Oral Microbiology Immunology 2006: 21: 53–60
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY
AND IMMUNOLOGY

Characterization of *Serratia marcescens* isolates from subgingival biofilm, extraoral infections and environment by prodigiosin production, serotyping, and genotyping

F. C. B. Barbosa[1], K. Irino[2], G. V. Carbonell[3], M. P. A. Mayer[1]
[1]Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP, [2]Section of Bacteriology, Institute Adolfo Lutz, São Paulo, SP, and [3]Section of Medical Biology, Institute Adolfo Lutz, Campinas, SP, Brazil

Barbosa FCB, Irino K, Carbonell GV, Mayer MPA. Characterization of Serratia marcescens *isolates from subgingival biofilm, extraoral infections and environment by prodigiosin production, serotyping, and genotyping.*
Oral Microbiol Immunol 2006: 21: 53–60. © Blackwell Munksgaard, 2006.

Introduction: *Serratia marcescens* is widely distributed in nature, and has emerged in the last years as an important nosocomial pathogen. The organism may also be found in subgingival biofilm in periodontitis patients. This study aimed to verify the subgingival prevalence of *S. marcescens* in different periodontal conditions and to evaluate whether the oral cavity would harbor strains similar to those causing infectious diseases.
Methods: The subgingival occurrence of *S. marcescens* was determined in 334 subjects. The phenotypic and genotypic diversity of 23 isolates from subgingival biofilm, 22 from extra-oral infections and 10 environmental strains, was compared by prodigiosin production, O and H serotyping and genotyping using polymorphic GC-rich repetitive sequences–polymerase chain reaction.
Results: *S. marcescens* was found more frequently in severe periodontitis patients (4.1%) than in gingivitis (3.2%) and healthy subjects (2.5%), but these differences were not statistically significant. Analysis of serotype distribution, prodigiosin production, and genotyping revealed that environmental strains were markedly different from most human isolates, either oral or extraoral.
Conclusion: These data suggest that *S. marcescens* isolates from subgingival biofilm are not just contaminants from the environment, but that the oral cavity may act as a reservoir of strains able to promote human infections. However, further studies are needed to elucidate the role of this bacterium in the pathogenesis of periodontal diseases.

Key words: genotyping; periodontal conditions; prodigiosin production, serotyping; *Serratia marcescens*

Marcia Pinto Alves Mayer, Ph.D, Avenue Professor Lineu Prestes, 1374, Edifício Biomédicas II, lab 110, São Paulo, SP 05508–900; Brazil
Tel.: + 55 11 30917348;
fax: + 55 11 30917354;
e-mail: mpamayer@icb.usp.br,
Accepted for publication August 10, 2005

Enteric rods and pseudomonads have been isolated from the oral cavity of healthy subjects (19), from periodontitis patients exhibiting different levels of attachment loss (2, 8, 31–33), and from peri-implant pockets in edentulous subjects with a past history of periodontitis (11). The prevalence of these organisms varies widely among different populations. We have previously shown that the prevalence of pseudomonads and enteric rods among periodontitis patients in São Paulo, Brazil, was higher than observed in USA, but lower than that in Sudan, Romania, and Dominican Republic (8). The higher prevalence in certain populations could be associated with contaminated drinking water or food, inadequate personal hygiene habits (31), or even the abusive use of antimicrobial agents (2).

Serratia marcescens was the most frequent organism among the

Enterobacteriaceae in periodontitis patients in Brazil, detected in almost 10.0% of the studied population (8). For many years it was considered a harmless saprophyte and has frequently been isolated from soil and water (9). However, it is now widely recognized as an opportunistic pathogen and as a cause of significant nosocomial infections of varying severity (15, 17), affecting mainly immunocompromised hosts, the elderly and neonatal infants (1, 10, 28, 34). Ocular infections due to this microorganism are a common cause of keratitis (17).

S. marcescens is relatively resistant to standard sterilization and disinfection protocols (10, 23) and is able to survive and grow under extreme conditions (17). Infections caused by this microorganism may be difficult to treat due to resistance to a variety of antimicrobials (1, 7–10, 36). Virulence is conferred by complement resistance, production of lipopolysaccharide, hemolysins, metalloproteases, serine proteases, cytotoxic factors, nuclease, chitinases, siderophores, and lipases (9, 16, 29, 40). *S. marcescens* also produces a wetting agent or surfactant called 'serrawettin', which is probably involved in biofilm formation (41), and it shows the ability to adhere to epithelial cells (25).

Studies revealed marked differences in the serotype distribution of environmental and clinical isolates of *S. marcescens* (6, 7). Expression of virulence factors, pigment production, and resistance to antimicrobial agents can also differ among the serotypes (7, 9). Thus, *S. marcescens* diseases may be of endogenous origin, and the gastrointestinal tract, but not the environment, seems to be the major reservoir of strains able to promote infectious diseases in humans (34).

To test the hypothesis that the subgingival biofilm could also act as reservoir of these organisms, the current investigation aimed to determine the prevalence of *S. marcescens* in subjects with different periodontal conditions, and to characterize isolates from subgingival biofilm, extraoral infections and the environment by prodigiosin production, O and H serotyping, and genotyping by polymorphic GC-rich repetitive sequences–polymerase chain reaction (PGRS–PCR).

