *S. marcescens* strains studied (64 of 85 strains). Three combinations have been regularly isolated from various clinical departments for a long time. The isolates belonged to biotype A5, ribotype 1 (19 strains from December 1987 to September 1989); biotype A8a, ribotype 1 (13 strains from December 1987 to March 1991); and biotype TT, ribotype 4 (9 strains from August 1988 to November 1989). Another combination, biotype TCT, ribotype 14, had a more limited distribution in the hospital (five strains), although it was persistently encountered from March 1988 to October 1991. The seven remaining combinations included two to four isolates. Several types showed some clustering of cases

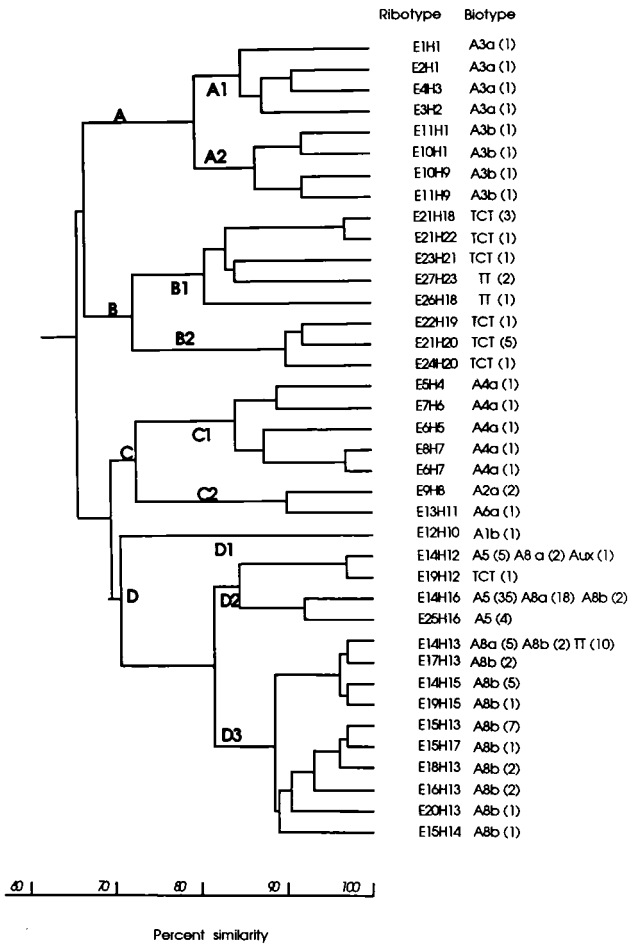


FIG. 4. Dendrogram of the average linkage percent similarity between the different ribotypes and correlation with classification of the strains in a particular biotype. Numbers of isolates are indicated in parentheses.

at certain times: biotype A5, ribotype 2 (four strains recovered from February 1989 through May 1989); biotype A5, ribotype 3 (two strains in November 1989); and biotype A8b, ribotype 4 (two strains in November 1987), ribotype 5 (two strains in October 1989), or ribotype 6 (three strains in October 1988). In each of these groups, the isolates originated from one or two wards. No clear clustering of cases was detected with the two last combinations because of disparity in time and space: biotype TCT, ribotype 15 (three strains originating from three wards and recovered in October 1988, July 1989, and September 1989), and biotype A2a, ribotype 36 (two strains from two departments, recovered in April 1989 and March 1991).

These data suggest an epidemic occurrence of single clones, although ribotyping is sometimes incapable of differentiating between strains of the same biotype that appear to be epidemiologically unrelated.

## DISCUSSION

Different methods have been used for the typing of *S. marcescens*. Biotyping provides a current way for discriminating between nosocomial strains, but only biotypes A5, A8a, A8b, and TCT are predominantly represented in hospital wards (13). Other methods, including serotyping (26), phage typing (9), testing for bacteriocin production or susceptibility (7, 8, 27, 28), and electrophoresis of outer membrane proteins (2) or total proteins (15, 20), have been found to be unsatisfactory because of insufficient discrimination and/or poor reproducibility. Restriction endonuclease analysis of DNA with conventional agarose gel electrophoresis has been successfully used for epidemiological fingerprinting. However, this method has a limited application because of the large number of bands and the poor resolution obtained in the gel (20). Recently, ribotyping was performed on a small series and provided efficient differentiation. This molecular typing marker appeared to be useful for studying the epidemiology of nosocomial *S. marcescens* isolates (3).

