INFECTION AND IMMUNITY, Jan. 1993, p. 204–212
0019-9567/93/010204-09$02.00/0

Vol. 61, No. 1

Genetic Structure of Populations of *Porphyromonas gingivalis* Associated with Periodontitis and Other Oral Infections

BRUNO G. LOOS,[1]† DAVID W. DYER,[1,2]* THOMAS S. WHITTAM,[3] AND ROBERT K. SELANDER[3]

Department of Oral Biology, School of Dental Medicine,[1] and Department of Microbiology, School of Medicine, 208A Sherman Hall,[2] State University of New York at Buffalo, Buffalo, New York 14214, and Institute of Molecular Evolutionary Biology, Mueller Laboratory, Pennsylvania State University, University Park, Pennsylvania 16802[3]*

Received 1 May 1992/Accepted 3 November 1992

One hundred isolates of the oral pathogenic bacterium *Porphyromonas gingivalis* were genetically characterized by determining the electrophoretic mobilities of 16 metabolic enzymes and the presence or absence of catalase activity. A total of 78 distinct electrophoretic types (ETs), representing multilocus genotypes, were identified, and cluster analysis placed them in three major phylogenetic divisions. Division I (71 ETs) included all 88 human isolates examined, most of which had been recovered from patients with periodontitis, together with 4 monkey isolates. The strains in division II (four ETs) and division III (three ETs) are strongly differentiated from those in division I and apparently represent two previously unclassified (cryptic) species. The mean genetic diversity per enzyme locus among the 92 isolates of division I (*P. gingivalis*, strict sense) was 0.321, and the strains were distributed among 14 phylogenetic clusters and single-ET lineages. The population structure is basically clonal, with some clonal genotypes being widespread, and even global, in distribution. There was no evidence of association between specific genetic lineages or clusters of ETs and the type of disease (periodontitis or root canal infections), invasive potential, serogroup, or fimbrial restriction fragment length polymorphism group. The finding that dental patients are infected by strains of a wide variety of chromosomal genotypes suggests that interstrain variation in pathogenicity is small. On the basis of the observed genetic structure of natural populations of *P. gingivalis*, we hypothesize that the role of this microorganism in the pathogenesis of periodontitis and other dental infections is largely opportunistic.

Porphyromonas gingivalis (formerly *Bacteroides gingivalis*) is an anaerobic, asaccharolytic, gram-negative coccobacillus (32) that is frequently a component of the flora of subgingival lesions of adult patients with periodontitis (6, 27, 35). *P. gingivalis* has also been isolated from root canal infections and other odontogenic abscesses (34, 37) and from the saliva and the surfaces of the tongues and tonsils of patients with periodontitis (38). The organism is often present in the oral cavities of periodontally healthy children and adolescents, but, because it occurs in densities below the detectable limit of anaerobic culturing (<1% of the total flora) (18, 26, 39), it is not routinely recovered from such individuals. Whether strains that colonize periodontally healthy hosts are genetically distinct from those associated with periodontitis is unknown.

Organisms identified as *P. gingivalis* have also been isolated from sheep with broken-mouth periodontitis (9) and monkeys with naturally occurring and ligature-induced periodontitis (5, 15). Isolates recovered from dogs and cats differ from human strains in their antigenic properties and in their production of catalase (8, 16, 25), but the overall genetic relatedness of these animal porphyromonads to those of human origin has not been determined.

Experimentally, the pathogenic potential of *P. gingivalis* has been previously examined with several animal models. Monoinfection of gnotobiotic rats with *P. gingivalis* induces alveolar bone loss (14), and implantation of this microorganism in the subgingivae of monkeys exacerbates periodontal breakdown (12). In the mouse model, certain strains are highly invasive and can produce fatal phlegmonous abscesses (4, 23, 36), whereas other strains cause only localized abscesses and no mortality in these mice. The differences in these strains that cause varying levels of invasiveness are unknown, but they suggest that certain *P. gingivalis* strains have unique abilities to cause diseases.

Little is known concerning the extent of phenotypic variation and genetic heterogeneity in natural populations and the evolutionary relationships of *P. gingivalis* strains. Three (7) or four (22) serogroups are currently recognized, and two biotypes have been distinguished on the basis of the presence or absence of catalase activity (16, 25). But it is now well established for bacterial species in general that similarity in serotypic, biotypic, and other phenotypic characters, including virulence factors, frequently does not indicate the overall genetic relationships of strains (31).