Material and methods

Subject selection

Subgingival biofilm was obtained from 334 subjects aged 14–80 years (133 males and 201 females; mean age 38.3 years). Of these subjects, 232 had severe or moderate periodontitis, 62 had gingivitis, and 40 were diagnosed as periodontally healthy. The selection criteria for plaque-induced periodontitis included the presence of at least two sites in distinct teeth with a probing pocket depth and attachment loss of at least 4 mm. Patients were diagnosed with severe or moderate periodontitis according to the criteria of Armitage (4, 5) based on the amount of clinical attachment loss. The radiographic pattern of alveolar bone destruction and reported medical history, obtained during anamnesis, were also analyzed. The clinical characteristics of plaque-induced gingivitis included the presence of gingival inflammation without loss of connective tissue attachment (3). Gingivitis and periodontitis patients were selected from among those referred for treatment at the Dental Schools of the University of São Paulo and the University Camilo Castelo Branco, both in São Paulo, SP, Brazil. Healthy periodontium subjects exhibited an absence of plaque-induced periodontal disease, fewer than 10.0% of sites with bleeding on probing, and no clinical signs of gingival inflammation, and were selected for sampling among second year dental students at the University of São Paulo. Subjects reporting periodontal treatment or antibiotic usage in the previous 6 months, or diseases associated with periodontitis such as diabetes and immunodeficiencies were excluded. The present study was approved by the ethical commission for research in humans of the Institute of Biomedical Sciences, University of São Paulo. Written consent after orally and written information about research purposes and methods was obtained from each individual. After the sampling procedures, the diseased subjects received dental treatment offered by the school clinics.

Clinical procedures

All clinical examinations were performed by one author (F.C.B.B.). Probing depth, clinical attachment loss and bleeding on probing were measured at all approximal, buccal, and lingual surfaces of every tooth to the nearest millimeter using a calibrated standard probe (GF-w, Hu-Friedy, Chicago, IL). Bone loss was confirmed by X-rays.

Bacterial sampling

In periodontitis patients, two or three sites in distinct teeth exhibiting the deepest periodontal pockets and clinical attachment loss ≥ 4 mm were selected for sampling. In gingivitis patients, three sites in distinct teeth exhibiting bleeding on probing and clinical evidence of inflammation were selected. Three sites at the approximal surfaces of molars were randomly selected for subgingival sampling in subjects with clinically healthy gingiva. Microbiological procedures were carried out using established techniques (30). After removal of supragingival dental plaque with sterile curettes, two sterile paper points (size 30, Endopoints, RJ, Brazil) were inserted subgingivally until firm resistance was felt, left in place for 10 s and transferred to VMGA III transport medium (24). The samples were maintained at room temperature and processed within 24 h after sampling.

Microbial isolation and speciation

The vials were placed in an incubator for 30 min at 37°C, and bacterial plaque was mechanically dispersed with a test tube mixer at the maximal setting for 60 s. Serial 10-fold dilutions were prepared and aliquots were plated on the surface of MacConkey agar (Oxoid, Basingstoke, Hampshire, UK). The plates were incubated aerobically at 37°C for 24 h. Each isolate was characterized according to colonial and cellular morphology and gram-stain characteristics.

Pigmented water isolates cultivated in nonselective agar were obtained from the State Agency for Environmental Control (CETESB, São Paulo, Brazil) and identified.

Subgingival and environmental isolates resembling *S. marcescens* were subcultured on MacConkey agar and analyzed for lactose fermentation and oxidase production (Strips for oxidase Newprov, Pinhais, Brazil). Colonies that did not ferment lactose and were cytochrome oxidase negative were submitted to EPM/MILi tests (38, 39), incubated aerobically at 37°C for 24 h to access production of urease, hydrogen sulfide, gas from glucose, and phenylalanine deaminase. Motility, indole production, and lysine decarboxylase were also tested. The strains negative for urease and phenylalanine deaminase, nonproducers of H_2S and indole, positive for lysine decarboxylase and motility, and producers or nonproducers of gas from glucose were speciated using the BBL CRYSTAL enteric/nonfermenter system (Becton Dickinson Microbiology Systems, Cockeysville, MD), according to the manufacturer's recommendations.

Bacterial strains

A total of 55 *S. marcescens* strains were analyzed by phenotyping and genotyping methods. Twenty-three isolates, listed in Table 1, were obtained from subgingival samples, comprising 16 strains isolated from individuals with different periodontal conditions in the present study and seven isolates obtained from periodontitis patients between 1996 and 1997 (8). Twenty-two isolates from extraoral infections (shown in Table 1) and 10 environmental strains were also tested. Two environmental isolates belonged to the collection of the Laboratory of Environmental Microbiology, Institute of Biomedical Sciences, University of São Paulo. The other eight environmental strains were isolated from samples of water for human consumption.

The reference strains *S. marcescens* CDC 4112, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 9027 were used as controls.

Pigmentation tests

All *S. marcescens* strains were grown in liquid medium (42) containing 1.0% glycerol, 0.5% ammonium citrate, 0.05% magnesium sulfate, 1% potassium phosphate, 0.5% sodium chloride, 0.005% ferric ammonium citrate, 0.1% yeast extract (Difco, Detroit, MI), and 0.2% peptone (Difco). The tubes were incubated at 28°C without shaking and observed for up to 7 days for pigment formation. *E. coli* ATCC 25922 was used as negative control.

Serotype identification

Serotyping was performed using standard methods (12, 20, 21). All somatic (O) and flagellar (H) antisera were prepared using *S. marcescens* reference strains received from Institute Pasteur, France (18). Somatic antigens were determined by the slide agglutination test (21). H antigen was identified after several serial passages in semisolid agar according to the method described by Le Minor & Pigache (20).

