AN ABSTRACT OF THE THESIS OF

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Title: Epidemiological typing of *Serratia marcescens*

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Serratia marcescens is a common cause of hospital acquired infections. Epidemiological studies are often performed on this and other potential pathogens to identify and possibly prevent future outbreaks. Of the many epidemiological techniques available, polymerase chain reaction (PCR) based typing techniques are commonly employed. Examples include randomly amplified polymorphic DNA (RAPD), repetitive extragenic palindromic (REP) elements, enterobacterial repetitive intergenic consensus (ERIC) sequences, polymorphic GC-rich repetitive sequence (PGRS), and ribotyping.

In this study, RAPD, REP, ERIC, PGRS, and ribotyping were used to compare the genetic relationship among 62 clinical *S. marcescens* isolates. After amplification, reaction products were analyzed by agarose gel electrophoresis and banding patterns among each isolate were compared using dendrograms. RAPD analysis using primer 1254 yielded 41 genotypic groups, primer 1283 resulted in 43 genotypic groups, primer 1060 amplified 37 genotypic groups, and the combination of primers 1254 and 1283 yielded 43 genotypic groups. REP amplified 54 genotypic groups, ERIC resulted in 19 genotypic groups, PGRS produced 60 genotypic groups, and ribotyping yielded 40 genotypic groups. Antimicrobial susceptibility patterns using antibiotic gradient plates were also used to differentiate these isolates. Data obtained were used to generate

37 phenotypic groupings based on dendrogram analysis. Of the techniques evaluated, PGRS provided the most discriminatory power to identify genomic differences among the 62 *S. marcescens* isolates.

Epidemiological Typing of *Serratia marcescens*

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Patton, Toni

Approved by Major Advisor

Approved by Committee Member

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Approved for the Graduate Council

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PREFACE

This thesis was prepared following the publication style of the American Society for Microbiology.

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Epidemiological typing

Epidemiological typing involves the use of laboratory techniques to identify different strains of organisms within a species (25). Practical clinical uses include determining if a patient has a recurring or relapsing infection, if certain strains are associated with specific clinical syndromes, tracing the source of contamination, preventing patient to patient spread, and to increase the understanding of the epidemiology of the infection (7, 31). Typing systems are best used to support or reject clinical hypotheses rather than replacing classic clinical analysis (10).

Typing methods can be classified into genotypic and phenotypic typing methods (25). Phenotypic methods are based on characteristics expressed by an organism (25) and include biotyping (15), antimicrobial susceptibility patterns (28), serotyping (29), bacteriophage typing (29), bacteriocin typing (27), polyacrylamide gel electrophoresis (39), immunoblotting (25), and multilocus enzyme electrophoresis (25). Drawbacks to phenotypic typing methods include a restricted number of characteristics that can be examined, lack of sensitivity, and the technique used may be species specific (38).

Genotypic typing methods are based on an organism's genetic make up (25). Any difference in the genome of one organism compared to another can allow for its differentiation (4). Genotypic methods include plasmid analysis (28), restriction endonuclease analysis of plasmids or chromosomal DNA (RFLP) (7), pulsed-field gel electrophoresis (9), polymerase chain reaction (PCR) techniques (31), and southern blot

analysis (38). Drawbacks to some genotyping procedures include requirements for large amounts of pure DNA or RNA and a high degree of technical skill (4).

Polymerase chain reaction

PCR is a technique that amplifies a specific DNA segment from a very complex genome (11). Amplification of a target sequence is accomplished in three basic steps. The first step, denaturation, involves separating the double stranded molecule into single strands. The second step, annealing, refers to the ability of gene specific primers to bind to specific sequences within the DNA template. The final step, elongation, involves extension of primers by Taq DNA polymerase. These three steps are repeated approximately 30 times, which results in the amplification of the desired gene sequence (11). There are several applications for the use of PCR including direct cloning of amplified fragments (11), mutagenesis (11), and genetic fingerprinting of complex genomes (4). PCR typing methods are simple, fast, and provide a comparative way to differentiate organisms (24).

Randomly amplified polymorphic DNA

Randomly amplified polymorphic DNA (RAPD) analysis is based on decreased annealing temperatures resulting in low stringency amplification of random DNA fragments within the genome of an organism (36). RAPD requires no previous knowledge of the DNA that is to be amplified and involves primers that are randomly selected and vary in length (31). The primers are generally shorter than primers used in conventional PCR. Since the number of annealing sites vary between strains,

amplification results in variable DNA patterns (31). Fingerprints generated from RAPDs provide the highest level of taxonomic resolution achieved by PCR methods (41). RAPDs have been used for fingerprinting the genomes of numerous organisms, including *Serratia marcescens* (17), *Candida albicans* (31), *Enterococcus faecium* (31), *Candida glabrata* (31), *Staphylococcus aureus* (31), *Haemophilus infleunzae* (19), and *Mycobacterium tuberculosis* (36).

Ribotyping

Ribotyping is based on the fact that ribosomal genes are highly conserved in all bacteria (25). The evolutionary conservation of ribosomal RNA (rRNA) sequences allows ribotyping to have broad spectrum applications (38). Since each organism has multiple copies of rRNA genes and the operons containing these genes have a spacer region of different length, amplification may result in a characteristic banding pattern unique to the strain in question (20).

Conventional ribotyping employs Southern blot analyses to detect RFLPs associated with the ribosomal operon (25). The drawback to this method is that it requires prolonged periods of time and a radioactive or fluorescent labeling system (21). PCR ribotyping uses DNA primers based on conserved sequences within the 16s and 23s rRNA genes to differentiate strains of bacteria (20). Random amplification is unlikely in PCR ribotyping because of primer specificity and higher annealing temperatures (20). Compared to ribotyping by Southern Blot analysis, PCR ribotyping is rapid, uses a safer detection method, has universal primers for eubacteria, and uses smaller amounts of DNA (21). Both methods of ribotyping have provided reproducible and reliable typing systems

for differentiation of numerous organisms, including *Serratia marcescens* (10), *Pseudomonas cepacia* (21), *Escherichia coli* (20), *Staphylococcus* species (32), *Enterococcus faecium* (20), *Enterobacter* species (20), *Enterococcus faecalis* (20), and *Mycobacterium tuberculosis* (35).

Enterobacterial repetitive intergenic consensus sequence

Enterobacterial repetitive intergenic consensus sequence (ERIC) PCR is based on a consensus sequence found in the Enterobacteriaceae family (31). ERIC sequences are repetitive elements that are 126 base pairs long (36) and contain a highly conserved central inverted repeat (40). ERIC sequences appear to be restricted to transcribed regions of the chromosome and their position seems to be variable in different species (36). ERIC-PCR has been used to successfully fingerprint numerous organisms, including *Serratia marcescens* (24), *Mycobacterium tuberculosis* (35), *Bartonella henselae* (34), *Salmonella enterica* (5), *Escherichia coli* (40), *Salmonella typhimurium* (40), and *Citrobacter diversus* (43).

Repetitive extragenic palindrome

Repetitive extragenic palindromic (REP) PCR is based on short extragenic repeat sequences found throughout the genome of bacteria (31). These sequences, which have a consensus 38 base pair sequence (40), appear to be highly conserved among many members of the family Enterobacteriaceae (43). Functions proposed for REP elements include roles in transcription termination, mRNA stability, and chromosomal domain organization (40). No examples of the REP sequence coding for a protein have been

reported (37). Even though the function of REP sequences are unclear, they are a significant part of the bacterial genome. REP-PCR is simple, rapid, and sensitive for discriminating between closely related strains (5). REP-PCR has been used in the typing of many organisms, including *S. marcescens* (8), *Mycobacterium tuberculosis* (22), *Bartonella henselae* (34), *Salmonella enterica* (5), *Escherichia coli* (40), *Salmonella typhimurium* (40), *Citrobacter diversus* (43), and *Bradyrhizobium* strains (41).

Polymorphic GC-rich repeat sequences

Polymorphic GC-rich repeat sequences (PGRS) are repetitive DNA elements that have been used primarily in the epidemiological typing of the *Mycobacterium tuberculosis* complex bacteria (2). This complex includes *M. tuberculosis, M. bovis, M. microti, M. gastri, M. gordonae, M. kansasii, M. szulgai,* and *M. africanum* (6, 30). PGRS elements have a 24 base pair core consensus sequence of CCGCCGTTGCCGCCGTTGCCGCCG (6). These elements are present in approximately 30 copies within the genome and are contained in GC-rich clusters of up to 1.3 kb in length (30). PGRS are thought to be related to interspersed repetitive prokaryotic elements such as REP sequences from the Enterobacteriaceae family (6).

Antimicrobial susceptibility patterns

Laboratories involved in epidemiological studies frequently perform antibiotic susceptibility tests on bacteria. Most of the testing is qualitative in terms of categorizing strains as susceptible, intermediate, or resistant (28). When determination of minimal inhibitory concentrations (MIC) is performed the method is considered quantitative.

Common approaches to antibiotic susceptibility testing include the Kirby-Bauer test (39), in which paper disks impregnated with different concentrations of antibiotics are placed in contact with bacterial lawns, or the use of antibiotic gradient plates (18).

Most strains isolated from the same hospital often share similar susceptibility patterns, resulting in poor discrimination of the isolates (13). The information acquired from these tests, however, may be helpful in the recognition of different bacterial strains (25) and in detecting early trends of elevating MIC among groups of organisms (28). Generally, determination of MIC is not suitable for epidemiological typing by itself. If used in combination with other typing methods it can further discriminate between strains (23) and be useful as an epidemiological tool (1). Antibiotic susceptibility testing is relatively inexpensive and generally adequate in most clinical settings (28).

Serratia marcescens

Bartolomeo Bizio identified *S. marcescens* in 1819, in which it appeared as blood in cornmeal mush (1). It was first thought to be a fungus, but later classified as a bacterium. *S. marcescens* is a gram negative rod shaped bacterium that belongs to the family Enterobacteriaceae. It is often associated with its characteristic red pigment, which has made it a part of history and religious folklore for appearing as blood. It was observed mainly on breads and other starchy foods as early as sixth century B.C (44). Until 1951, *S. marcescens* was considered benign (1) and the pigmented organism was used as a biological marker (16). Studies involving *S. marcescens* included drifting and settling experiments in hospitals and to show that respiratory tract infections could be transmitted by hand. Additionally, other uses of this organism included studies in

classrooms to demonstrate the dispersal of bacteria, aerosolization experiments to demonstrate germ warfare techniques, and to show how talking, coughing, and sneezing projects bacteria into the air (44).

In 1951 the first case of a nosocomial infection caused by *S. marcescens* was reported (16). Today, *S. marcescens* is commonly associated with hospital acquired infections, especially affecting immunocompromised and surgical patients (13). Specific examples of *S. marcescens* infections include urinary tract infections, respiratory infections, endocarditis, burn infections, septicemia, and pneumonia (1). This organism is ubiquitous in nature and is different from the other members of the family Enterobacteriaceae as it produces the extracellular enzymes DNase, gelatinase, and lipase (1).

