# Comparison of serotype, biotype and bacteriocin type with rDNA RFLP patterns for the type identification of *Serratia marcescens*

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## SUMMARY

Variations in rDNA gene loci in DNA digests of 209 clinical isolates of *Serratia marcescens* were determined with an *Escherichia coli* rRNA probe. Forty-one restriction fragment length polymorphism patterns (ribotypes) were identified, based on the size of 4–14 (mean 7.5) hybridization bands. The patterns differed by more than a single band in 98% of pair-wise comparisons. On a subset of 76 isolates, ribotyping proved to be marginally more discriminating than biotyping (discrimination index 0·92 *v.* 0·89) followed by serotyping (0·87) and bacteriocin typing (0·74). About one-third of isolates belonged to unique ribotypes and only two ribotypes exceeded 5% in frequency (23·0 and 6·4% respectively). A combination of serotype or biotype with ribotyping defined a similar number of strains, although none of the methods alone was sufficiently discriminatory to identify strains. We conclude that due to the accessibility of biotyping and the lack of commercially available antisera for *S. marcescens*, the biotype and ribotype together provide reliable markers of strain identity.

## INTRODUCTION

In Europe and North America, *Serratia marcescens* is an infrequent cause of bacteraemia but outbreaks of infection are occasionally reported [1–3]. These infections occur most often in the immunocompromised host and neonates, and are often accompanied by resistance to antibiotics active against other Gram-negative bacteria [4]. The epidemiology of *S. marcescens* in hospitals is unclear, due in part, perhaps, to the lack of highly discriminatory methods of strain type identification.

A number of methods have been described for the typing of *S. marcescens* including serotyping and phage typing [5, 6], biotyping [7], bacteriocin typing [8],

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whole-cell protein fingerprinting [9], multilocus enzyme typing and plasmid profiles [10] and restriction endonuclease analysis of chromosomal DNA [11]. The reference methods of serotyping and phage typing are restricted to a small number of reference centres and the other techniques are most suitable for comparative typing or fingerprinting of isolates within outbreaks.

In the last 5 years there has been considerable interest shown in the applicability of indexing variation in the loci of rRNA genes in bacterial DNA for subspecies identification [12]. This technique, ribotyping, has been used for epidemiological studies of many bacterial species including *S. marcescens* [13] with varying degrees of success in terms of discrimination between strains. We have applied ribotyping to a collection of isolates of *S. marcescens* from clinical episodes of infections in different hospitals, and compared the results of selected strains with those obtained by serotyping, bacteriocin sensitivity and biotyping in order to determine the most suitable methods or combination of methods for the type identification of strains.

## MATERIALS AND METHODS

*Bacterial strains.* A collection of 209 isolates, recovered from five hospitals in Madrid, was made in the period 1988–90. Approximately half of the total collection was isolated from urine and the remainder from respiratory tract infections, surgical wounds, blood, catheter tips and CSF. A subset of 76 isolates from different patients in various hospitals and wards was assembled for the comparison of typing techniques. All isolates were identified by the API 20E system (API, BioMérieux) and *S. marcescens* ATCC 8100 was used as a reference strain.

*Biotyping.* Isolates were tested for growth on single carbon sources [7], as well as tetrathionate reduction, production of prodigiosin and haemolysis of horse blood.

*Serological typing.* Serotypes were determined by an enzyme-linked immunosorbent assay [14] with a panel of absorbed O antisera [5].

*Bacteriocin typing.* Isolates were tested for susceptibility to the ten producer strains of Traub and co-workers [8] and bacteriocin types were designated according to that scheme. Novel type patterns were given Roman numerals.

*Ribotyping.* Isolates were grown in lactose broth overnight at 37 °C and following centrifugation, DNA was extracted by the method of Pitcher and colleagues [15]. Additional steps with chloroform-pentanol and protease K were included to eliminate autodegradation of the DNA [16]. DNA samples (5–8 μg) were digested with different restriction endonucleases (Pharmacia, Boehringer Mannheim and BRL) with the buffers and reaction conditions recommended by the manufacturer. Electrophoresis, Southern transfer and hybridization with cDNA to *Escherichia coli* (16S + 23S) rRNA probe was performed as described previously [17].