Table 1. Distribution of 23 oral and 22 extraoral isolates of *S. marcescens* according to the sampling site

Source	No. of isolates
Oral	
Severe periodontitis	18
Moderate periodontitis	2
Gingivitis	2
Healthy	1
Extraoral	
Urinary tract infection	5
Ocular secretion	1
Oropharynx	2
Abscess	2
Wound secretion	2
Ascitic liquid	1
Respiratory tract	3
Blood	3
Ankle secretion	2
Semen	1
Total	45

Genotyping

Chromosomal DNA of *S. marcescens* isolates and *P. aeruginosa* ATCC 9027 was extracted by a method similar to that described by Hejazi et al. (15). Briefly, bacterial pellets of 1.5 ml of overnight bacterial cultures in TSB (Tryptic Soy Broth, Merck, Darmstadt, Germany) were suspended in 567 µl Tris EDTA buffer (TE) (10 mM Tris; 1 mM EDTA; pH 7.5) and added to 30 µl of 10% dodecyl sodium sulfate (SDS) and 6 µl proteinase K solution (20 mg/ml; Gibco BRL, Paisley, UK). The suspensions were incubated at 37°C for 1 h and 100 µl of 5 M NaCl and 80 µl CTAB/NaCl solution (CTAB: Sigma Chemical Co, St. Louis, MO) were added, followed by incubation at 65°C for 10 min. DNA was extracted with phenol/chloroform/isoamylalcohol (PCI) (25 : 24 : 1), precipitated with isopropanol, washed once with 70% cold ethanol, dried at room temperature, suspended in 100 µl of TE buffer and maintained at −20°C until use. The concentration and purity of DNA were determined by measuring the absorbance in a Gene Quant Pro spectrophotometer (Applied Biosystems, Cambridge, UK) at 260 nm/280 nm.

Genotyping was performed by PGRS–PCR using the primer PGRS (5′-CCG CCG TTG CCG CCG TTG CCG CCG-3′) described by Patton et al. (26). Typical reactions consisted of 125 ng of chromosomal DNA and 50 pM primer, 2.5 µl of 10 × PCR buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP and 1 U of Taq polymerase (Invitrogen, Carlsbad, CA) in a final volume of 25 µl. Amplification was carried out on the termocycler model GeneAmp PCR System 2400 (Applied Biosystems, Foster City, CA) in the following conditions: 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min. An initial denaturation step at 94°C for 5 min and a final extension at 72°C for 5 min were used (26). Aliquots of 20 µl of each product were submitted to electrophoresis in 2% agarose gel in Tris-acetate buffer (TAE) (40 mM; pH 8.5; EDTA 2 mM). Ethidium bromide stained gels were photographed (Epson, Hemel Hempstead, UK).

The band profiles in agarose gels were analyzed visually and with the aid of GENETOOLS software (1998–2000 Synoptics Ltd) and scored using a binary code. Similarity among strains was calculated through SM coefficient using NTSYS pc 2.0 program (Exeter Software, Setauket, NY) and a dendrogram was generated by using the algorithm UPGMA (Unweighted Pair Group Method using Arithmetic Average) (35).

Statistical analysis

The prevalence of *S. marcescens* in periodontitis, gingivitis, and healthy subjects was compared by using the Chi-squared test. Differences were considered significant at the $P < 0.05$ level.

Results

The clinical characteristics of the subjects and sites selected for bacterial sampling are shown in Table 2.

Oxidase-negative colonies that did not ferment lactose and resembled *S. marcescens* were subcultured; 16 strains from periodontium and eight environmental strains were identified as *S. marcescens*.

S. marcescens was isolated from 11 (3.3%) of the 334 subjects studied. The occurrence of *S. marcescens* according to the periodontal condition of the host is shown in Table 3. According to the Chi-squared test, the higher prevalence of

Table 2. Number and age in years of 334 subjects with different periodontal conditions, and clinical characteristics of the sites selected for bacterial sampling

	Severe periodontitis $n = 194$	Moderate periodontitis $n = 38$	Gingivitis $n = 62$	Healthy $n = 40$
Mean age (years) (range)	42.6 ± 10.9 (18–80)	38.1 ± 1.6 (21–53)	33.2 ± 10.6 (14–58)	24.6 ± 5.4 (18–37)
Mean probing depth (mm) (range)	7.4 ± 1.7 (4.0–13.0)	5.1 ± 0.04 (4.0–6.0)	2.4 ± 0.4 (2.0–3.0)	(1.8 ± 0.4) (1.0–2.0)
% bleeding	100.0	100.0	100.0	0.0

± = standard deviation.

Table 3. Occurrence of *S. marcescens* in individuals with different periodontal conditions

Organism	Severe periodontitis $n = 194$	Moderate periodontitis $n = 38$	Gingivitis $n = 62$	Healthy $n = 40$	Total $n = 334$
S. marcescens	8 (4.1)	0 (0.0)	2 (3.2)	1 (4.5)	11 (3.3)*

(%) of occurrence in each periodontal condition.
$\chi^2 = 0.29$ ($P > 0.05$). * no statistically significant differences.

S. marcescens in subjects with severe periodontitis (4.1%) than in subjects with other periodontal conditions was not statistically significant ($P > 0.05$). The organism was isolated from two or more sites from four severe (2.0%) periodontitis patients; three of these patients harboring *S. marcescens* exhibited high levels of the organism (over 4×10^4 colony-forming units (CFU)/sample), whereas the positive sites from subjects with other periodontal conditions exhibited lower levels of *S. marcescens*. The clinical characteristics of the subjects and sites infected with *S. marcescens* are shown in Table 4.

All environmental strains were pigmented, whereas 22 of 23 (95.7%) of the subgingival strains and 13 of 22 (59.1%) isolates from extraoral infections were nonpigmented. Serotype O6,14:H2 was found in 60.0% of environmental strains. In all, 65.2% of oral isolates belonged to serotype O6,14:H12. Extraoral strains exhibited a more diverse serotype profile, the most prevalent being O6,14:H10 (22.7%). The results of serotyping are described in Table 5.

