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Restriction Endonuclease Analysis of *Eikenella corrodens*

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Eikenella corrodens **is a gram-negative facultative bacillus commonly found in the oral cavity. Although the role of *E. corrodens* in periodontitis is not clear, its isolation from extraoral infections attests to its pathogenic potential. Previous studies suggested that this species is phenotypically diverse. In the present study, we used restriction endonuclease analysis (REA) to assess the genetic diversity of this species and to explore the applicability of REA in studying the transmission of *E. corrodens*. Two groups of *E. corrodens* isolates were used in this study. Group 1 included 47 epidemiologically independent isolates recovered from dental plaques in periodontally healthy subjects and periodontitis patients and from extraoral infections in different geographic areas. Group 2 *E. corrodens* included 40 isolates recovered from two periodontitis patients and two periodontally healthy subjects. The results indicated that *E. corrodens* is genetically heterogeneous, as determined by REA. The majority of the group 1 *E. corrodens* isolates exhibited strain-specific restriction patterns. Forty restriction patterns were distinguishable among the 47 isolates. Analyses of group 2 isolates revealed that three of four subjects harbored more than one clonal type of *E. corrodens*. In one instance, a periodontitis patient was found to be colonized by six different clones. Furthermore, two different clonal types of *E. corrodens* were recovered from a single periodontal pocket in this patient. The results indicated that REA may be a useful tool in the epidemiologic investigation of *E. corrodens* infections.**

Eikenella corrodens is a facultative gram-negative bacillus commonly found in the oral cavity (4, 9) and in the intestinal and genital tracts (7, 23). This organism is considered a candidate periodontal pathogen based on various clinical studies showing that elevated numbers of this species are associated with periodontitis (13, 17, 22). *E. corrodens* may cause serious extraoral infections in which it is recovered either as the sole infecting organism or in combination with other bacteria (3, 20, 21). Although results from DNA hybridization studies indicate that *E. corrodens* strains are rather homogeneous (6, 10; C.-K. C. Chen, T. V. Potts, and M. E. Wilson, J. Periodontal Res., in press), evidence for phenotypic diversity has been obtained. *E. corrodens* exhibits variability with respect to nitrate reduction, aminopeptidase and lipase production (10), lysine decarboxylase and catalase activity, colony morphology (Chen et al., in press), outer membrane protein and lipopolysaccharide profiles (C.-K. C. Chen and M. E. Wilson, J. Infect. Dis., in press), and resistance to serum complement bactericidal activity (5).

Given the presence of *E. corrodens* in the normal oral microflora as well as its association with periodontal and extraoral infections, it is reasonable to question whether some strains might be relatively harmless commensals while other strains might be more virulent and thus capable of causing infections. Methods which could discriminate between different *E. corrodens* strains and which could be used to study the transmission of *E. corrodens* among humans, between different anatomic sites, or between different periodontal sites in the same subject would be helpful in resolving this question. Restriction endonuclease analysis (REA) has been increasingly used in epidemiologic studies of bacterial transmission. Restriction endonucleases recognize and cut specific nucleotide sequences along DNA strands. DNA fragments generated after endonuclease digestion may be separated by agarose gel electrophoresis. Heterogeneity in DNA sequences between strains is exemplified by different restriction patterns. In the present study, we used REA to examine the genetic diversity among *E. corrodens* isolates and to determine the applicability of this technique in studying the transmission of *E. corrodens* infections.

MATERIALS AND METHODS

Strains. Two groups of *E. corrodens* isolates were examined in the present study. Group 1 consisted of 47 epidemiologically independent *E. corrodens* isolates (Table 1). Group 2 consisted of 40 *E. corrodens* isolates recovered from two periodontally healthy subjects and two periodontitis patients in Buffalo (Table 1). One *E. corrodens* isolate per site was examined. However, in a few instances, two isolates per site were examined. *E. corrodens* strains were maintained on sheep blood agar (tryptic soy agar supplemented with 5% sheep blood, 0.001% menadione, 5 μg of hemin per ml, and 0.1% yeast extract) at 37°C in a humidified chamber with 5% CO_2. All strains were examined for purity and identified as *E. corrodens* on the basis of both phenotypic characteristics and biochemical activities as previously described (4).