*Analysis of data.* The molecular weights of DNA hybridization bands were determined from migration distances with reference to *Hind* III digests of phage lambda (Gibco-BRL Ltd). All observed band positions were recorded and scored as 0 or 1 for computer-assisted analysis. Pair-wise comparisons of similarity were

made using the simple matching coefficient (Sm) which applies equal weight to positive and negative tests results and cluster analysis was performed by the unweighted pair group matching average (UPGMA) of the data [18]. The discriminatory indices of the typing systems were calculated according to Hunter and Gaston [19] and separation values (sv) of bands were the number of positive reactions multiplied by the number of negative reactions [20].

### RESULTS

Preliminary experiments showed that of ten restriction endonuclease enzymes tested, *Ssp* I produced hybridization fragments over a wide range of molecular sizes and formed clear and resolvable bands (Fig. 1). Using this enzyme, 14 hybridization band positions were identified among the 209 isolates. Table 1 shows the frequency of occurrence of the bands and reproducibility in terms of molecular size. One band (N) was common to all isolates and the frequency of other bands ranged from 3·4 % (band K) to 94·0 % (J). Band positions were highly reproducible and varied only by ±0·6 kb for DNA above 12 kb in size. In terms of separation values, band G was the most discriminating followed by bands D and E. Bands J, K and N were of the least value for distinguishing between patterns.

The presence or absence of a band was recorded for all isolates and 41 patterns (ribotypes) were distinguished if any difference in profile was considered significant. The number of bands ranged from 4 to 14 (mean 7·5) in the ribotypes identified. A pair-wise comparison of all differences within the 41 types was made and of 615 total comparisons, as many as 600 pairs differed by more than one single band reaction (< 92 % similarity). This is reflected in the clustering of the profiles shown in Fig. 2.

Ribotype patterns were distributed unequally among the unselected isolates. Pattern R21 accounted for approximately 23 % of all isolates followed by R15 (7·7 %) and R38 (5.5 %). Twelve ribotypes were represented by 2 or 3 isolates each and 26 patterns were unique.

### Comparison of ribotyping with other methods

The discrimination of ribotyping was compared with serotype, biotype and bacteriocin type on a panel of 76 isolates. Table 2 gives the percentage frequency of distribution of each type. Eleven serotypes were identified and three serotypes O14, S1254 and S3255 accounted for two-thirds of the isolates. Biotyping gave a similar discrimination index as serotyping but half of the isolates fell into the three most frequent types. The discrimination of bacteriocin typing was relatively poor as only eight type patterns were recognized and over 40 % of the isolates failed to produce a bacteriocin active on the indicator strains. The percentage distribution of ribotypes was similar to that seen with the unselected isolates and this method gave the highest discrimination index.

Table 3 shows the distribution of ribotype patterns among isolates of the more frequent (> 5 %) serotypes and biotypes. It is clear that none of the three systems defined homogeneous populations. The most common serotype (O14) could be further divided by 15 ribotype patterns and the common biotype A8b by 11 ribotypes. Conversely, 4 serotypes and 7 biotypes were grouped as ribotype R21.
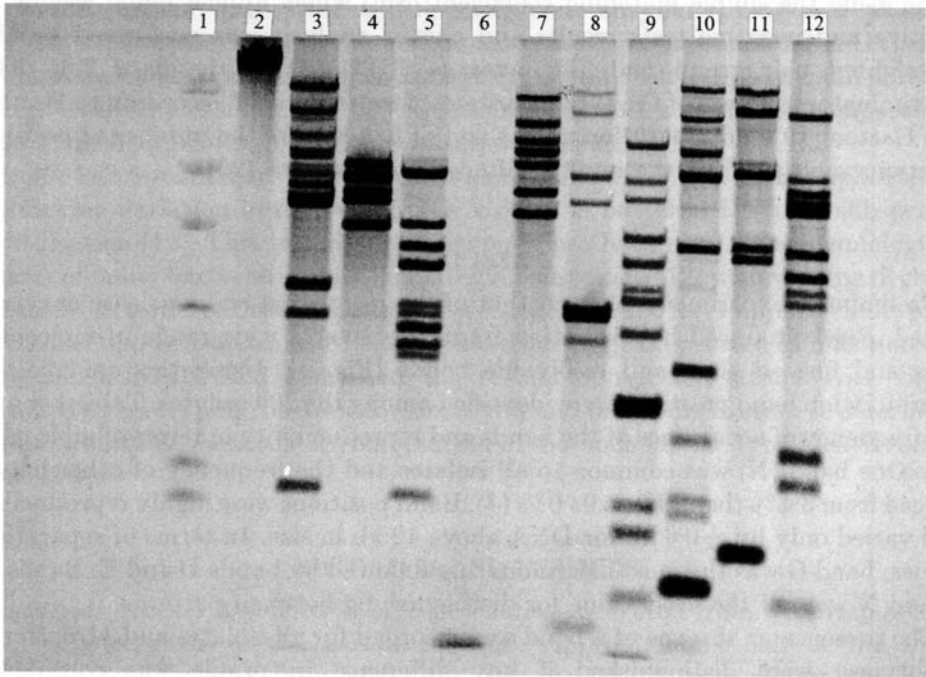
Fig. 1. rRNA hybridization profiles of DNA of *S. marcescens* ATCC 8100 digested with different restriction endonucleases. Lane (1) lambda DNA digested with *Hin*d III, (2) undigested DNA, (3) DNA digested with *Bg*E II, (4) *Pst* I, (5) *Sal* I, (6) *Hae* III, (7) *Bam*H I, (8) *Xho* I, (9) *SSp* I, (10) *Dra* I, (11) *Hin*d III, (12) *Eco*R I.