Genotyping by PGRS-PCR resulted in amplification of fragments of 0.3–6.5kb (Fig. 1A–C). Fifty-six genotypes of *S. marcescens* were identified, one for each isolate. A dendrogram was built based on cluster analysis (Fig. 2). Most *S. marcescens* isolates were clustered in group A, except for two environmental strains that were in group B, formed also by the outgroup strain *P. aeruginosa*. Interestingly, these strains of *S. marcescens* (Env.VI and Env.IX) were mannitol negative, differing from all other studied strains of *S. marcescens*.

Group A strains were further divided into four clusters (Fig. 2). Most environmental strains ($n = 8$) belonged to cluster I, formed also by two isolates from periodontitis patients. Fifteen isolates from the periodontium (65.2%) and six strains from extraoral infections were located in cluster II. Cluster III was formed only by isolates from the periodontium, while 14 of 22 strains from extraoral infections (63.6%) were located in group IV, formed also by an isolate from severe periodontitis, and by CDC 4112, isolated from human infection.

Table 4. Number and age in years of the subjects with different periodontal conditions infected with *S. marcescens*, and clinical characteristics of the sites selected for bacterial sampling

	Severe periodontitis $n = 8$	Gingivitis $n = 2$	Healthy $n = 1$
Mean age (years)	36.7 ± 8.9	30 ± 6.0	18
(range)	(23–52)	(24–36)	0.0
Mean probing depth (mm)	6.7 ± 1.7	2.1 ± 0.4	(1.3 ± 0.5)
(range)	(5–10)	(2.0–3.0)	(1.0–2.0)
% bleeding	100.0	100.0	0.0

± = standard deviation.

Table 5. Distribution of *S. marcescens* isolates according to serotype

Strains	Serotypes (%)
Environmental $n = 10$	O6, 14:H2(60.0); O6, 14:H8(20.0); O6, 14:H10(10.0); O4:HNT*(10.0)
Extraoral $n = 22$	O6, 14:H10 (22.7); O4; H1 (13.6); O1:H7(9.0); O6, 14:H2(9.0); O5; H1 (4.5); O5:H2 (4.5); O5:H3 (4.5); O5:H8 (4.5); O6, 14:H4 (4.5); O12:H23 (4.5); O6, 14:H12 (4.5); O12:H12 (4.5); O3:H2 (4.5); O2:H1 (4.5)
Subgingival $n = 23$	O6, 14:H12(65.2); O5:H4 (17.4); O8:H12 (4.3); O6, 14:H8 (4.3); O6, 14:H4 (4.3); O6, 14:H- (4.3)

(%) of occurrence among strains of the same origin.
*NT = antigen untypeable.

Most pigmented extraoral strains were located in cluster IV, with the majority of extraoral isolates, and not with the pigmented environmental strains. Multiple subgingival isolates from four subjects were studied, and one single serotype was observed per subject. However, multiple isolates from three of these subjects were located in different clusters in the dendrogram, indicating that a subject may be colonized by more than one genotype of *S. marcescens*.

Discussion

Despite its occurrence in nature, *S. marcescens* is now a largely recognized opportunistic pathogen, causing a wide range of nosocomial infections (1, 10, 18, 28, 34). In the present study, *S. marcescens* was isolated from the subgingival biofilm of subjects with different periodontal conditions, suggesting a possible role of the subgingival biofilm as a reservoir of these opportunistic pathogens (Table 3). The number of periodontitis patients harboring *S. marcescens* (3.4%) is lower than observed in our previous study (8), but higher than observed among periodontitis patients in the USA (31). The occurrence of *S. marcescens* was higher among severe periodontitis patients (4.1%) than in other subjects; however, statistical analysis revealed that this difference was not significant. Although these opportunistic pathogens may also be detected in the subgingival biofilm of patients without attachment loss, only low levels of *S. marcescens* were found in these subjects, whereas some severe periodontitis patients positive for *S. marcescens* were heavily colonized, suggesting an association of *S. marcescens* with certain cases of severe disease. A wide age range of subjects was selected for this study and, interestingly, eight of 11 subjects infected with *S. marcescens* (72.7%) were less than 36 years old (Table 4).

Thus, the oral isolates of *S. marcescens* were compared to isolates from extraoral infections and from the environment in order to evaluate whether the oral cavity would be merely contaminated by environmental organisms, or could act as a reservoir of strains able to promote human diseases.

Prodigiosin production differs according to the source of the *S. marcescens* isolate. Usually pigmented isolates are found in the environment and are rarely detected among clinical human strains. Our data corroborate these findings, since 59.1% of extraoral isolates and 95.7% of subgingival isolates