Ribotyping refers to the use of Southern blot analysis to detect polymorphisms associated with the ribosomal operon(s). *E. coli* 16 and 23S rRNA can be used to probe complementary segments contained on restriction length fragments with a wide range of cultivable species, because ribosomal sequences are highly conserved in bacteria. The use of a commercial nonradioactive probe detection system greatly facilitates the procedure and allows this method to be used in any well-equipped laboratory.

In the study described here, we found that ribotyping yields reproducible and easily readable patterns. All 139 *S. marcescens* nosocomial strains studied were characterized; *Eco*RI digests produced more bands and more rDNA profiles ($n$ = 27) than *Hin*dIII ($n$ = 23). Each distinct combination of patterns identified 38 ribotypes, and, theoretically, additional enzymes could further increase the discriminatory power.

Interpretation of clonality based on restriction fragment length polymorphism analysis by ribotyping is not well defined. Koblavi et al. (16) considered that any difference in *Vibrio cholerae* patterns indicated individual clones, while Tenover et al. (25) assigned *Staphylococcus aureus Cla*I and *Hin*dIII ribotypes that differed by a single band to subtypes. The similarity, although not identity, of isolates could be interpreted as indicating possible variants because a change in a fragment might occur, e.g., by mutation or by acquisition of a transposon. A single band difference in the *Eco*RI and *Hin*dIII combined rDNA pattern seems sufficient to us for characterizing *S. marcescens* strains. Indeed, the stability over time of the patterns yielded by isolates from the same patient and the results of other epidemiological methods, such as multilocus enzyme electrophoresis of esterases or alkaline phosphatase and genome macrorestriction analysis by pulsed-field electrophoresis (data not shown), support this hypothesis.

Clustering of the ribopatterns compared by the Dice coefficient showed from about 65 to 97% similarity, allowing discrimination within that range. The dendrogram also revealed four main clusters and nine subclusters of ribopatterns clearly distinct at approximately $\leq$80% similarity. A good correlation between clustering by ribotyping and belonging to biotype A1b, A3a, A3b, or A4a was observed. More variability was noted for the other subclusters which did not group all the strains of one biotype or included isolates representative of two or more biotypes. However, most strains in each predominant biotype were arranged in one subcluster.

Ribotyping indicated more differences between strains (38 ribopatterns) than did biotyping (11 biotypes and 1 nontypeable group). The combined results of the two methods had a very high discriminatory power. When one strain per patient was retained, 120 strains displayed 44 different groups. Several groups were predominantly represented. They principally characterized isolates within hospital biotypes A5, A8a, TCT, and TT. Biotype A8b, which is also confined to hospitalized patients, and biotypes which were seldom encountered, such as A4a, A3a, and A3b, appeared very heterogeneous. The simi-

larity of some strains could result from the spread of a single clone, but it may be impossible for ribotyping to verify such possible outbreaks because even epidemiologically unrelated isolates were identical. For instance, in biotype A8b, one strain yielding ribotype 5 was isolated in 1986 at the Brugmann University Hospital in Brussels. The same ribotype characterized two isolates taken 3 years later from the University Hospital in Liège, 100 km away. In such cases, however, no technique is likely to be sufficient, and investigation would have to rely on the overall clinical context. Typing systems can provide potentially useful information, but they are best used to support or reject clinical hypotheses rather than to replace classic clinical or epidemiological analysis (19).

Although ribotyping is time-consuming, it provides a highly reproducible and reliable system for typing nosocomial strains of *S. marcescens*. It adds discrimination to biotyping and is an interesting technique for the epidemiologist.

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