The objectives of this study were to generate DNA fingerprints for 62 clinical *S. marcescens* isolates and to compare different PCR typing techniques for use in genotypic analysis. Also, antimicrobial susceptibility patterns for each isolate were generated. Antimicrobial susceptibility patterns were accomplished using a gradient plate technique with various antibiotics. The PCR typing methods used included RAPD (31), REP (34), ribotyping (20), ERIC (34), and PGRS (12).

Methods and Materials

Bacterial species and growth conditions

Sixty-two *Serratia marcescens* isolates were obtained from Dr. Sue Katz, Department of Basic Sciences, Midwestern University, Glendale, Arizona. The organisms were routinely propagated on Luria-Bertani (LB) agar media at 37°C and stored at 4°C. All chemicals were obtained from either Fisher Scientific (St. Louis, MO) or Sigma (St. Louis, MO) unless otherwise noted.

Chromosomal DNA isolation

Chromosomal DNA was isolated from each *S. marcescens* strain using a modification of an established procedure (33). Briefly, *S. marcescens* isolates were inoculated individually into 6 ml of brain heart infusion (BHI) broth contained in 20 ml test tubes and incubated for approximately 14 h at 37°C with shaking at 250 rpm in a New Brunswick Scientific Series 25-incubator shaker (Edison, NJ). Cultures were transferred to 15 ml conical tubes and cells isolated by centrifugation at 4000 rpm for 5 min in a Sorvall GLC-1 tabletop centrifuge (Newton, CT). The supernatant was discarded and cells resuspended in 2 ml of tris-ethylenediaminetriacetate (TE) buffer. Cell lysis was accomplished by adding 100 µl of 20% sodium dodecyl sulfate (SDS) to a final concentration of 1%. Following mixing by inversion, the solution was incubated at 60°C for 15 min. To degrade RNA, 20 µl of 10 mg/ml RNase (final concentration 1 µg/ml) was added and the solution incubated at 37°C for 20 min. Subsequently, 15 µl of 10 mg/ml proteinase K (final concentration 14 µg/ml) was added and the solution

incubated at 60°C for 1 h to degrade protein. To remove additional contaminating proteins, two phenol extractions were performed by adding 2 ml of saturated phenol (pH 8) to the solution. After mixing by inversion for approximately 2 min, the solution was centrifuged for 5 min and the top (aqueous) layer removed. A second phenol extraction was performed as described. Following the final phenol extraction, 2 ml of chloroform/isoamyl alcohol (24:1) was added and extraction performed as described above for the phenol extractions. DNA contained in the aqueous phase was transferred to a clean 15 ml conical tube and 10 ml of 95% ethanol was added. The resulting solution was mixed by inversion and precipitated DNA removed with a glass Pasteur pipet and placed in clean 1.5 ml microcentrifuge tube. The DNA was dried using a Savant Iss110 Integrated Speed Vac System (Holbrook, NY) and resuspended in 200 µl TE. DNA samples were stored at 4°C.

Quantification of chromosomal DNA

Quantification of chromosomal DNA was determined using a Beckman DU530 spectrophotometer (Fullerton, CA). The DNA was diluted 100 fold by mixing 990 µl sterilized water and 10 µl DNA in a Fisherbrand quartz cuvette. Absorbance was measured at 260 nm and 280 nm. The quantity of DNA was determined by the following equation: (260 nm reading)(dilution factor)(50 µg/ml) = µg/ml chromosomal DNA. The 260 nm/280 nm ratio represented the DNA purity.

Polymerase chain reaction

Chromosomal DNA from *S. marcescens* isolates was amplified using the polymerase chain reaction (PCR) on a MJ Research Minicycler (Watertown, Mass). The PCR techniques used were: randomly amplified polymorphic DNA (RAPD) (31), repetitive extragenic palindromic (REP) elements (34), enterobacterial repetitive intergenic consensus (ERIC) sequences (34), polymorphic GC-rich repetitive sequence (PGRS) (12), and ribotyping (20). Primers and amplification conditions used are listed in Table 1. Typical reaction mixtures consisted of 500 ng DNA, 200 μM deoxynucleotide triphosphates (dNTPs), 1.5 μM $MgCl_2$, 0.5 μl Taq polymerase, and 50 nM of each primer in buffer containing 50 mM TrisCl, 50 mM KCl, and 0.01% Triton-X100 in a final volume of 100 μl. The PGRS reaction mixture was similar as above except 1.75 mM $MgCl_2$ was used.

Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate PCR amplification products and to obtain DNA fingerprints of the *S. marcescens* isolates. Preparation of agarose gels and electrophoretic conditions used are listed in Table 2. The electrophoretic chambers included a Minicell EC370M and Maxicell EC360M (Fisher) powered by a Bio-RAD model 250/2.5 power supply (Bio-RAD; Hercules, CA). Agarose gels were visualized using a UV Intensity Transilluminator (Fisher) and documented with Panasonic CCD Ultra Lum camera and scion image software (Ultra Lum; Paramount, CA).

Generation of dendrograms

Agarose gels were analyzed visually and scored using a binary code. Data were entered into Microsoft EXCEL and SPSS Windows release 9.0 standard version was used to analyze the binary code and generate dendrograms based on the euclidean distance coefficient and unweighted pair group method average (UPGMA).

Antibiotic resistance profiles

Antibiotic gradient plates were made by combining 10 ml of BHI agar and the appropriate amount of antibiotic (concentrations variable) into a 50 ml conical tube. After mixing by inversion, the solution was poured into a Petri dish tilted on a 5 mm thick glass rod. After solidification, the Petri dish was laid flat and another 10 ml of BHI agar, without antibiotic, was poured on the agar and allowed to solidify. Antibiotics used were ampicillin, gentamicin, tetracycline, and chloramphenicol. To determine antibiotic resistance profiles, bacterial isolates were streaked in a straight line 70 mm across the dish from low concentration to high concentration with 5 different isolates per Petri dish (Fig. 1). Following incubation at 37°C for 18 h, bacterial growth was measured until individual colonies were more than 3 mm apart or growth was not apparent on the media. The concentration at which each isolate was susceptible was calculated with the following formula:

(antibiotic concentration (μg/ml)/ 70 mm) X measured bacterial growth (mm)

Following the calculations, isolates were grouped according to their antibiotic susceptibility. The grouping parameters are shown in Table 3.

TABLE 1. PCR primers and reaction conditions

Reaction	Primer	Amplification conditions[a]
1283	5'GCGATCCCCA3'	94°C 1 min 39°C 1 min 72°C 2 min
1254	5'CCGCAGCCAA3'	94°C 1 min 39°C 1 min 72°C 2 min
1254/1283	5'CCGCAGCCAA3' 5'GCGATCCCCA3'	94°C 1 min 37°C 1 min 72°C 2 min
1060	5'CCCGGGATAA3'	94°C 1 min 39°C 1 min 72°C 2 min
ERIC	5'GTGAATCCCCAGCAGCTTACAT3'	94°C 1 min 52°C 1 min 72°C 1 min
REP[b]	5'REP1R-Dt: 5'NCGNCGNCATCNGGC3' 5'REP 2D: 5'RCGYCTTATCMGGCCTAC3'	94°C 1 min 52°C 1 min 72°C 1 min
GC-rich	5'CCGCCGTTGCCGCCGTTGCCGCCG3'	94°C 1 min 55°C 1 min 72°C 1 min
Ribotyping	23s: 5'GGTACCTTAGATGTTTCAGTTC3' 16s: 5'TTGTACACACCGCCCGTCA3'	94°C 1 min 55°C 1 min 72°C 1 min

[a]Conditions listed represent one cycle. Thirty cycles were used. All PCR conditions included and initial 94°C for 5 min and ERIC, ribotyping, REP, and GC-rich contained a final 72°C for 5 min.

[b]N = A,C,G, and T; M = A and C; R = A and G; Y = C and T

TABLE 2. Agarose gel sizes, ingredients, and electrophoretic conditions

Gel size	Ingredients	Conditions
12 wells	0.21 g agarose	98 V
	30 ml TAE[a]	45 min
	1 µl ethidium bromide	250 ml TAE[a] (running buffer)
60 wells	3 g agarose	120 V
	200 ml TAE[a]	1.5 h
	6 µl ethidium bromide	1.75 L TAE[a] (running buffer)

[a]1X TAE prepared from a 50X stock (242 g tris, 57.1 ml acetic acid, 4 ml 0.5 M EDTA/1 L)

FIG. 1. Antibiotic gradient plate. Brain heart infusion agar supplemented with the appropriate antibiotic was allowed to solidify as indicated in the methods and materials. Plates were inoculated with five different *S. marcescens* isolates 70 mm across the plate from low concentration to high concentration. After incubation at 37°C for 16 h, plates were examined for growth. Estimation of antibiotic resistance levels was made according to the calculation in the materials and methods.

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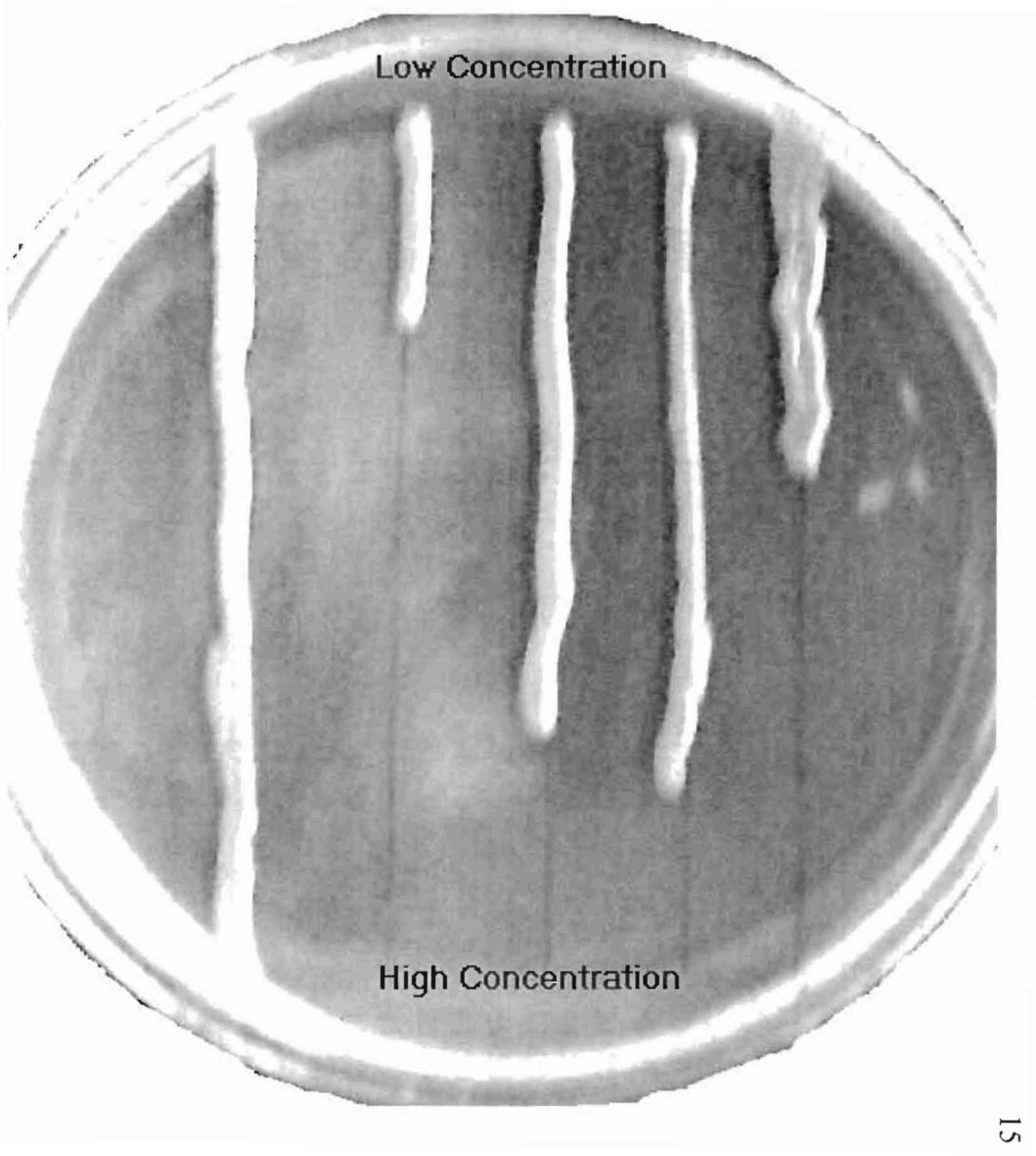