Table 1. *Size, frequency of distribution and separation values of rRNA hybridization bands of DNA digests of 209 isolates of* S. marcescens

| Band | Molecular size (kb) | Frequency (%) value* | Separation |
|------|---------------------|----------------------|------------|
| A | 20·0 ± 0·6 | 77·8 | 364 |
| B | 17·6 ± 0·6 | 11·1 | 288 |
| C | 15·4 ± 0·6 | 80·3 | 330 |
| D | 11·6 ± 0·3 | 21·4 | 378 |
| E | 10·4 ± 0·3 | 62·4 | 378 |
| F | 9·3 ± 0·2 | 50·4 | 348 |
| G | 8·1 ± 0·2 | 30·8 | 414 |
| H | 6·3 ± 0·2 | 85·5 | 348 |
| I | 5·4 ± 0·2 | 46·1 | 330 |
| J | 4·7 ± 0·2 | 94·0 | 180 |
| K | 4·4 ± 0·2 | 3·4 | 180 |
| L | 3·8 ± 0·2 | 86·3 | 330 |
| M | 3·3 ± 0·2 | 19·6 | 364 |
| N | 2·9 ± 0·2 | 100·0 | 40 |

* After Rypka and co-workers [20].

Thus, ribotyping, in common with O-serotyping, failed to discriminate between individual strains. A combination of two systems greatly increased discrimination, for example, serotyping and ribotyping distinguished 55 strains within the panel of 76 isolates and biotyping with ribotyping identified 59 strains.
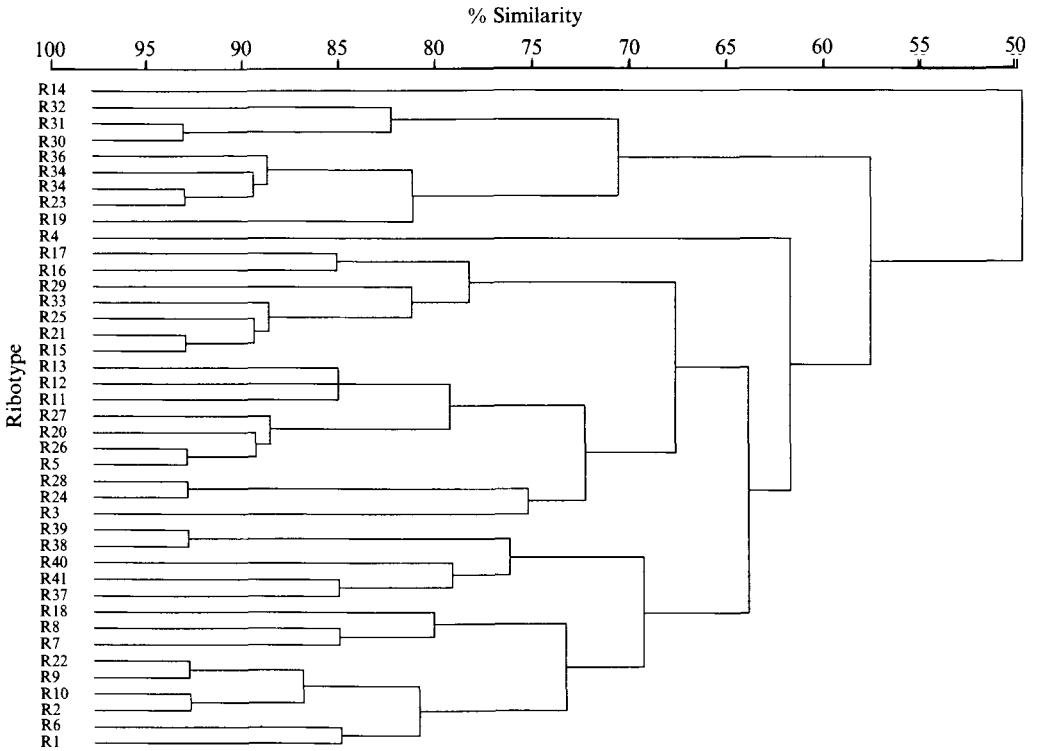
Fig. 2. Dendrogram of cluster analysis of pair-wise comparisons of similarity between 41 ribotype patterns of *S. marcescens*.