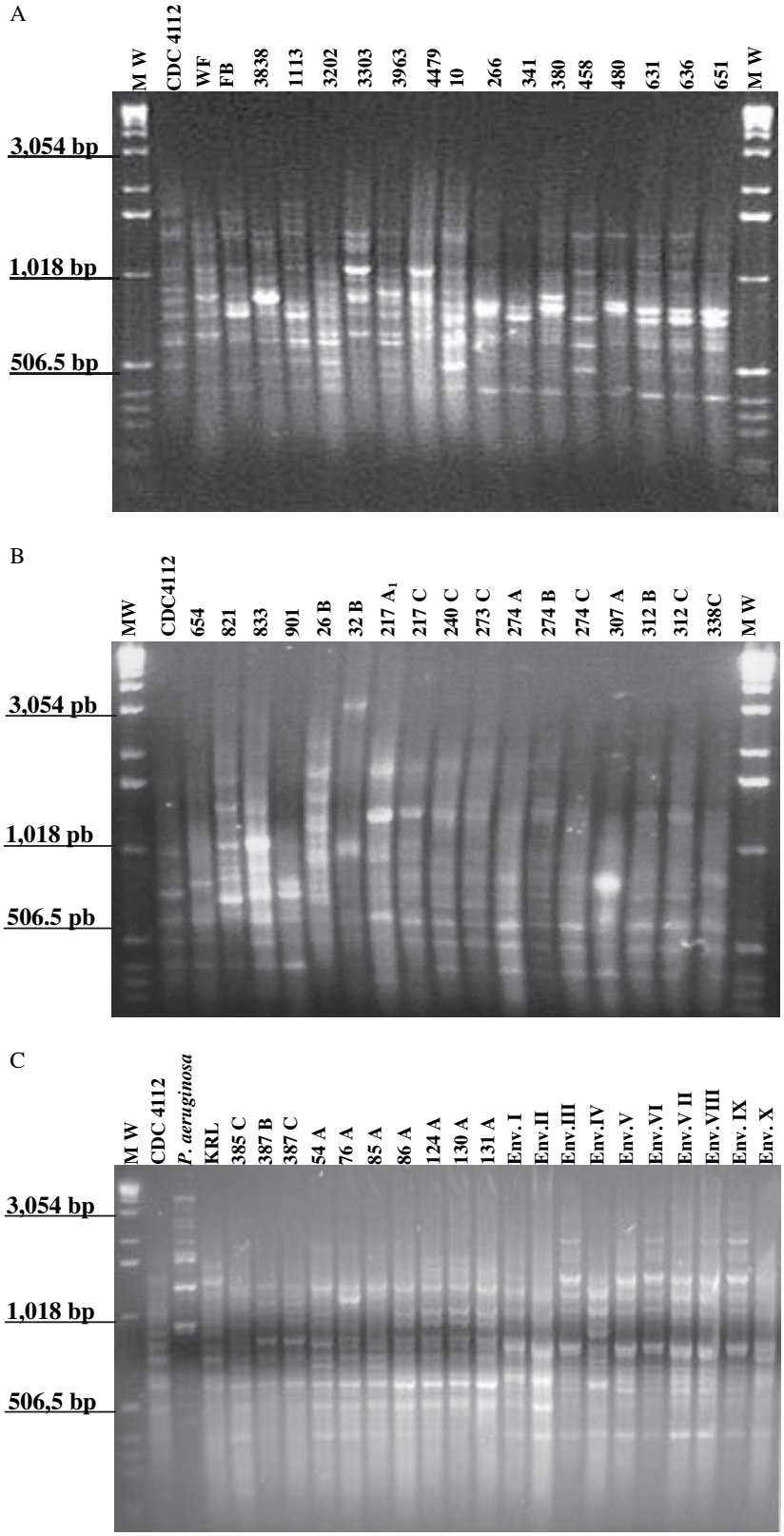

Fig. 1. Digital images of ethidium bromide stained 2% agarose gels in TAE after electrophoresis showing amplicons produced by PGRS–PCR using template DNA from *S. marcescens* isolates. MW, molecular weight (1 kb DNA ladder).

in this study were nonpigmented, whereas all environmental strains were pigmented. However, the high number of pigmented extraoral human isolates does not represent the prevalence of pigmented nosocomial strains, as some pigmented extraoral isolates obtained from the collection of the Laboratory of Microbiology, Hospital of the School of Medicine of Ribeirão Preto, São Paulo, Brazil, were selected for this analysis. In the Brazilian study in which most nosocomial isolates used in this study was obtained, pigmented strains were isolated with low frequency (3.4%) and caused infection in only 31.0% of cases (9).

Prodigiosin is a secondary metabolite produced by *S. marcescens* growing at 27–30°C that is not essential for bacterial growth (37) and has no defined role in the physiology of producing strains of *Serratia*. This product does not appear to be an important virulence factor for *S. marcescens*, as most serotypes of this bacterium associated with human disease are nonpigmented (7). In addition, the ability to produce prodigiosin is correlated with the absence of plasmids, suggesting that plasmids with virulence or resistance factors could be involved in the evolutionary divergence of the prodigiosin-producing and -nonproducing isolates (13). The absence of prodigiosin production by most oral strains suggests that this factor does not play a role in periodontal diseases, indicating that oral strains may have virulence or resistance factors similar to extraoral strains. However, further studies are necessary to elucidate possible correlations between absence of prodigiosin and pathogenicity.

The similarity between oral and extraoral isolates was further supported by serotyping results (Table 5). If the environment was the natural reservoir of oral strains, the frequencies of epidemiologic markers such as serotype should be similar between the two sources. On the other hand, differences in frequency distribution imply that the epidemiologic markers are associated in some way with the fitness of the bacteria to colonize humans and produce human diseases, or to grow in the environment (7). In this study, the prevalence of serotypes differed according to the source of *S. marcescens*. It has already been reported that isolates from human infections differ from environmental strains (6). Most environmental isolates were classified as serotypes O6,14:H2 and O6,14:H8. The isolates from extraoral infections presented a much higher diversity in somatic and flagellar antigens than