Genotypic characterization of strains has revealed extensive heterogeneity in natural populations of *P. gingivalis*. Restriction fragment length polymorphism (RFLP) analysis with the *fimA* gene distinguished 25 patterns among 39 strains (17), and fingerprinting (restriction endonuclease analysis) of genomic DNA revealed 29 distinct patterns among 33 strains (19). However, because these studies did not yield quantitative estimates of overall genetic relationships among strains, they did not provide a basis for constructing a phylogenetic framework within which to organize research and to evaluate data on host occurrence, disease associations, and putative virulence factors.

In the present study, we have used multilocus enzyme electrophoresis to further examine genetic variation in natural populations of *P. gingivalis* and to estimate genetic relationships among strains. Our analysis indicates that

* Corresponding author.

† Present address: Department of Periodontology, Academic Center Dentistry Amsterdam, Louwesweg 1, 1066 EA Amsterdam, The Netherlands.

dental patients are infected by strains of a wide variety of chromosomal genotypes and strongly suggests that certain isolates infecting animals represent two undescribed cryptic species.

MATERIALS AND METHODS

Bacterial strains. We examined a total of 100 isolates of *P. gingivalis* recovered from humans (88 isolates) and animals (12 isolates) in 11 countries on five continents (Table 1). Each strain was originally identified as *P. gingivalis* by the investigator from whom it was obtained. Most of the human isolates were recovered from adult patients with periodontal disease, but some isolates were obtained from root canal, periapical, or other odontogenic infections. Three isolates from healthy subjects (adolescents) were also included. The animal isolates were recovered from a dog, a cat, sheep, and two species of monkey (Table 1).

Culture conditions. Upon receipt, the strains were revived on blood agar plates (Difco Laboratories, Detroit, Mich.) enriched with 5% defibrinated sheep blood (Crane Laboratories, Syracuse, N.Y.), hemin (5 μg/ml), and vitamin K_1 (1 μg/ml). Cultures were incubated for several days at 37°C in an anaerobic chamber (Forma Scientific, Marietta, Ohio) in an atmosphere of 5% CO_2–10% H_2–85% N_2. At this point, the cultures were inspected for purity, production of characteristic nonfluorescent, black-pigmented colonies, and catalase activity (see below). The API ZYM profile (Analytab Products, Plainview, N.Y.) of each isolate in our sample matched the pattern characteristic of *P. gingivalis* (19). Stocks of each strain, prepared by suspending freshly plated culture cells in sheep blood, were stored in liquid nitrogen.

To obtain large numbers of cells for enzyme extraction, a 5% inoculum from a dense starter culture was used to inoculate 400 ml of Trypticase soy broth (Difco) enriched with hemin (5 μg/ml) and vitamin K_1 (1 μg/ml). These cultures were incubated anaerobically for 24 to 48 h in 1-liter sidearm flasks (Bellco, Vineland, N.J.), optical density readings at A_{600} were periodically made with a spectrophotometer (Spectronic 21; Milton Roy, Rochester, N.Y.), and purity was assessed by Gram staining and subculturing on both aerobic and anaerobic blood agar plates.

Preparation of enzyme extracts. Broth cultures in the logarithmic or early stationary phase were harvested by centrifugation at 12,000 × *g* at 4°C for 20 min in 500-ml-volume bottles. The supernatant was removed by aspiration. During these procedures, we noticed that some strains yielded tight, dense cell pellets, while other strains produced loose, flocculent pellets after being centrifuged. The reasons for this are not known. Individual cell pellets were weighed, and the cells were stored at −20°C.

Pellets were thawed and the cells were kept on ice at all times during the course of further manipulation. The cells were resuspended in phosphate-buffered saline (0.137 M NaCl, 2 mM KCl, 4 mM $Na_2HPO_4 \cdot 7H_2O$, 1 mM KH_2PO_4 [pH 7.4]) at 1 ml/0.5 g (wet weight) for tight pellets or at 1 ml/1 g (wet weight) for loose pellets. The suspension was transferred to a 50-ml-volume centrifuge tube, and the volume was estimated. The cells were lysed by the addition of the nonionic detergent *n*-octyl β-D-glucopyranoside (Sigma Chemical Co., St. Louis, Mo.) (11) from a 1 M stock solution to a final concentration of 50 mM with constant stirring for 15 min. The cleared viscous suspension was transferred to a 12-ml-volume centrifuge tube and then sonicated twice for 15 s (small tip, setting 6, 50% duty cycle) (Sonifier model 350; Branson, Danbury, Conn.) to decrease viscosity. Finally, the sonicated suspension was centrifuged at 20,000 × *g* at 4°C for 20 min, and the clear supernatant was stored in aliquots of 200 μl at −70°C.