Low Concentration
High Concentration

Table 3. Antimicrobial susceptibility groupings[a]

Group #	Ampicillin Concentration	Gentamicin Concentration	Chloramphenicol Concentration	Tetracycline Concentration
1	91-100	0-10	0-10	71-80
2	91-100	0-10	21-30	71-80
3	31-40	0-10	11-20	21-30
4	91-100	0-10	11-20	0-10
5	21-30	0-10	11-20	0-10
6	91-100	0-10	11-20	51-60
7	41-50	0-10	21-30	41-50
8	61-70	0-10	11-20	11-20
9	91-100	0-10	21-30	41-50
10	91-100	0-10	0-10	41-50
11	91-100	0-10	11-20	21-30
12	91-100	0-10	11-20	11-20
13	31-40	0-10	0-10	11-20
14	0-10	0-10	21-30	11-20
15	91-100	0-10	21-30	21-30
16	31-40	0-10	11-20	0-10
17	91-100	0-10	11-20	31-40
18	91-100	11-20	11-20	11-20
19	41-50	0-10	11-20	31-40
20	91-100	0-10	21-30	31-40
21	51-60	11-20	11-20	11-20
22	91-100	0-10	0-10	21-30
23	71-80	0-10	0-10	11-20
24	91-100	0-10	0-10	31-40
25	91-100	0-10	0-10	0-10
26	51-60	0-10	0-10	21-30
27	41-50	0-10	11-20	21-30
28	71-80	0-10	0-10	31-40
29	41-50	11-20	21-30	0-10
30	11-20	0-10	0-10	11-20
31	11-20	0-10	11-20	21-30
32	71-80	0-10	11-20	21-30
33	91-100	11-20	11-20	31-40
34	0-10	0-10	0-10	0-10
35	61-70	0-10	11-20	41-50
36	91-100	0-10	21-30	51-60
37	91-100	11-20	0-10	41-50

[a]Antibiotic concentrations are in μg/ml.

Results

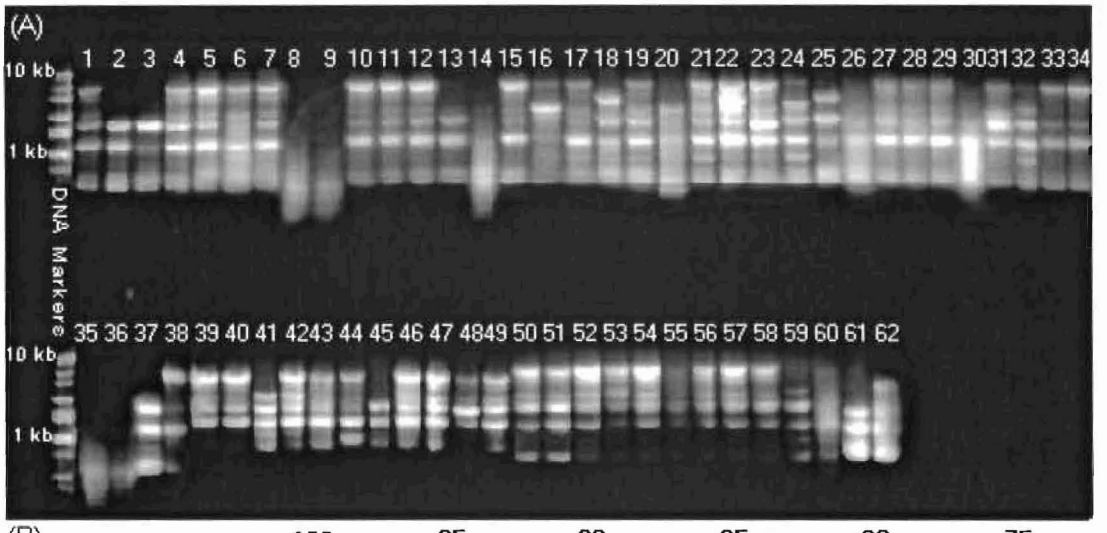
Chromosomal DNA isolation

Chromosomal DNA from 62 *S. marcescens* clinical isolates was successfully isolated. The concentration and purity of the DNA was determined with spectrophotometry by measuring the absorbance at 260 nm and 280 nm. Each DNA sample was standardized to 500 ng/μl for use in PCR.

Characterization using RAPD

Randomly amplified polymorphic DNA analysis was performed on the 62 *S. marcescens* isolates using three arbitrary primers designated 1254, 1283, and 1060 (Table 1). Primer 1254 generated 23 different sized bands as determined by agarose gel electrophoresis (Fig. 2A), as compared to primer 1283, which generated 38 different sized bands (Fig. 3A). Isolate number 43 was unable to be characterized with primer 1283. Dendrogram profiles were generated for primers 1283 and 1254 (Fig. 2B and Fig. 3B). The dendrograms revealed 40 genotypic groupings with primer 1254 and 58 groupings for primer 1283. When primers 1254 and 1283 were used in combination, they generated 25 different sized bands (Fig. 4A) and the dendrogram yielded 43 groups (Fig. 4B). Agarose gel electrophoresis revealed 19 different sized DNA bands from primer 1060 (Fig. 5A) and the dendrogram profile revealed 40 genotypic groupings (Fig. 5B).