DNA purification. Bacteria were grown in Todd-Hewitt broth (30 g/liter) supplemented with 2 mg of KNO_3, 50 μg of L-cysteine hydrochloride, and 5 μg of hemin per ml. Cells were harvested by centrifugation and suspended in Tris-EDTA buffer (50 mM Tris hydrochloride, 50 mM EDTA [pH 8.0]). Total cellular DNA was extracted by a modification of the procedures of Silhavy and co-workers (19) as previously described (Chen et al., in press). Briefly, cells were lysed with 1% sodium dodecyl sulfate (SDS) at 60°C for 1 h, digested with proteinase K (50 μg/ml) at 60°C for 1 h, and deproteinized by extraction twice with Tris-buffered phenol and once with chloroform. The DNA was precipitated by the addition of 2 volumes of cold ethanol and 0.1 volume of 3 M sodium acetate and was dissolved in 50 mM Tris hydrochlo-

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TABLE 1. Sources and origins of *E. corrodens* isolates

Group and source	No. of strains (geographic origin [*n*])
1 (*n* = 47)	
Sputum	2 (Georgia [1], Tennessee [1])
Periodontitis patients (plaque)	27 (New York [18], Massachusetts [9])
Periodontally healthy subjects (plaque)	7 (New York)
Blood	7 (Virginia [1], Nebraska [1], Mississippi [2], Delaware [1], Georgia [1], Kansas [1])
Subdiaphragmatic abscess	1 (Mississippi)
Neck abscess	1 (Colorado)
Bite wound	2 (Washington, D.C. [1], Alabama [1])
2 (*n* = 40)[a]	
Subject D1	
Buccal mucosa	1
Tongue	1
Tonsil	1
Saliva	1
Plaque	12
Subject D4, plaque	13
Subject H5, plaque	7
Subject H8	
Plaque	3
Tonsil	1

[a] All were from the State University of New York at Buffalo. Subjects D1 and D4 were adult periodontitis patients. Subjects H5 and H8 were periodontally healthy subjects.

ride (pH 7.5)–1 mM EDTA. Subsequently, the DNA suspension was digested with RNase A (200 μg/ml) overnight at 4°C. The DNA suspension was reextracted with phenol and chloroform, precipitated with ethanol, and dissolved in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA (TE buffer). The DNA concentration was determined by measuring A_{260}. The DNA concentration was adjusted to 200 μg/ml with TE buffer, and the samples were stored at 4°C.

Restriction endonuclease digestion. The following restriction endonucleases were used in this study: *Eco*RI, *Bam*HI, *Hin*dIII, *Pst*I, *Apa*I, *Asp*718, *Cfo*I, *Eco*RV, *Pvu*II, *Sty*I, *Sac*I, *Xho*I, *Cla*I, *Dra*I, *Bst*EII, and *Not*I (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.) and *Bst*BI, *Sfi*I, and *Xba*I (New England BioLabs, Inc., Beverly, Mass.). Approximately 4 μg of DNA in a 20-μl volume was digested to completion in accordance with manufacturer recommendations. Following the addition of 0.1 volume of 10× loading buffer (0.1 M EDTA, 1% [wt/vol] SDS, 50% [vol/vol] glycerol, 0.1% [wt/vol] bromophenol blue), the restricted DNA samples were heated to 60°C for 10 min and stored at 4°C prior to analysis by gel electrophoresis.