Table 2. *Percentage frequencies of type determined by serotyping, biotyping, bacteriocin typing and ribotyping among 76 patient isolates of* S. marcescens

| Serotype | (%) | Biotype | (%) | Bacteriocin | (%) | Ribotype | (%) |
|---|---|---|---|---|---|---|---|
| 2 | 3·9 | A1a | 2·6 | M1 | 19·6 | R2 | 2·6 |
| 4 | 1·3 | A1b | 3·9 | M2 | 6·0 | R10 | 2·6 |
| 6 | 7·8 | A2a | 5·2 | M3 | 1·6 | R11 | 2·6 |
| 10 | 2·6 | A2b | 3·9 | M4 | 1·3 | R12 | 2·6 |
| 14 | 29·4 | A3a | 14·3 | M5 | 1·3 | R15 | 6·5 |
| 16 | 3·9 | A3b | 14·3 | V11 | 14·7 | R21 | 23·4 |
| 20 | 3·9 | A3c | 1·3 | XL | 9·2 | R22 | 3·9 |
| 21 | 3·9 | A4a | 2·6 | LV111 | 2·2 | R23 | 3·9 |
| 22 | 3·9 | A4b | 2·6 | NT | 43·6 | R25 | 3·9 |
| S1254 | 19·2 | A6a | 9·1 | | | R27 | 2·6 |
| S3255 | 17·9 | A6b | 6·5 | | | R31 | 2·6 |
| NT | 2·6 | A8a | 5·2 | | | R38 | 3·9 |
| | | A8b | 22·1 | | | R39 | 2·6 |
| | | TC | 1·3 | | | R40 | 3·9 |
| | | TCT | 2·6 | | | Distinct | 31·2 |
| | | TT | 1·3 | | | | |
| 0·87* | | 0·89 | | 0·74 | | 0·92 | |

* Discrimination index.

Table 3. *Distribution of ribotype patterns among serotypes and biotypes of* S. marcescens

| | Total | R2 | R10 | R11 | R12 | R15 | R21 | R22 | R23 | R25 | R27 | R31 | R38 | R39 | R40 | Other |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Serotype** | | | | | | | | | | | | | | | | |
| O6 | 4 | | | | | | | | | | | | | | 3 | 1 |
| O14 | 23 | 1 | 1 | | | | 6 | | | 3 | 2 | | | | | 10 |
| S1254 | 15 | | | | 1 | | 10 | | | | | | 1 | | | 3 |
| S3255 | 16 | | | 2 | | 2 | | | 3 | | | | 1 | | | 8 |
| Other | 18 | 1 | 1 | | | 2 | 2 | 2 | 3 | | | 2 | 1 | 2 | | 2 |
| **Biotype** | | | | | | | | | | | | | | | | |
| A2a | 4 | | 1 | | | | 1 | | | | | | | | | 2 |
| A3a | 11 | 2 | 1 | 2 | 1 | | | | | | | | 1 | 2 | 1 | 1 |
| A3b | 11 | | | | | | 3 | | 1 | | 2 | 2 | | | | 3 |
| A6a | 7 | | | | 1 | 1 | 3 | | | | | | | | | 2 |
| A6b | 5 | | | | | | 1 | | | 1 | | | | | | 3 |
| A8a | 4 | | | | | | | 1 | | 2 | | | 1 | | | |
| A8b | 17 | | | | | 1 | 6 | 2 | | | | | | | 2 | 6 |
| Other | 17 | | | | | 2 | 1 | 2 | 2 | 2 | | | 1 | | | 7 |