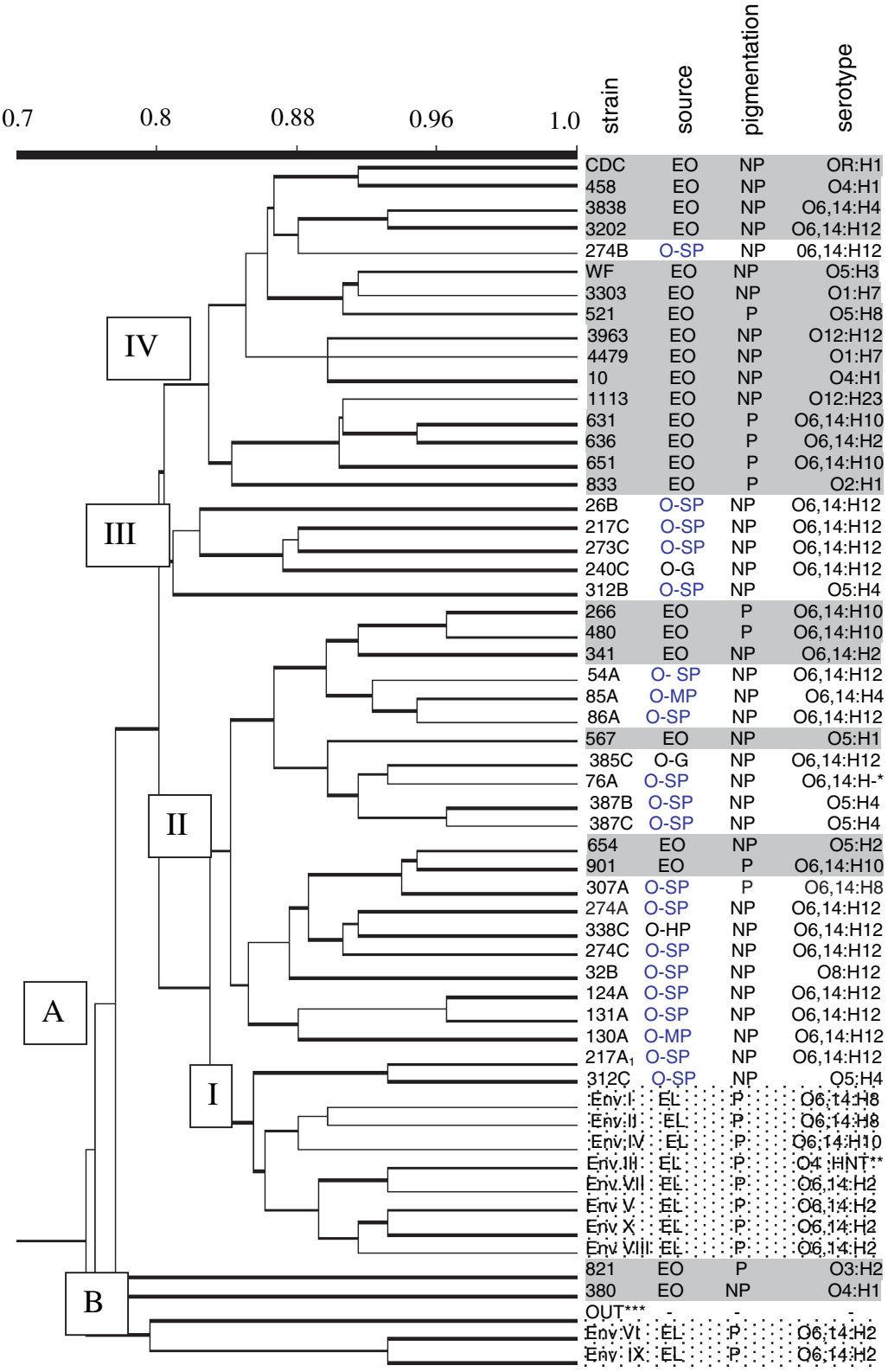

Fig. 2. Cluster analysis of *S. marcescens* isolates from different origins based on PGRS–PCR data, calculated based on the SM Coefficient of Similarity by UPGMA algorithm. The strains according to allocation in the dendrogram, as well as their source (EL, environmental; EO, extraoral infection; O, oral [G = gingivitis, HP = healthy periodontium, SP = severe periodontitis, MP = moderate periodontitis]), production of prodigiosin (P = pigmented and NP = nonpigmented) and serotype are listed. *H–, nonmotile; **NT, untypeable antigen; ***OUT, = outgroup (*P. aeruginosa*).

the other sources and only two common serotypes were observed between environmental and extraoral strains. Serotypes O6,14:H12 and O5:H4 constituted over 80.0% of oral isolates. The serotypes O6,14:H4 and O6,14:H12 were observed both among oral and extraoral isolates and these serotypes were also found among nosocomial isolates in Brazil, occurring in a study in more than 70.0% of these isolates (18). Serotypes O1:H7 and O4:H1 were found exclusively among isolates from extraoral infections, and have also been implicated in nosocomial infections in the US (14, 28).

Genotyping based on repetitive elements has been used successfully in a wide array of organisms including *S. marcescens* (10, 22). A detailed investigation on genotyping methods based on PCR for typing clinical *S. marcescens* isolates demonstrated that PGRS–PCR had a higher discriminatory power than REP-PCR or ERIC-PCR, probably due to the high GC content in the genome of this bacterium (26).

Most studies employing genotyping methods for *S. marcescens* have analyzed nosocomial isolates; to our knowledge, this is the first report of PGRS-PCR being applied for genotyping of *S. marcescens* strains isolated from periodontium and environment. The reproducibility of this technique was good since the band pattern of the reference strain remained constant throughout the experiments (Fig. 1A–C). PGRS-PCR data revealed correlation between band patterns and the source of *S. marcescens* isolates. According to the cluster distribution (Fig. 2), environmental *S. marcescens* strains were markedly different from most isolates from human infections. In addition, strains from subgingival biofilm were mainly located in two clusters, formed also by isolates from extraoral infections (II and III), although most of strains from extraoral infections belonged to a separate cluster (IV).