Fig. 2. Characterization of *S. marcescens* by RAPD using primer 1254. (A) 1.5% agarose gel of amplified DNA products. DNA markers are labeled and lane numbers represent isolate numbers 1-34 and 35-62 which contain the amplified products from the 62 isolates. Agarose gel electrophoresis revealed 23 different sized bands. (B) Dendrogram analysis of the RAPD data . Forty genotypic groupings were revealed.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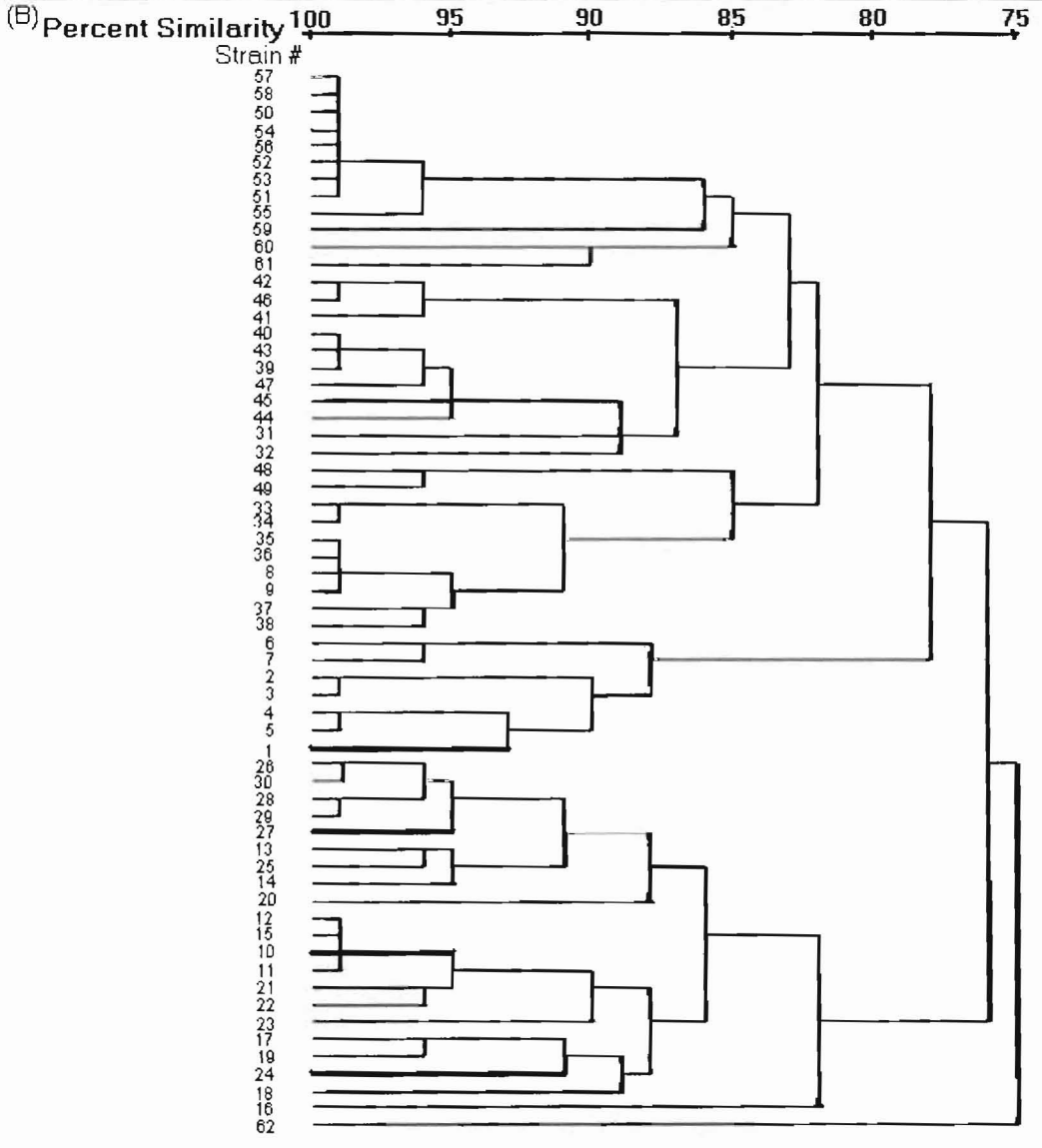
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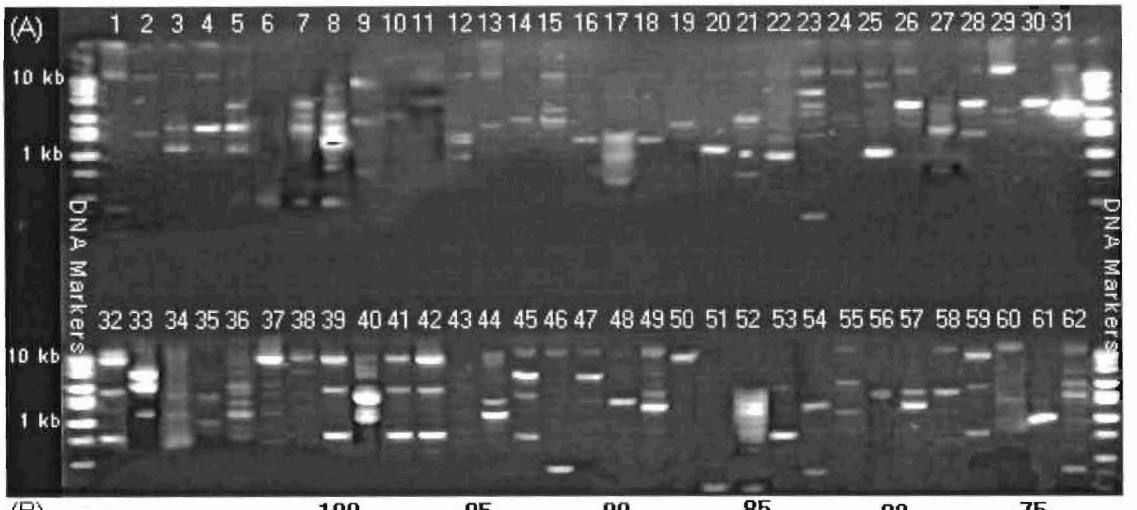
Fig. 3. Characterization of *S. marcescens* by RAPD using primer 1283. (A) 1.5% agarose gel of amplified RAPD products. DNA markers are labeled and lane numbers represent isolate numbers 1-31 and 32-62 which contain the amplified DNA products from the 62 isolates. Agarose gel electrophoresis revealed 38 different sized bands. (B) Dendrogram analysis of the RAPD data. Fifty-eight genotypic groupings were revealed.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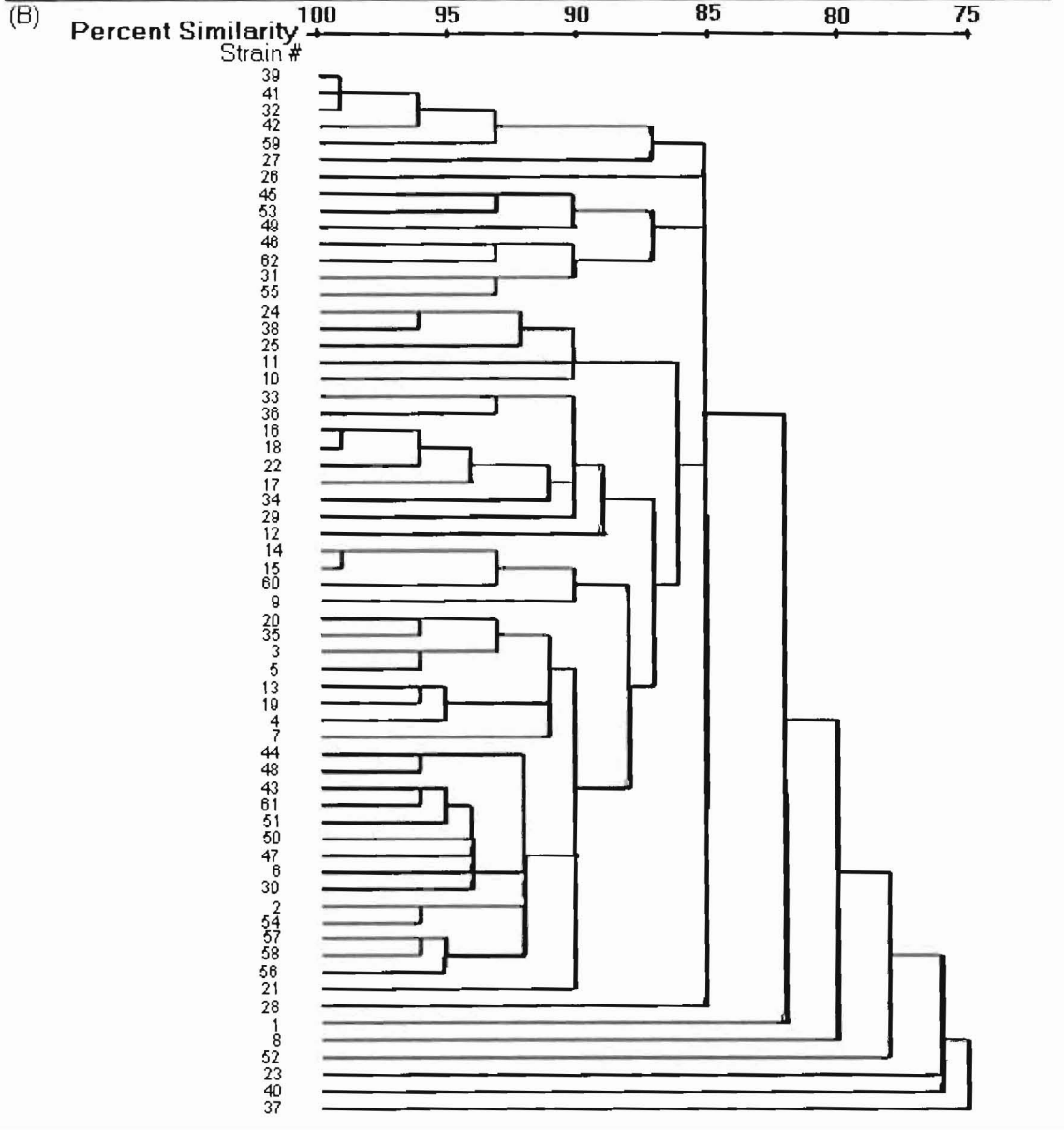
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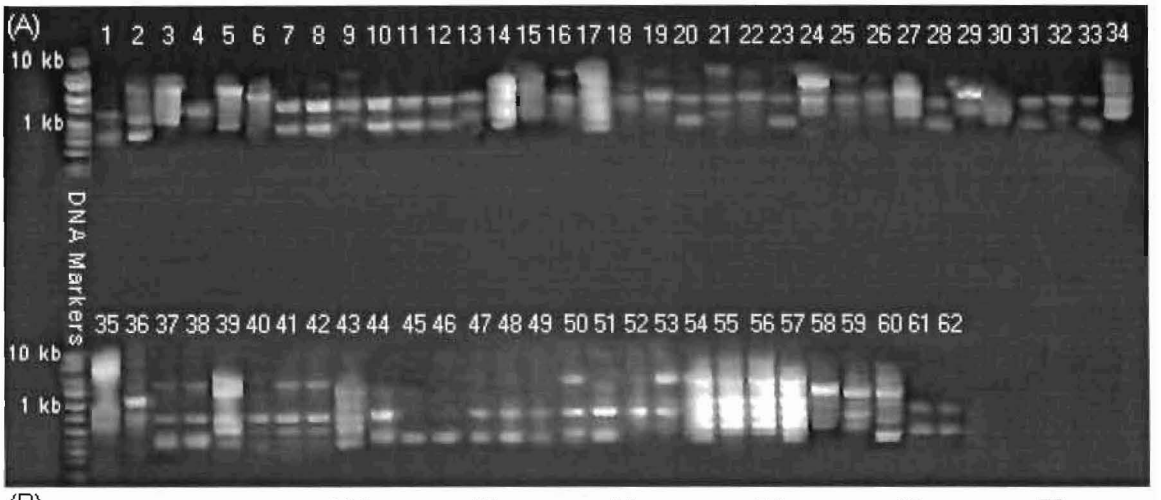
Fig. 4. Characterization of *S. marcescens* by RAPD using primers 1254 and 1283. (A) 1.5% agarose gel of amplified RAPD products. DNA markers are labeled and lane numbers represent isolate numbers 1-34 and 35-62 which contain the amplified DNA products from the 62 isolates. Agarose gel electrophoresis revealed 25 different sized bands. (B) Dendrogram of the RAPD data. Forty-three genotypic groupings were generated.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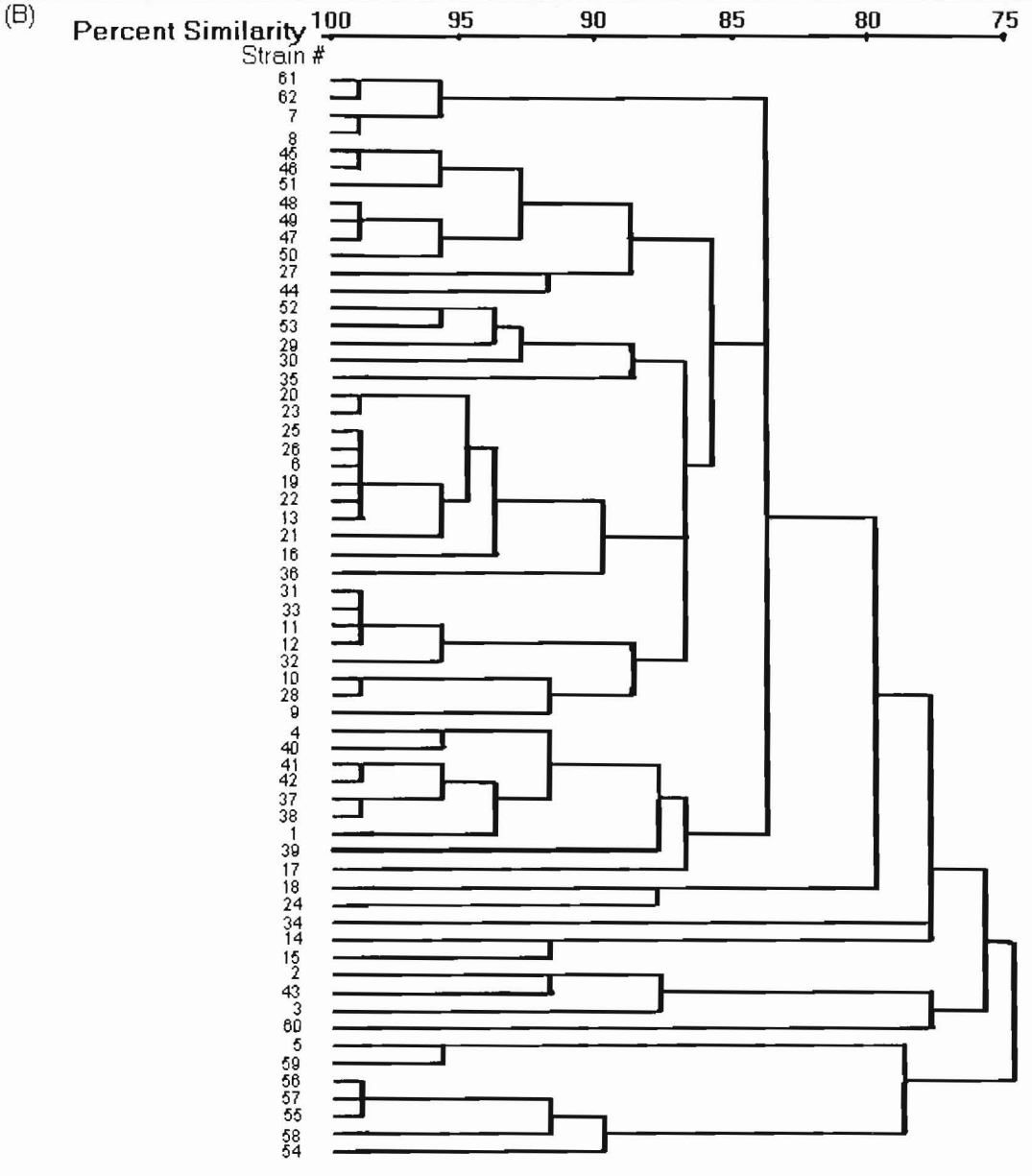
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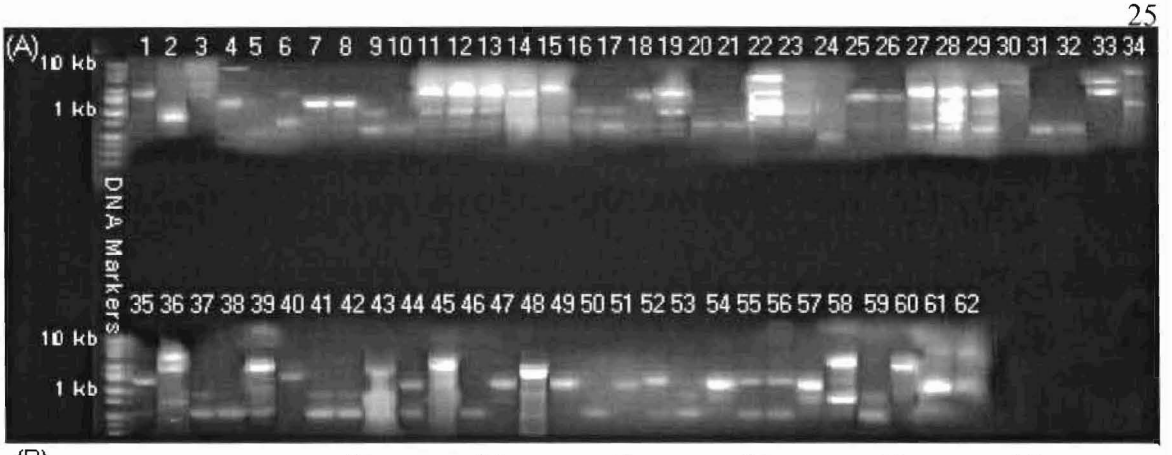
Fig. 5. Characterization of *S. marcescens* by RAPD using primer 1060. (A) 1.5% agarose gel of amplified RAPD products. DNA markers are labeled and lane numbers represent isolate numbers 1-34 and 35-62 which contain the amplified DNA products from the 62 isolates.Agarose gel electrophoresis revealed 19 different sized bands. (B) Dendrogram of the RAPD data. Forty genotypic groupings were generated.