Gel electrophoresis. Electrophoresis was carried out on a horizontal slab gel apparatus (Max Horizontal Submarine Agarose Gel; Hoefer Scientific Instruments, San Francisco, Calif.). Twenty-microliter samples (containing approximately 4 μg of DNA) were separated on a 0.7% agarose gel in 0.1 M Tris-borate (pH 8.3)–2 mM EDTA–0.5 μg of ethidium bromide per ml at 100 V for 5.5 h. The gel concentration and conditions of electrophoresis were chosen after preliminary experiments with various gel concentrations (from 0.55 to 1%) and voltages (from 35 to 120 V). Following electrophoresis, UV-illuminated gels were photographed with a Polaroid camera. DNA size markers (DNA molecular weight marker III; Boehringer) were included in each run. Undigested DNA was also electrophoresed to determine the integrity of the total cellular DNA and to determine the presence of plasmids. For assessment of the identity or nonidentity of the restriction patterns between any two given strains, the patterns in each lane were visualized and compared two at a time independently by two of the authors. To avoid variability from different electrophoresis runs, we used only samples run on the same gels for the determination of identity or nonidentity. Subsequently, strains with similar or apparently identical restriction patterns were further confirmed by running the samples on the neighboring lane of the same gel.

RESULTS

Selection of restriction endonucleases for the genetic analysis of *E. corrodens*. *E. corrodens* ATCC 23834[T] and a clinical isolate, UB 344, were used initially to determine which restriction endonucleases provided clear and interpretable restriction patterns. From the initial experiments, it was determined that *Cla*I, *Dra*I, *Bst*EII, *Not*I, *Pvu*II, *Sty*I, *Eco*RV, *Pst*I, *Eco*RI, and *Hin*dIII yielded relatively clear restriction patterns with bands ranging from 1 to 20 kilobase pairs. Subsequently, DNA samples from eight randomly selected strains (including ATCC 23834[T], six oral isolates, and one nonoral isolate) were digested with these endonucleases to determine which enzyme generated strain-specific restriction patterns. Three endonucleases (*Eco*RI, *Bst*EII, and *Pvu*II) produced unambiguous strain-specific restriction patterns. Accordingly, these three endonucleases were further used in subsequent experiments. Electrophoresis of undigested total DNA revealed that 1 isolate (UB 190) of the 87 isolates examined contained a plasmid (data not shown).

Assessment of genetic diversity among *E. corrodens* isolates. Total genomic restriction profiles of reference and clinical isolates of *E. corrodens* of diverse origins (Table 1, group 1 *E. corrodens*) were compared to assess the potential genetic diversity among strains of this species. Figure 1 depicts the restriction profiles of *Eco*RI digests of DNA prepared from 24 representative strains of this group. The restriction profiles of isolates from subgingival plaque samples of periodontitis patients exhibited marked diversity (Fig. 1A). Similar diversity was noted in the restriction profiles of extraoral isolates and isolates from plaques samples of periodontally healthy individuals (Fig. 1B). There was no apparent common restriction pattern shared by the *E. corrodens* isolates recovered from similar clinical conditions (periodontally healthy subjects, periodontitis patients, or patients with extraoral infections). This result would tend to disfavor the hypothesis that clonal types of *E. corrodens* capable of causing extraoral infections or periodontitis are unique. Consistent with this interpretation, seven pairs of *E. corrodens* isolates were found to exhibit almost indistinguishable restriction patterns (Fig. 2; each pair is indicated by a thick horizontal bar above the lanes). In a number of instances, isolates exhibiting very similar restriction patterns had diverse anatomic or geographic origins. These results were confirmed with a second restriction enzyme (*Bst*EII; data not shown). The remaining 33 isolates in group 1 exhibited unique restriction profiles. Thus, a total of 40 restriction patterns could be differentiated among the 47 *E. corrodens* isolates.