No correlation was found between cluster allocation and serotype, since the same serotype was observed in isolates belonging to different clusters. The lower discriminatory power of serotyping than genotyping (PFGE) was previously described (9). Pigmented isolates from extraoral infections exhibited the same serotype as environmental pigmented strains, but belonged to different genotypic groups, evidence that despite prodigiosin production, these strains exhibited more chromosomal similarity with other human strains than with environmental strains. Multiple strains isolated from different periodontal sites in the same subject were classified in the same serotype, but strains from each of three among four subjects with multiple studied strains were located in different clusters, indicating that the subgingival biofilm may harbor more than one genotype of *S. marcescens*.

Virulence depends on a combination of properties that enable the organism to adhere and to colonize specific niches and to promote tissue destruction. Although prodigiosin induces apoptosis in eukaryotic cells, it is a marker of environmental strains (7). Resistance to human serum is restricted to certain serotypes detected among environmental and clinical isolates (7, 9), suggesting that this factor is not of major importance in human infections. On the other hand, culture filtrates of most clinical isolates of *S. marcescens*, whether pigmented or not, led to cytopathic effects on several eukaryotic cell lines (9). In addition, properties such as agglutination with red blood cells and expression of type 1 fimbriae by certain serotypes were more prevalent among clinical isolates than in environmental strains (7). The genetic determinants for these differences among isolates of different sources are still unknown. Extra-chromosomal elements such as plasmids were detected in *S. marcescens*; these were usually not related to resistance to antimicrobials, but their role was not established (9). Other structures such as pathogenicity islands encoding virulence factors such as adhesins, present in clinical isolates but not in most environmental strains, as shown for other organisms such as *Vibrio cholerae* (27), have still not been demonstrated for *S. marcescens*.

These results suggest that *S. marcescens* isolates from subgingival biofilm are not just contaminants from the environment, but that the oral cavity may act as a reservoir of strains able to promote human diseases. However, more detailed studies involving virulence factors of *S. marcescens* strains isolated from periodontium are needed.

Acknowledgments

This study was supported by FAPESP Grant 02/01242–3. We are also grateful to the Dental Schools of the University of São Paulo and University Camilo Castelo Branco.

We thank M. I. Sato from CETESB and R. Gamba from the Laboratory of Environmental Microbiology of the Institute of Biomedical Sciences (University of São Paulo) for providing environmental strains, and R. Prisco for statistical analysis.

References

1. Alfizah H, Nordiah AJ, Rozaidi WS. Using pulsed-field electrophoresis in the molecular investigation of an outbreak of *Serratia marcescens* infection in an intensive care unit. Singapore Med J 2004: **45**: 214–218.
2. Ali RW, Bakken V, Nilsen R, Skaug N. Comparative detection frequency of 6 putative periodontal pathogens in Sudanese and Norwegian adult periodontitis patients. J Periodontol 1994: **65**: 1046–1052.
3. Armitage GC. Clinical evaluation of periodontal diseases. Periodontol 2000 1995: **7**: 39–53.
4. Armitage GC. Development of a classification system for periodontal diseases and conditions. Ann Periodontol 1999: **4**: 1–6.
5. Armitage GC. Periodontal diagnoses and classification of periodontal diseases. Periodontol 2000 2004: **34**: 9–21.
6. Aucken HM, Pitt TL. Different O and K serotype distributions among clinical and environmental strains of *Serratia marcescens*. J Med Microbiol 1998: **47**: 1097–1104.
7. Aucken HM, Pitt TL. Antibiotic resistance and putative virulence factors of *Serratia marcescens* with respect to O and K serotypes. J Med Microbiol 1998: **47**: 1105–1113.
8. Barbosa FCB, Mayer MPA, Saba-Chufji E, Cai S. Subgingival occurrence and antimicrobial susceptibility of enteric rods and pseudomonads from Brazilian periodontitis patients. Oral Microbiol Immunol 2001: **16**: 306–310.
9. Carbonell GV, Della Colleta HHM, Yano T, Darini ALC, Levy CE, Fonseca BAL. Clinical relevance and virulence factors of pigmented *Serratia marcescens*. FEMS Immunol Med Microbiol 2000: **28**: 143–149.
10. Cimolai N, Trombley C, Wensley D, LeBlanc J. Heterogeneous *Serratia marcescens* genotypes from a nosocomial pediatric outbreak. Chest 1997: **111**: 194–197.
11. Danser MM, van Winkelhoff AJ, van der Velden U. Periodontal bacteria colonizing oral mucous membranes in edentulous patients wearing dental implants. J Periodontol 1997: **68**: 209–216.
12. Ewing WH. Edwards' and Ewing's Identification of Enterobacteriaceae, 4nd edn. New York: Elsevier Science, 1986: 423–441.
13. Gargallo-Violla D. Enzyme polymorphism, prodigiosin production, and plasmid fingerprints in clinical and naturally occurring isolates of *Serratia marcescens*. J Clin Microbiol 1989: **27**: 860–868.
14. Grimont PAD, Grimont F, Le Minor S, Davis B, Pigache F. Compatible results obtained from biotyping and serotyping in *Serratia marcescens*. J Clin Microbiol 1979: **10**: 425–432.
15. Hejazi A, Keane CT, Falkiner FR. The use of RAPD-PCR as a typing method for *Serratia marcescens*. J Med Microbiol 1997: **46**: 913–919.