(A)
10 kb
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DNA Markers
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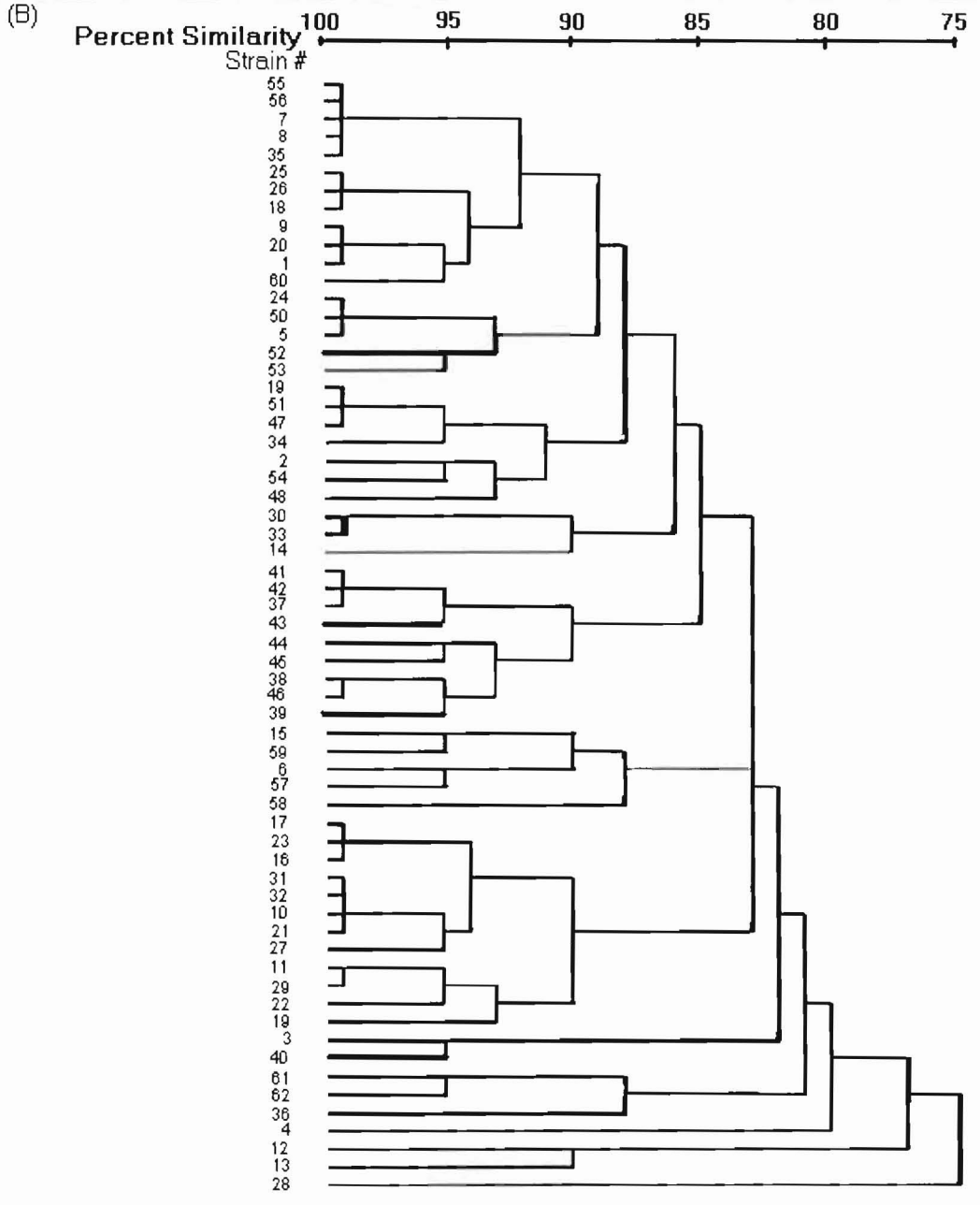
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Characterization using ERIC and REP

Primers based on ERIC and REP PCR were designed to amplify these random repetitive sequences (Table 1). Using the ERIC primer, 12 unique bands were generated with a common band being amplified at approximately 250 bp (Fig. 6A). Dendrogram analysis of these data revealed 19 genotypic groupings (Fig. 6B). REP primers generated 39 unique amplicons (Fig. 7A). Upon dendrogram analysis, 54 genotypic groupings were revealed (Fig. 7B). These data suggest REP-PCR is much more discriminatory than ERIC-PCR.

Characterization using ribotyping

Primers used to target rRNA gene sequences of *E. coli* were used in PCR ribotyping of the 62 *S. marcescens* isolates (Table 1). Analysis of amplification patterns by agarose gel electrophoresis yielded 19 unique bands based (Fig. 8A). A common 850 bp band was amplified in all isolates. Dendrogram analysis revealed 40 genotypic groupings (Fig. 8B).

Characterization using PGRS

PGRS is designed to target repetitive DNA elements found in the *Mycobacterium tuberculosis* complex bacteria (12). Using PGRS primers (Table 1) 49 unique bands were amplified (Fig. 9A) resulting in 60 genotypic groupings (Fig. 9B).

Fig. 6. Characterization of *S. marcescens* by ERIC. (A) 1.5% agarose gel of amplified ERIC products. DNA markers are labeled and lane numbers represent isolate numbers 1-31 and 32-62 which contain the amplified DNA products from the 62 isolates.Agarose gel electrophoresis amplified 12 unique bands. (B) Dendrogram of ERIC data. Nineteen genotypic groupings were generated.