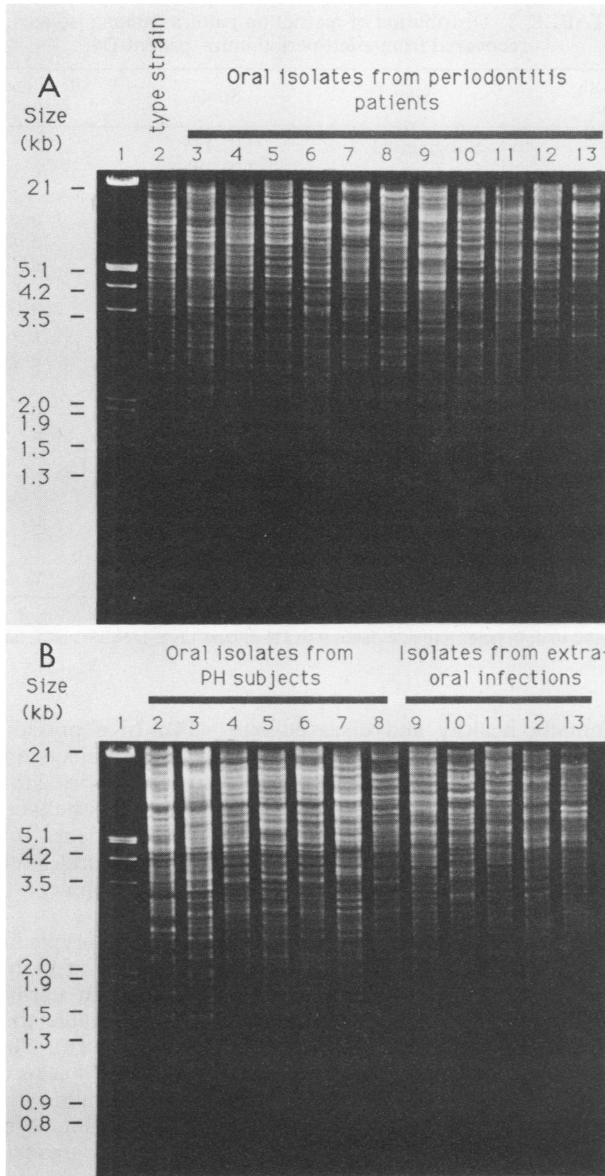

FIG. 1. (A) Restriction patterns of group 1 *E. corrodens* isolates generated with *Eco*RI. The type strain of *E. corrodens* is shown in lane 2; isolates recovered from plaque samples of periodontitis patients are shown in lanes 3 to 13. Lane 1 contains DNA size markers. (B) Restriction patterns of oral isolates generated with *Eco*RI. Lanes: 2 to 8, isolates from plaque samples of periodontally healthy (PH) subjects; 9 to 13, isolates from extraoral infection sites. Lane 1 contains DNA size markers. kb, Kilobase pairs.

Clonal diversity among *E. corrodens* isolates from individual subjects. To determine whether individual subjects are colonized by one or more clones of *E. corrodens*, we compared the restriction patterns of multiple isolates recovered from two periodontally healthy subjects and two adult periodontitis patients. The restriction patterns (generated with *Eco*RI) of 13 strains isolated from an adult periodontitis patient (D1) are depicted in Fig. 3. At least three distinct restriction patterns were evident, suggesting that this subject was colonized by more than one clone. Oral isolates from a second adult periodontitis patient (D4) also exhibited considerable diversity, with six different restriction patterns

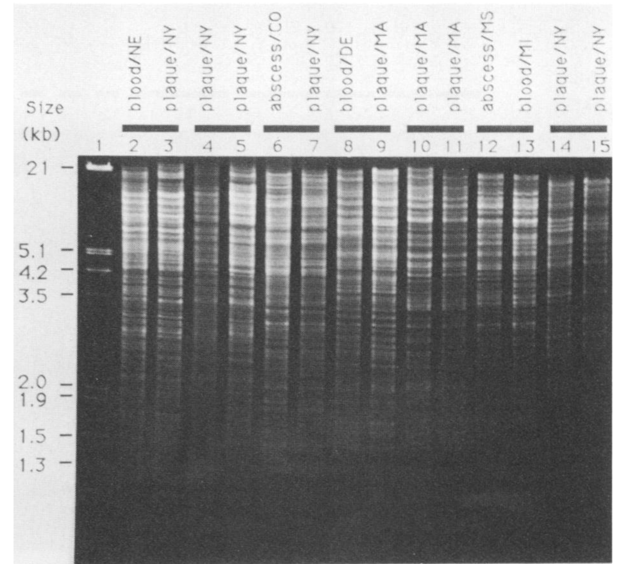

FIG. 2. Restriction patterns shared by epidemiologically independent *E. corrodens* isolates. Lane 1, DNA size markers. The seven groups of *E. corrodens* isolate pairs are indicated by the thick horizontal bars above the lanes. The sources of isolation and geographic areas are also indicated. kb, Kilobase pairs.