16. Hines DA, Saurugger PN, Ihler GM, Benedik MJ. Genetic analysis of extracellular proteins of *Serratia marcescens*. J Bacteriol 1988: **170**: 4141–4146.
17. Hume EB, Willcox MD. Emergence of *Serratia marcescens* as an ocular surface pathogen. Arch Sociedad Español Oftalmol 2004: **10**: 1–3.
18. Irino K, Vaz TMI, Landgraf IM, Brandileone MCC, Vieira VSD. Sorotipos de *Serratia marcescens* em infecções humanas. Rev Inst Adolfo Lutz 1989: **49**: 107–115.
19. Kue IM, Samaranayake LP, van Heyst EN. Oral health and microflora in an institutionalised elderly population in Canada. Int Dent J 1999: **1**: 33–40.
20. Le Minor S, Pigache F. Etude antigénique de souches de *Serratia marcescens* isolées en France. I. Antigène H: individualisation de six nouveaux facteurs H. Ann Microbiol (Paris) 1977: **128**: 207–214.
21. Le Minor S, Pigache F. Etude antigénique de souches de *Serratia marcescens* isolées en France. II. Caractérisation des antigènes O et individualisation de 5 nouveaux facteurs, fréquence des sérotypes et désignation des nouveaux facteurs H. Ann Microbiol (Paris) 1978: **129**: 407–423.
22. Liu PY-F, Lau Y-J, Hu B-S, Shir J-M, Cheung M-H, Shi Z-Y, et al. Use of PCR to study epidemiology of *Serratia marcescens* isolates in nosocomial infection. J Clin Microbiol 1994: **32**: 1935–1938.
23. Marrie TJ, Costerton JW. Prolonged survival of *Serratia marcescens* in chlorhexidine. Appl Environ Microbiol 1981: **42**: 1093–1102.
24. Möller AJR. Microbiological examination of root canals and periapical tissues of human teeth. Odontol Tidskr 1966: **S74**: 380.
25. Obana Y, Shibata K, Nishino T. Adherence of *Serratia marcescens* in the pathogenesis of urinary tract infections in diabetic mice. J Med Microbiol 1991: **35**: 93–97.
26. Patton TG, Katz S, Sobieski RJ, Crupper SS. Genotyping of clinical *Serratia marcescens* isolates. a comparison of PCR-based methods. FEMS Microbiol Lett 2001: **194**: 19–25.
27. Rivera ING, Chun J, Huq A, Sack RB, Colwell RR. Genotypes associated with virulence in environmental isolates of *Vibrio cholerae*. Appl Environ Microbiol 2001: **67**: 2421–2429.
28. Schaberg DR, Alford RH, Anderson R, Farmer JJ 3rd, Melly MA, Schaffner W. An outbreak of nosocomial infection due to multiply resistant *Serratia marcescens*: Evidence of interhospital spread. J Infect Dis 1976: **134**: 181–188.
29. Scheffer J, König W, Braun V, Goebel W. Comparison of four hemolysin-producing organisms (*Escherichia coli, Serratia marcescens, Aeromonas hydrophila,* and *Listeria monocytogenes*) for release of inflammatory mediators from various cells. J Clin Microbiol 1988: **26**: 544–551.
30. Slots J. Rapid identification of important periodontal microorganisms by cultivation. Oral Microbiol Immunol 1986: **1**: 48–55.
31. Slots J, Feik D, Rams TE. Prevalence and antimicrobial susceptibility of *Enterobacteriaceae*, *Pseudomonadaceae* and *Acinetobacter* in human periodontitis. Oral Microbiol Immunol 1990: **5**: 149–154.
32. Slots J, Feik D, Rams TE. Age and sex relationships of superinfecting microorganisms in periodontitis patients. Oral Microbiol Immunol 1990: **5**: 305–308.
33. Slots J, Rams TE, Feik D, Taveras HD, Gillespie GM. Subgingival microflora of advanced periodontitis in the Dominican Republic. J Periodontol 1991: **62**: 543–547.
34. Smith PJ, Brookfield DSK, Shaw DA, Gray J. An outbreak of *Serratia marcescens* infection in a neonatal unit. Lancet 1984: **21**: 151–153.
35. Sneath PHA, Sokal R. R. In: Numerical taxonomy. San Francisco: WF Freeman, 1973: 1–15.
36. Stock I, Grueger T, Wiedemann B. Natural antibiotic susceptibility of strains of *Serratia marcescens* and the *S. liquefaciens* complex: *S. liquefaciens* sensu stricto, *S. proteamaculans* and *S. grimesii*. Int J Antimicrob Agents 2003: **22**: 35–47.
37. Sunaga S, Li H, Sato Y, Nakagawa Y, Matsuayama T. Identification and characterization of the *pswP* gene required for the parallel production of prodigiosin and serrawettin W1 in *Serratia marcescens*. Microbiol Immunol 2004: **48**: 723–728.
38. Toledo MRF, Fontes CF, Trabulsi LR. MILi: um meio para a realização dos testes de motilidade, indol e lisina descarboxilase. Rev Microbiol 1982: **13**: 230–235.
39. Toledo MRF, Fontes CF, Trabulsi LR. EPM: Uma modificação do meio de Rugai para a realização simultânea dos testes de produção de gás a partir de glicose, H_2S, urease, e triptofano desaminase. Rev Microbiol 1982: **13**: 309–315.
40. Vinogradov E, Petersen BO, Duus J, Radziejewska-Lebrecht J. The structure of the polysaccharide part of the LPS from *Serratia marcescens* serotype O19, including linkage region to the core and the residue at the non-reducing end. Carbohydr Res 2003: **338**: 2757–2761.
41. Wei Y-H, Lai H-C, Chen S-Y, Yeh M-S, Chang J-S. Biosurfactant production by *Serratia marcescens* SS-1 and its isogenic strain SMΔR defective in SpnR, a quorum-sensing LuxR family protein. Biotechnol Lett 2004: **26**: 799–802.
42. Williams RP, Gott CL, Qadri SMH, Scott RH. Influence of temperature of incubation and type of growth medium on pigmentation in *Serratia marcescens*. J Bacteriol 1971: **106**: 438–443.