(A)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31
10 kb
1 kb
DNA Markers
32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62
10 kb
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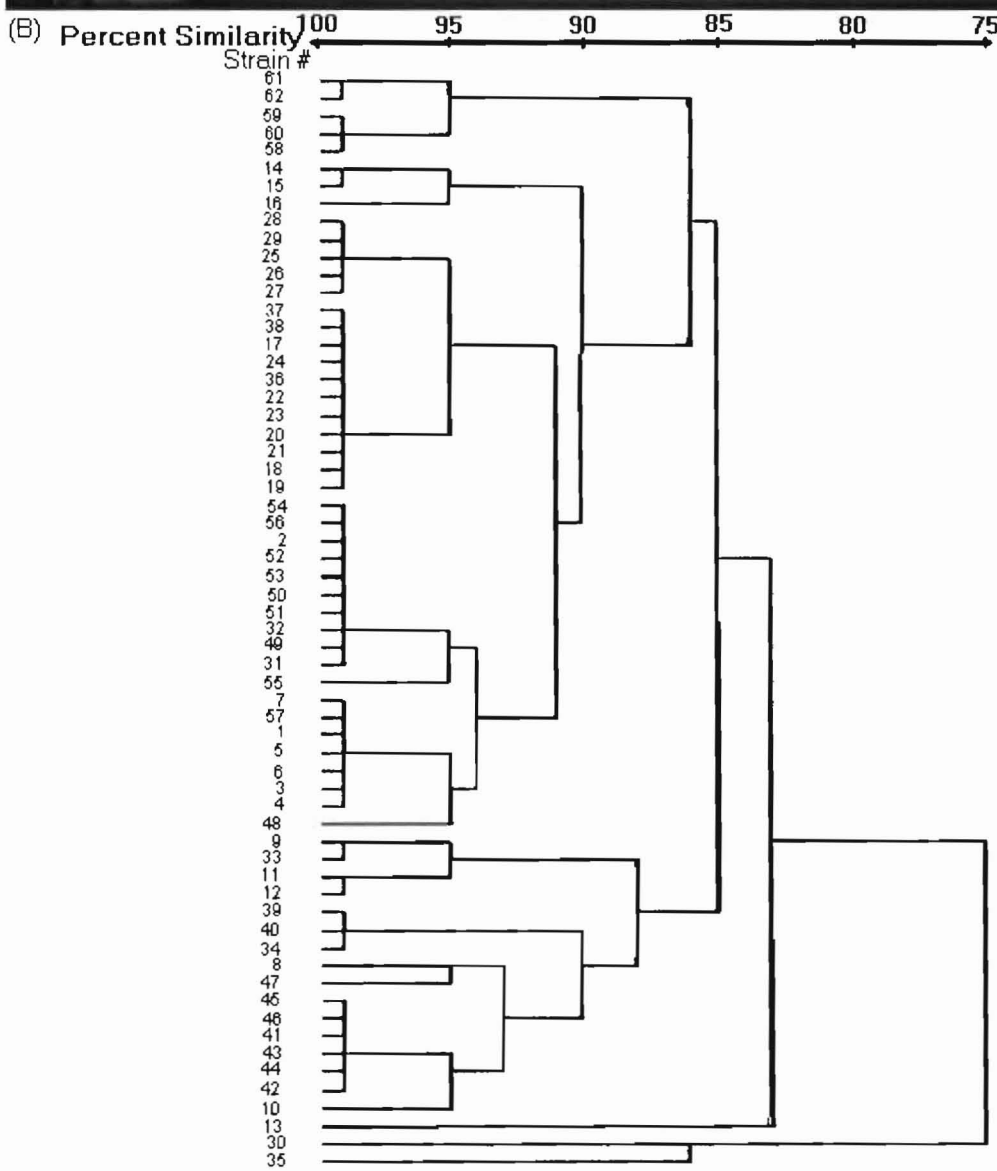
(B) Percent Similarity
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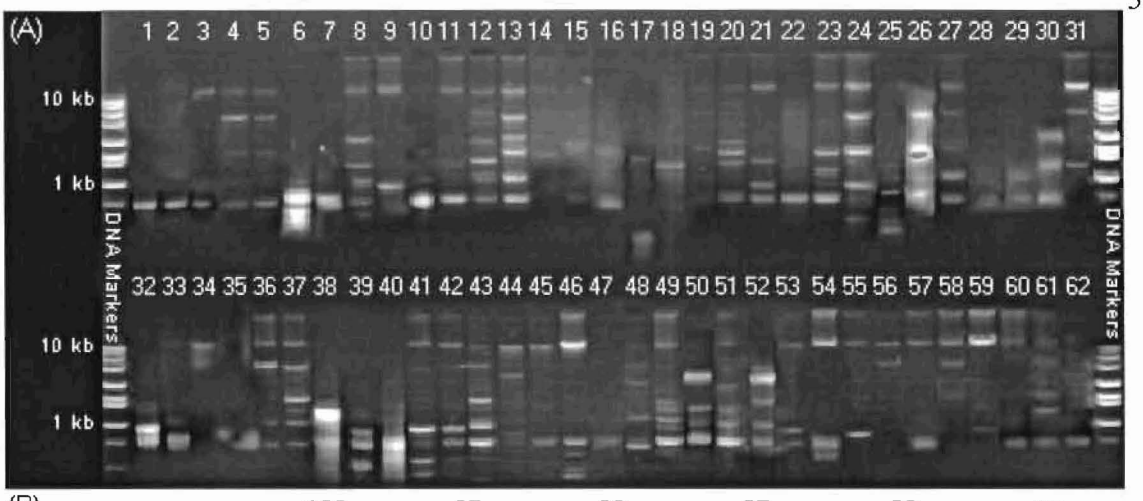
Fig. 7. Characterization of *S. marcescens* by REP. (A) 1.5% agarose gel of amplified REP products. DNA markers are labeled and lane numbers represent isolate numbers 1-31 and 32-62 which contain the amplified DNA products from the 62 isolates. Agarose gel electrophoresis yielded 39 different sized bands. (B) Dendrogram of REP data. Fifty-four genotypic patterns were generated.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DNA Markers

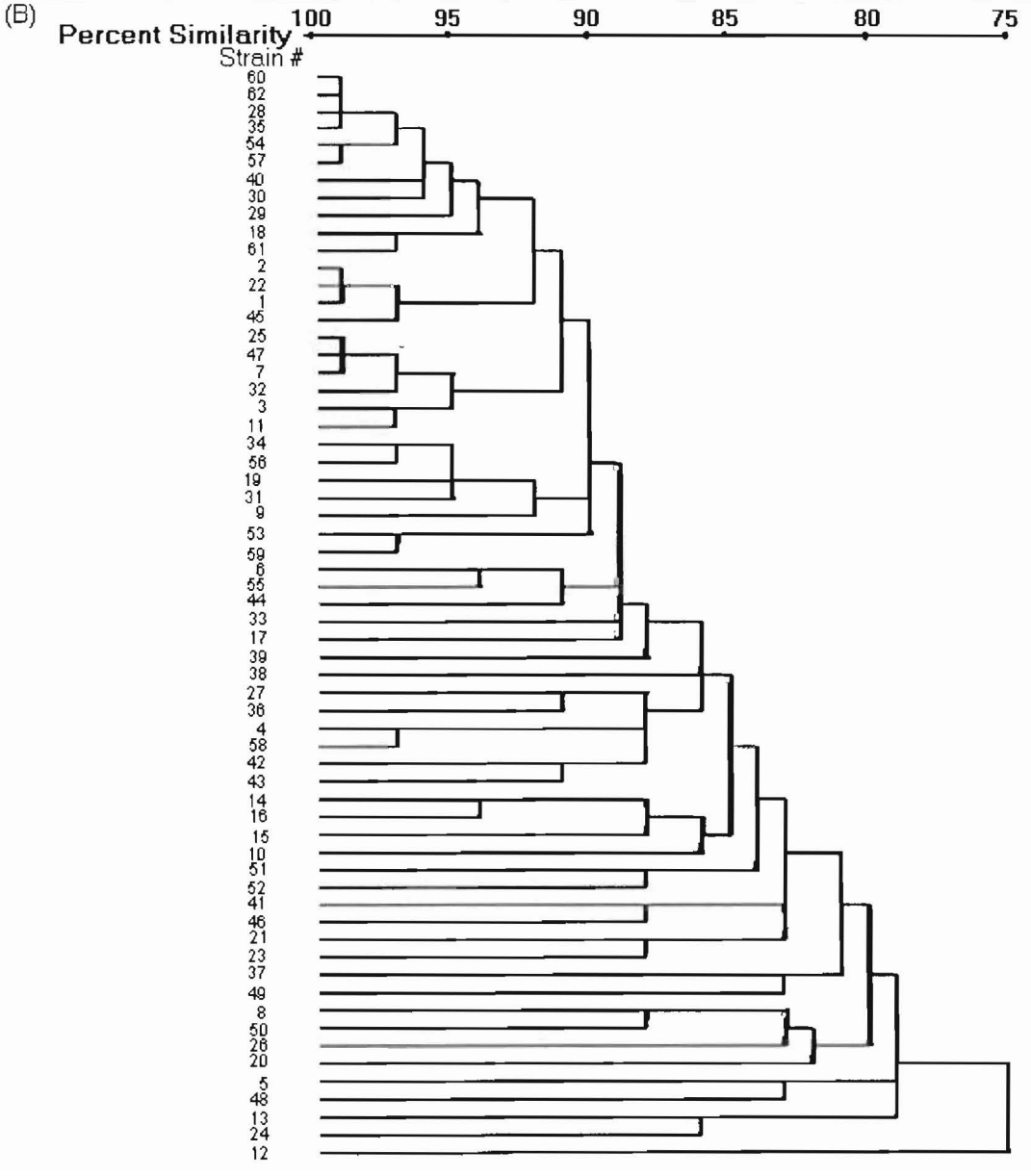
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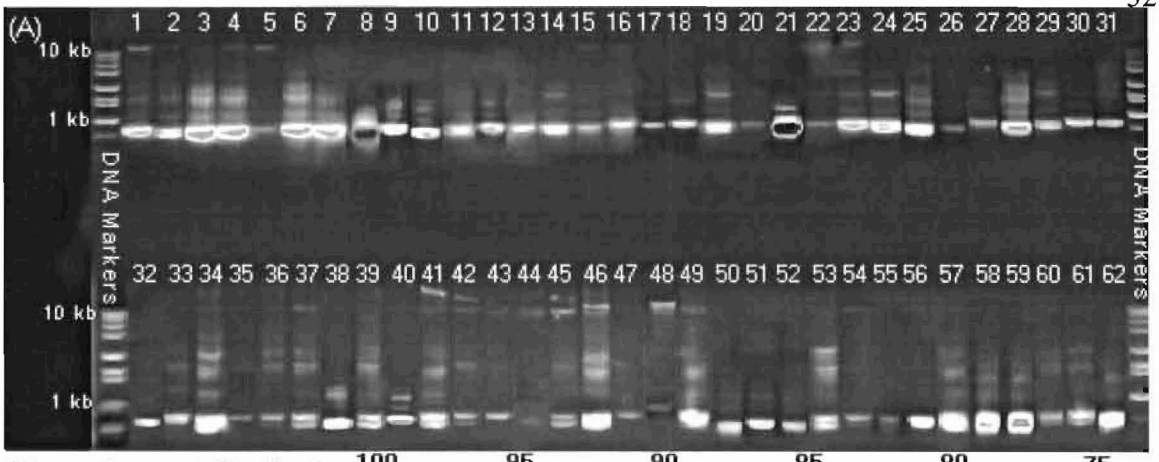
Fig. 8. Characterization of *S. marcescens* by ribotyping. (A) 1.5% agarose gel of amplified ribotyping products. DNA markers are labeled and lane numbers represent isolate numbers 1-31 and 32-62 which contain the amplified DNA products from the 62 isolates. Agarose gel electrophoresis yielded 19 different sized bands. (B) Dendrogram of ribotyping data. Forty genotypic patterns were generated.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DNA Markers