being distinguished among 13 strains examined (Fig. 4). Similar results were obtained when a second restriction endonuclease (*Pvu*II) was used to analyze the isolates from this patient (data not shown). In a previous study, three of the isolates from patient D1 were examined for outer membrane protein and lipopolysaccharide profiles by SDS-poly-

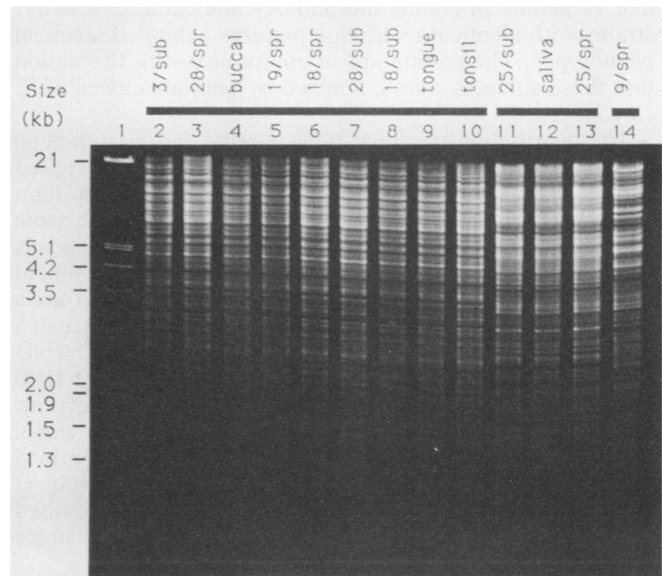

FIG. 3. Restriction patterns of oral isolates from periodontitis patient D1. The sources of isolation are indicated. The periodontal sites are identified by tooth number and anatomic site (sub, subgingival plaque; sup, supragingival plaque). Isolates exhibiting identical restriction patterns are indicated by thick horizontal bars above the lanes. Three additional isolates from this patient were also examined and exhibited a pattern identical to that in lanes 2 to 10 (data not shown). kb, Kilobase pairs.

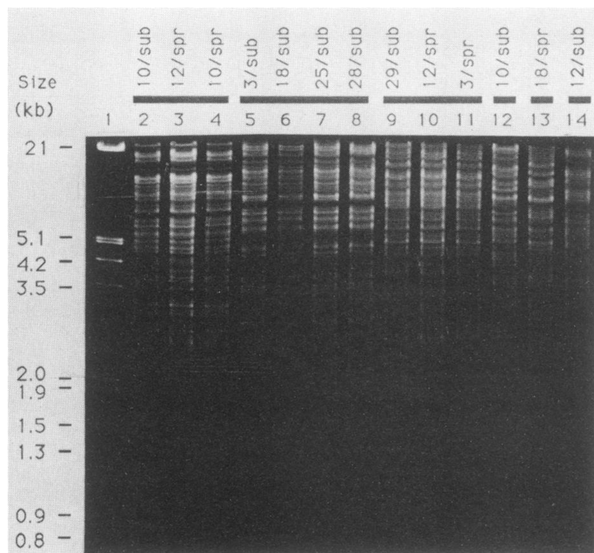

FIG. 4. Restriction patterns of oral isolates from periodontitis patient D4. The anatomic site (sub, subgingival plaque; supra, supragingival plaque) and tooth number from which each strain was isolated are indicated. The six restriction pattern groups, are listed in Table 2, are indicated by the thick horizontal bars above the lanes. These were assigned letters a through f, as follows: lanes 2 to 4, pattern a; lanes 5 to 8, pattern b; lanes 9 to 11, pattern c; lane 12, pattern d; lane 13, pattern e; and lane 14, pattern f. kb, Kilobase pairs.