## DISCUSSION

*S. marcescens* is a heterogeneous species which probably contains a limitless number of different strains. A typing method should therefore be able to differentiate between these strains accurately and reproducibly. Although several methods have been applied to the typing of this species, none of them is sufficiently discriminating and reproducible to be used alone for epidemiological studies. Some methods such as serotyping, biotyping and bacteriocin typing appear to make broad distinctions or groups within *S. marcescens* while others, e.g. phage typing, offer increased discrimination especially when combined with serotyping [6].

The O serotyping scheme for *S. marcescens* is undergoing revision in the light of increasing knowledge of the chemical basis of the serological reactions [21]. Some antigens traditionally considered to be O lipopolysaccharide antigens are now known to contain, in addition, acidic capsular polysaccharides which cross-react extensively within the species. These findings offer an explanation as to the inconsistency of agglutination results of O type sera with whole cells and the reactions of purified lipopolysaccharide O antigens in ELISA [22]. Absorbed sera are therefore necessary for accurate O type determination [5].

For improved discrimination between isolates from incidents of infection in hospitals, isolates of the same O serotype may be further divided by their sensitivity to phages. This hierarchical approach has proved relatively useful for strain typing but due to poor reproducibility, phage patterns tend to overlap and this makes it difficult to assess the similarity of isolates [6]. Other methods have been utilized with varying degrees of success but none, either alone, or in combination, has been adopted universally.

The technique of ribotyping was first applied to *S. marcescens* by Bingen and colleagues [13], who showed that it allowed the differentiation of strains within a paediatric hospital. They found four different RFLP patterns with *Eco*R I and *Hin*d III digests of DNA from apparent outbreak isolates and three unrelated strains were distinct. In our preliminary studies we found enzyme *Ssp* I to be the most suitable in terms of hybridization fragment sizes and their distribution throughout the gel, but we can confirm that both *Eco*R I and *Hin*d III enzymes give a range of fragments appropriate for RFLP analysis.

As the size reproducibility on repeated testing of a reference strain was adequate, we allocated type numbers to the ribotype patterns of isolates primarily for convenience of comparison. Nevertheless, we recognize that such type designations cannot be extended to other studies and therefore we have not presented a scheme of type-patterns for reference.

The main advantage of patterns based on a small number of bands (or tests) with an average of seven reactions per type pattern is that overlapping of patterns is reduced. The similarity matrix showed that > 95 % of all pair-wise comparisons of patterns were less than 90 % similar and most were in the region of 50–70 % similarity. Thus, there was wide diversity in the populations defined by ribotype patterns. Based on separation values, it appears that some bands are more significant in determining the discrimination of the type pattern than others and perhaps their presence or absence should receive added weight. A lack of

correspondence, therefore, between bands of low separation value might be discounted and only 'major' band differences would contribute to the pattern. This may lead to a loss of discrimination which might be acceptable to the user if there was a concomitant increase in reproducibility.

In terms of discrimination, serotyping and/or biotyping combined with ribotyping were of a similar level. However, given the lack of commercial sources of O antisera and the comparatively low incidence of *S. marcescens* in clinical infections, the combination of biotype and ribotype must be favoured. Biotyping by the method of Grimont [12] is accessible to most laboratories and would serve logically as a screen to identify strains distinct from an index strain. Ribotyping could then be performed to confirm or deny the homogeneity of the index strain.

In conclusion, none of the methods described is optimal for strain definition in *S. marcescens*. Ribotyping proved to be reasonably discriminating and stable, although, as with serotyping, it tended to produce groups within this species rather than identifying strains. However, if used in combination with biotyping or serotyping, it should be adequate for epidemiological investigations of type identity.

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