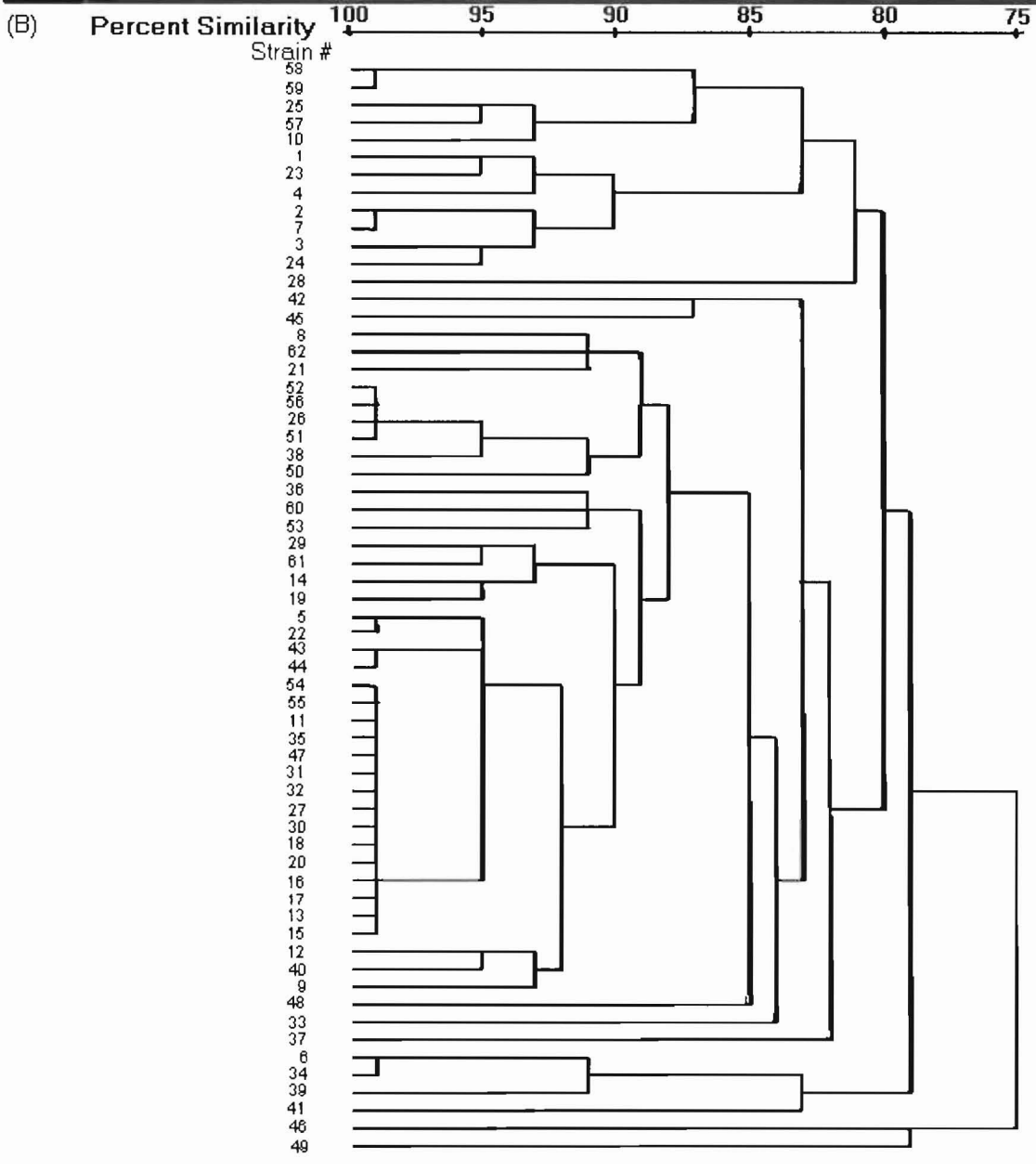
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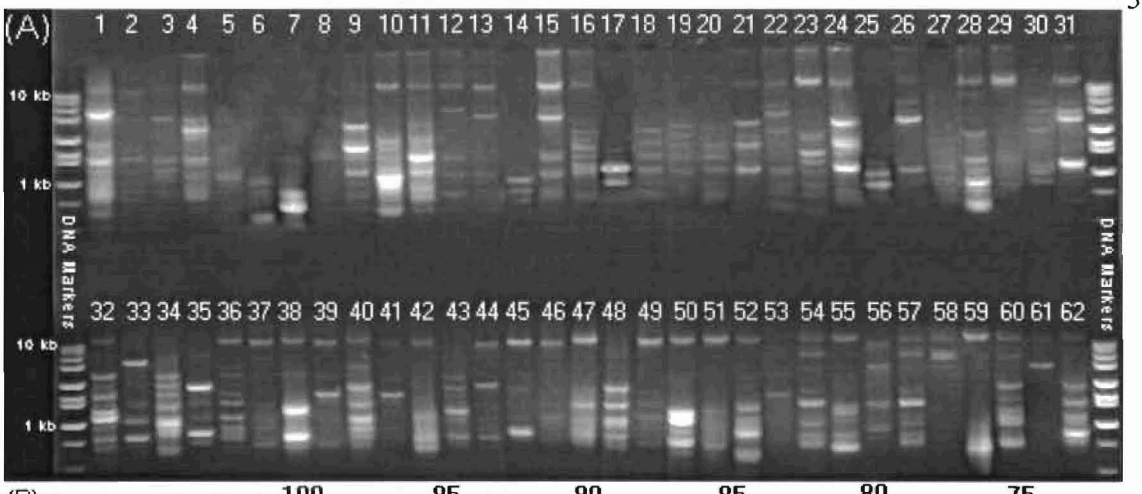
Fig. 9. Characterization of *S. marcescens* by PGRS. (A) 1.5% agarose gel of amplified PGRS products. DNA markers are labeled and lane numbers represent isolate numbers 1-31 and 32-62 which contain the amplified DNA products from the 62 isolates. Agarose gel electrophoresis revealed 49 different sized bands. (B) Dendrogram of PGRS data. Sixty genotypic patterns were generated.


(A)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31
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DNA Markers
32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62
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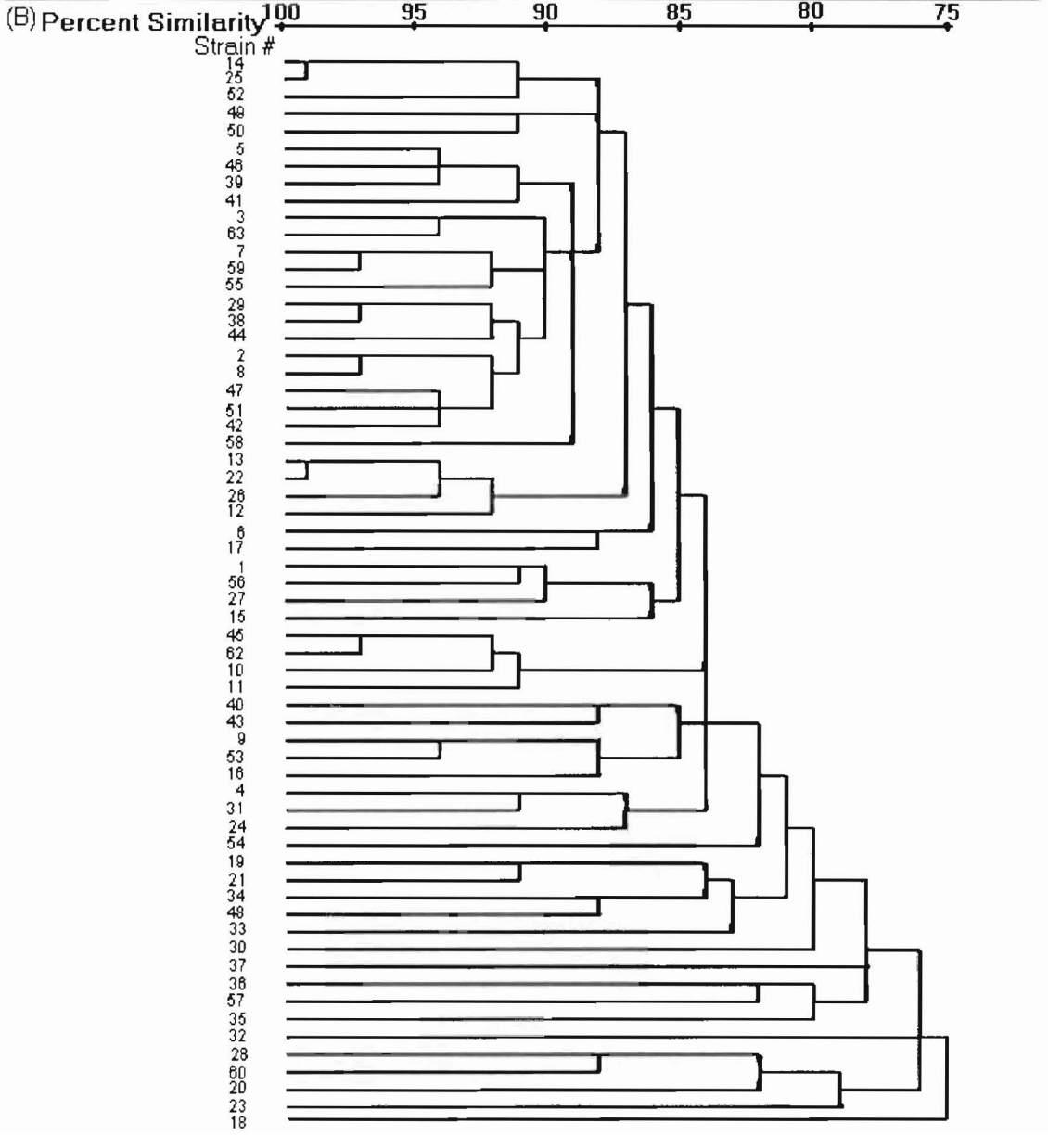
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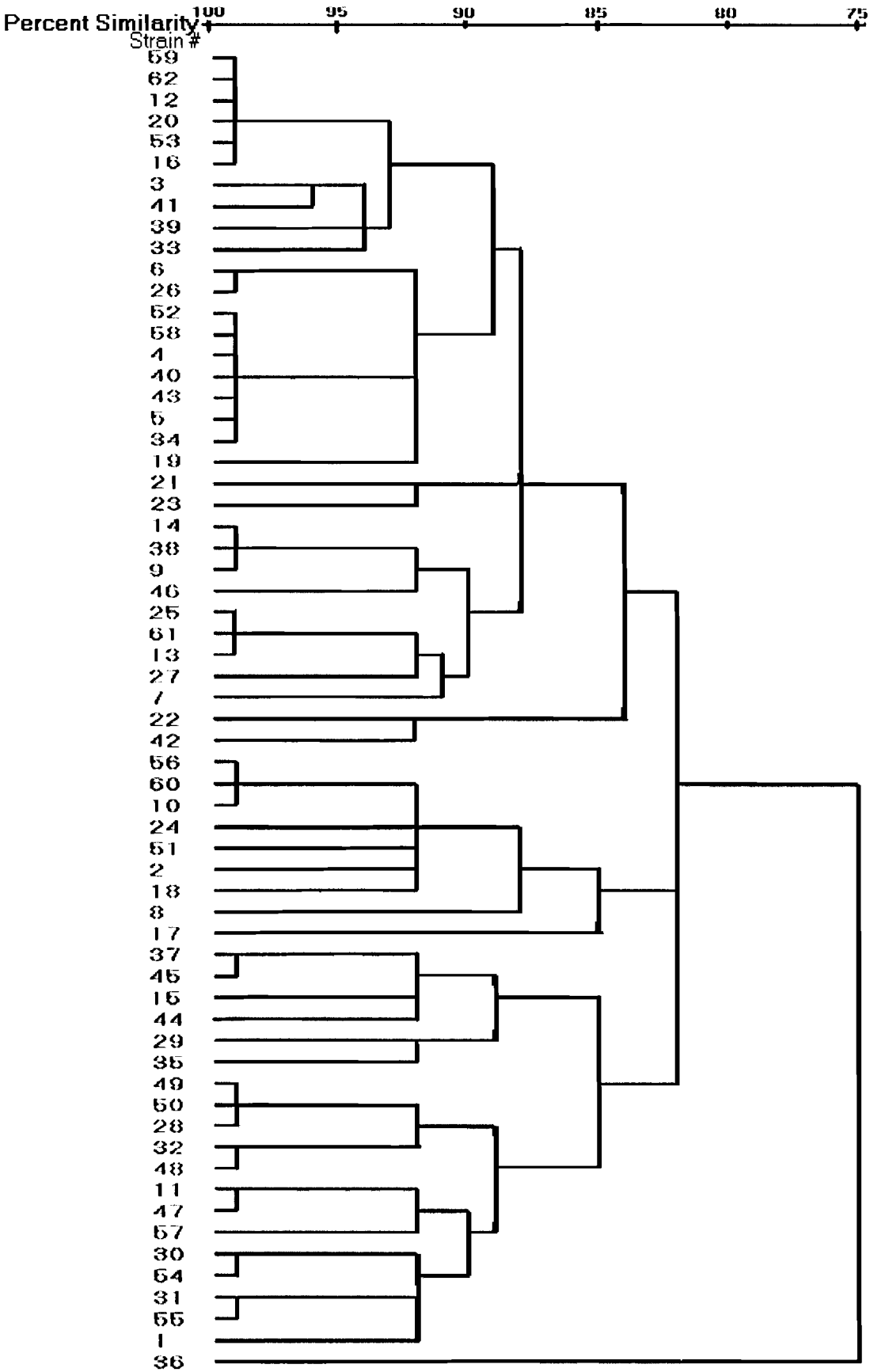
Characterization using antimicrobial susceptibility patterns