acrylamide gel electrophoresis. The SDS-polyacrylamide gel electrophoresis analysis indicated that strains with different restriction patterns also differed in outer membrane protein and lipopolysaccharide patterns (Chen and Wilson, in press). In addition, although the 13 isolates from patient D4 exhibited variability in colony morphology and catalase activity, strains with identical restriction patterns exhibited identical phenotypes. The results are in conformity with the notion that these subjects were colonized by multiple clones of *E. corrodens*.

The distribution of clonal types among various sampling sites of patient D4 appeared to be random, and *E. corrodens* isolates recovered from supra- and subgingival plaque samples from a single tooth did not necessarily have the same restriction patterns (Table 2). Furthermore, *E. corrodens* isolates recovered from a single periodontal pocket exhibited different restriction patterns. Analysis of isolates from a periodontally healthy subject (H5) revealed the presence of a single clonal type among seven isolates examined, while isolates from a second periodontally healthy subject (H8) exhibited two restriction patterns (Table 3). Three of four individuals were found to be colonized by multiple clonal types (Table 3). Although only one clonal type was identified among seven isolates from a periodontally healthy subject (H5), it cannot be concluded on the basis of our limited sample size that this subject was colonized with a single strain of *E. corrodens*.

DISCUSSION

The role of *E. corrodens* in the etiology and pathogenesis of human periodontal disease has not been defined. While some investigators have observed increased numbers of *E. corrodens* in periodontally diseased sites (13, 17, 22), others have found comparable numbers of this organism in periodontally healthy and diseased sites (14). In a previous cross-sectional study of periodontally healthy subjects and patients with juvenile or adult periodontitis, we reported that the number of cultivable *E. corrodens* did not differ between healthy and diseased sites (4). Hence, despite evidence that *E. corrodens* is capable of causing serious extraoral infections, the role of this organism in the development of periodontal disease remains questionable.

We have observed that *E. corrodens* is a phenotypically diverse species. Studies based on the analysis of stable phenotypic markers have provided evidence that certain types of bacterial infection appear to be attributable to a limited number of clones of this species (8, 11, 16). This evidence prompted us to consider whether certain strains of *E. corrodens* are associated with periodontitis while other strains represent part of the normal oral flora. Under such conditions, while the number of cultivable *E. corrodens* might be similar in periodontally healthy and diseased subjects, the clonal types associated with health and disease may differ.

Various approaches have been used to assess phenotypic and/or genotypic variation within a given bacterial species. Analysis of outer membrane protein and/or lipopolysaccharide electrophoretic mobility on SDS-polyacrylamide gels (15), electrophoretic properties of multilocus enzymes (18), and REA of total genomic DNA (2) have been used to assess

TABLE 2. Distribution of restriction patterns among isolates recovered from adult periodontitis patient D4

Tooth no.	Site	Strain	Restriction pattern[a]
3	Subgingival	UB 135	b
	Supragingival	UB 143	c
10	Subgingival	UB 127	a
		UB 126	d
	Supragingival	UB 144	a
12	Subgingival	UB 140	f
	Supragingival	UB 130	a
		UB 132	c
18	Subgingival	UB 137	b
	Supragingival	UB 133	e
25	Subgingival	UB 138	b
28	Subgingival	UB 139	b
29	Subgingival	UB 128	c

[a] Designated a through f according to the order of appearance in Fig. 4: a, lanes 2 to 4; b, lanes 5 to 8; c, lanes 9 to 11; d, lane 12; e, lane 13; and f, lane 14.

TABLE 3. Restriction patterns of *E. corrodens* strains recovered from individual subjects

Subject (diagnosis)[a]	Total no. of strains examined	No. of restriction patterns	Presence of different clonal types from a single site
D1 (AP)	16	3	−
D4 (AP)	13	6	+
H5 (PH)	7	1	−
H8 (PH)	4	2	−

[a] AP, Adult periodontitis; PH, periodontally healthy.

bacterial phenotypic diversity and to define the clonal nature of bacterial infections. Among these techniques, REA has the advantage of being very sensitive to subtle genomic differences while being largely independent of environmentally related phenotypic changes. Such qualities have prompted the use of REA in studying the epidemiology of bacterial infections.

In this investigation, we performed REA of total genomic DNA from 87 isolates of *E. corrodens* with the following questions in mind. (i) Is *E. corrodens* a genotypically diverse species? (ii) Are certain clones of *E. corrodens* associated with extraoral infections and/or periodontal disease while other strains are present only in healthy subjects? (iii) Are individuals harboring this organism colonized by a single clone or multiple clones of *E. corrodens*? (iv) Is REA applicable to the study of *E. corrodens* transmission?

Despite prior evidence (from DNA hybridization techniques) that *E. corrodens* is a homogeneous species (6, 10; Chen et al., in press), restriction fragment patterns of 47 strains of diverse anatomic and geographic origins revealed the presence of marked genetic diversity among *E. corrodens* strains. The clonal diversity among *E. corrodens* strains contrasts with the results of a similar study of *Actinobacillus actinomycetemcomitans* in which a limited number of genotypes were identified (24) but is reminiscent of the diversity among *Streptococcus mutans* (11) and *Bacteroides gingivalis* strains (B. G. Loos, J. A. Herweijer, M. Shlossman, R. J. Genco, and D. P. Dickinson, J. Dent. Res. **67**:369, abstr. no. 2049, 1988).

No evidence was obtained to indicate that certain genotypes of *E. corrodens* are unique to extraoral or periodontal infection sites. Among 47 isolates from various oral and extraoral sites, 7 pairs of *E. corrodens* isolates exhibited similar or identical restriction patterns (Fig. 2). In several instances, strains exhibiting similar or apparently identical restriction patterns were isolated from distinct anatomic sites (plaque versus blood, abscess versus blood, abscess versus plaque) and geographic locations.

We also performed REA on multiple strains of *E. corrodens* isolated from individuals subjects to determine whether these subjects were colonized by single or multiple clonal types. In contrast to the results of a similar study indicating that a single clone of *B. gingivalis* is present in the oral cavity of a given subject (Loos et al., J. Dent. Res.), we observed that three of four subjects (two adult periodontitis patients and one healthy subject) were colonized by multiple *E. corrodens* clones. Further, in one of the periodontitis patients, different clonal types were recovered from a single periodontal pocket. Such findings are not unprecedented. For example, the coexistence of multiple clonal types of *S. mutans* has been reported in subjects harboring this organism (12). A single restriction pattern was observed among seven isolates derived from an additional periodontally healthy subject. The limited number of samples obtained from this individual precludes definitive determination of whether this subject was, in fact, colonized by a single clone of *E. corrodens*.

Some of the strains included in this study were also used in a previous serologic study (1) and in a recent study assessing outer membrane protein and lipopolysaccharide electrophoretic heterogeneity among *E. corrodens* strains (Chen and Wilson, in press). It is interesting to note that *E. corrodens* strains which were of the same serogroup or which exhibited identical outer membrane protein patterns could differ in their restriction patterns (data not shown). Similar findings have been observed with respect to *A. actinomycetemcomitans*, in which serotype b strains consist of at least two different REA groups (24).

The results of this study indicate that substantial genotypic diversity exists among *E. corrodens* strains. For this reason, REA may be useful as a tool for epidemiologic studies of *E. corrodens* infections. Moreover, until the relationship between genotypic characteristics and virulence has been defined, the marked genotypic diversity among *E. corrodens* strains casts doubt upon the feasibility of establishing the periodontopathic potential of this species solely on the basis of total cultivable *E. corrodens* present.

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