Antimicrobial susceptibility to gentamicin, chloramphenicol, tetracycline, and ampicillin was determined using antibiotic gradient plates for the 62 *S. marcescens* isolates as illustrated in Figure 1. Table 3 illustrates the grouping parameters used to generate the dendrogram profile showing 37 phenotypic groupings (Fig. 10). The two most common patterns observed were group number four, which contained seven isolates and group number eleven, which contained six isolates.

Fig. 10. Characterization of *S. marcescens* by antimicrobial susceptibility patterns. Data for dendrogram were obtained as indicated in the Materials and Methods. Thirty-seven phenotypic groupings were generated (Table 1).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Discussion

The theory behind epidemiological typing schemes is that strains in one epidemiological cluster came from a common precursor and these strains will share certain characteristics that can differentiate them from other epidemiologically unrelated strains of the same species (31). There are several different typing methods used in epidemiological studies, two of which include PCR based typing methods and antimicrobial susceptibility patterns. PCR typing methods are genotypic techniques and are fairly new. PCR methods have improved the ability to study fastidious pathogens and pathogens that cannot be cultivated, allowing for such pathogens to undergo epidemiological studies (25). Antimicrobial susceptibility patterns are phenotypic typing methods and have been current for sometime. This type of analysis is routinely performed in most microbiology laboratories (25).

Serratia marcescens is a known causative agent of nosocomial infections and therefore, it is essential to perform epidemiological studies to prevent future outbreaks (4). To control nosocomial infections, it is important to determine if the strain is epidemic or sporadic and to identify a possible source of the infection (13). The purpose of this research was to differentiate 62 clinical *S. marcescens* isolates by PCR-fingerprinting and compare different PCR typing techniques. Additionally, antimicrobial susceptibility patterns were generated for these isolates and data obtained were compared to PCR typing techniques. To demonstrate the discriminatory power of each technique, an isolate was traced through each dendrogram for each technique presented. Table 4 summarizes these findings.

Table 4. Percent similarity of the isolates to isolate number 43

PCR	100% similarity	95% similarity	90-87% similarity
RAPD 1254	40, 39	47, 45, 44	31,32
RAPD 1283		51	20, 35, 3, 5, 13, 19, 4, 7, 44, 48, 61, 51, 50, 47, 6, 30, 2, 54, 57, 58, 56, 21
RAPD 1254 & 1283			2, 3
RAPD 1060		41, 42, 37	44, 45, 38, 46, 39
ERIC	45, 46, 41, 44, 42	10	39, 40, 34, 8, 47
REP			4, 58
Ribotyping	44	54, 55, 11, 35, 47, 31, 32, 27, 30, 18, 20, 16, 17, 13, 15, 5, 22	29, 61, 14, 19, 12, 40, 9
PGRS			40

RAPDs are straightforward, do not require previous knowledge of the DNA in question, quick, relatively inexpensive, and convenient to perform (17). For these reasons, this method is a good typing technique for use in epidemiological studies. However, this procedure has been questioned in terms of its reproducibility, but appears not to be a problem if the technique is standardized. Standardization protocols include using consistent volumes and concentrations of reagents, using the same thermal cycler, and standardizing procedures for visualization of fingerprints (31).

RAPD analysis was used in this research with three arbitrary primers designated 1254, 1283, and 1060. The primers 1254 and 1283 were used in a previous study involving RAPDs on *S. marcescens* (17). In this study, the authors found RAPDs had high discriminatory power for typing this organism and reproducibility did not appear to be a problem (17). In contrast, the 62 *S. marcescens* isolates examined in this study were differentiated the best with primer 1283. Strain specific differences among the isolates likely account for the observed differences between this study and the previous study (17). Interestingly, primer 1283 did not amplify DNA in isolate number 43, possibly due to sequence divergence of the chromosomal DNA corresponding to the 3' end of the primer. The possibility of contaminated DNA from isolate number 43 leading to no amplification can also be ruled out since the DNA amplified with other primers. Tracing isolate 43 through the dendrogram profiles of the other RAPDs demonstrated it is 100% similar to isolates 40 and 39 using primer 1254. With primer 1060, isolate 43 was 95% similar to isolates 41, 42, and 47. When primers 1254 and 1283 were used in combination, isolate 43 was 95% similar to isolate 4 and 88% similar to isolate 40.

REP and ERIC-PCR are widely used typing methods to differentiate isolates among the family Enterobacteriaceae because they target specific repetitive elements, which are highly conserved within this family (40). REP and ERIC provide PCR-fingerprints using relatively high annealing temperatures similar to standard PCR reactions to exclude random binding of primers (31).

A previous study using ERIC-PCR differentiated 17 groupings from a total of 22 *S. marcescens* isolates sampled from the same hospital (24). Thus, this typing technique demonstrated high discriminatory power. In contrast, this investigation demonstrated ERIC-PCR discriminated the *S. marcescens* isolates poorly. Poor discrimination by ERIC-PCR demonstrates the principle that primer selection is an important aspect in PCR based typing techniques. With ERIC-PCR, isolate 43 was 100% similar to isolates 42, 44, 41, 46, and 45.

REP-PCR did successfully differentiate the 62 *S. marcescens* isolates. A previous study also demonstrated that REP-PCR had high discriminatory power (8). In this study five *S. marcescens* isolates were used to verify that three of these isolates were related. Following isolate 43 through the dendrogram profile with REP-PCR, it was demonstrated that it was 91% similar to isolate 42. Compared to ERIC-PCR, these 62 *S. marcescens* isolates REP-PCR were better differentiated.

PCR ribotyping targets the 16s and 23s intergenic spacer region of the bacterial rRNA genes (20). PCR-ribotyping is possible because the ribosomal genes are highly conserved for all bacteria (25). Amplification occurs with specific primers using high annealing temperatures which minimizes the chances for random amplification (20). PCR-ribotyping generally involves digestion of the DNA with restriction enzymes prior

to PCR (24). PCR-ribotyping in this manner has the advantage of easier interpretation because total DNA is not being amplified (24). Drawbacks, however, include an extra step added to the whole process and potentially more bands to be analyzed (20). Both approaches have shown to successfully discriminate various organisms (20). For example, PCR-ribotyping with 46 *Enterobacter cloacae* and *E. aerogenes* clinical isolates yielded 11 discrete banding patterns (20). The authors suggest this method does not have as high discriminatory power as other methods, but can determine the relatedness of multiple samples if species specific primers are used (20). Another study on 22 *S. marcescens* isolates using previously digested DNA differentiated 17 distinct ribotypes which correlated with the 17 types observed in ERIC-PCR (24). Ribotyping results in this study suggest the 62 isolates are not closely related because there was good discrimination between the isolates. Isolate 43 is 100% similar to isolate 44 in the ribotyping dendrogram and is also 100% similar to isolate 44 using ERIC-PCR. In contrast to ERIC-PCR isolate 43 was also 95% similar to isolates 5, 22, 54, 55, 11, 35, 47, 31, 32, 27, 30, 18, 20, 16, 17, 13, and 15 using ribotyping-PCR. Thus, in this study ribotyping was more discriminatory than ERIC-PCR.

PGRS-PCR is generally used to type *Mycobacterium tuberculosis* complex bacteria (2) and no data exist using *S. marcescens*. PGRS-PCR targets GC-rich repetitive sequences that appear to be abundant in *Mycobacterium tuberculosis* complex bacteria. PGRS has shown to have higher discriminatory power as compared to direct repeat and insertion sequences (1). PGRS is thought to be related to REP sequences, which had high discriminatory power against the 62 *S. marcescens* isolates. Also, the genome of *S. marcescens* contains a high GC content (14), therefore it was assumed that PGRS-PCR

would be discriminatory. This method differentiated the 62 *S. marcescens* isolates the best as compared to all other PCR techniques used. Sixty groupings were generated with PGRS-PCR. Using PGRS, isolate 43 was 88% similar to isolate 40.

Antimicrobial susceptibility patterns are based on minimal inhibitory concentrations of an antibiotic against an organism and are commonly used in epidemiological studies (23). This method is not considered to be a suitable typing method because antibiotic susceptibility is not a stable characteristic (25). Use of this method with other typing methods and several antibiotics, however, can increase the suitability and discrimination of this technique. Antimicrobial susceptibility is generally conducted using a disk diffusion technique and measuring zones of inhibition to determine if the organism is susceptible, intermediate, or resistant to the antibiotic (23). This method was used in a previous study to separate 69 *S. marcescens* isolates into three epidemiological types (23). In contrast, another study conducted showed antibiotic resistance patterns could not be used to discriminate nosocomial *S. marcescens* isolates (13). Most epidemiological studies include antimicrobial susceptibility patterns as either a way to group the isolates or as a comparison to typing techniques. The data in this study showed that antimicrobial patterns can discriminate between isolates that were not necessarily isolated from the same area or at the same time. Following isolate 43 on the dendrogram profile it was 100% similar to isolates 40, 5, 4, 58, 52, and 34.

Comparing the discriminatory power of the PCR methods and the antimicrobial susceptibility patterns, PGRS was shown to have the highest resolutions for demonstrating genetic variation. However, all PCR methods did demonstrate high genetic variation, with the exception of ERIC. The typing techniques used here may be

useful in a clinical setting to identify sources and types of organisms to prevent further spread. PCR methods are simple, quick, reliable, and can be applied to any species for which DNA can be prepared.

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