Electrophoresis of enzymes. Methods of horizontal starch gel electrophoresis and the demonstration of specific enzyme activities were as described previously by Selander et al. (29).

Electromorphs (mobility variants) were determined for the following 16 metabolic enzymes: adenosine deaminase (ADA), esterase 1 (ES1), esterase 2 (ES2), guanine deaminase (GDA), NAD-dependent glutamate dehydrogenase (GLU), indophenol oxidase 1 (IP1), indophenol oxidase 2 (IP2), malate dehydrogenase (MDH), nucleoside phosphorylase (NSP), glycine-phenylalanine peptidase 1 (GP1), glycine-phenylalanine peptidase 2 (GP2), glycine-phenylalanine peptidase 3 (GP3), leucine-glycine-glycine peptidase (LGG), phosphoglycerate dehydrogenase (PGD), phosphoglucose isomerase (PGI), and phosphoglucomutase (PGM). In addition, we scored each isolate for the presence or absence of catalase (CAT) activity as described previously (19), without, however, determining the electrophoretic mobilities of this enzyme in the positive strains.

Statistical analysis. Electromorphs for each enzyme were equated with alleles at the corresponding structural gene loci so that each bacterial strain was fully characterized by its multilocus genotype (allele combination) for the 17 enzyme-encoding loci assayed (29). In our analyses, absences of activity (null phenotypes) were treated as missing data, except in the case of CAT. Distinctive multilocus genotypes were designated electrophoretic types (ETs) (29) and were numbered sequentially by their inferred relationships from a cluster analysis based on the average linkage algorithm (presented as dendrograms in Fig. 1 and 2) (40). For this analysis, a matrix of genetic distances between all pairs of ETs was calculated from comparisons of electrophoretic profiles. Each entry in the distance matrix was equal to the proportion of mismatches (i.e., the number of enzyme loci with different alleles divided by the number of loci compared) between two ETs. For each comparison, null states were not included.

The genetic diversity for each enzyme locus (h) among isolates was calculated as $h = 1 - \Sigma x_i^2 [n/(n-1)]$, where x_i is the frequency of the ith allele at the locus and n is the number of isolates (29). Mean genetic diversity per locus (H) is the arithmetic average of the h values for all loci.

Genetic differences between various subgroups of isolates were assessed by partitioning the total genetic diversity into within- and between-group components (40). The within-subgroup diversity (H_S) was calculated as the unweighted arithmetic average of the genetic diversity values across subgroups. Total genetic diversity among subgroups (H_T) was tabulated for each locus by using the average allele frequencies across subgroups and the total number of isolates in the formula above. Coefficients of genetic differentiation among subgroups (G_{ST}) were calculated as $(H_T - H_S)/H_T$, which equals the ratio of the between-subgroup component of diversity to the total genetic diversity across subgroups of isolates (40).

RESULTS

Genetic diversity and relationships among all isolates. In the collection of 100 isolates phenotypically and biochemically characterized as *P. gingivalis*, all 17 enzymes assayed were polymorphic, with an average of 4.5 alleles per locus (range, 2 to 8) (Table 2). By comparing the allele profiles of all strains, we identified 78 distinctive ETs (multilocus enzyme

TABLE 1. Source information for 100 isolates of 78 ETs of *P. gingivalis*

Cluster or lineage and ET	Isolate[a]	Host	Type of oral infection	Site of isolation	Geographic locality
Division I					
A					
1	HG 756	Human	Periodontitis	Tongue	Amsterdam, The Netherlands
2	OMG 511	Human	Periodontitis	Subgingival	Gothenburg, Sweden
	B262	Human	Postjuvenile periodontitis	Subgingival	Umeå, Sweden
	OMG 434	Human	Periodontitis	Subgingival	Kenya, Africa
3	B42	Human	Odontogenic abscess	Drainage incision	Hofu, Japan
4	JKG 2	Human[b]	Periodontitis	Subgingival	Ann Arbor, Mich.
	JKG 8	Human	Periodontitis	Subgingival	Ann Arbor, Mich.
5	295-1	Human	Periodontitis	Subgingival	Umeå, Sweden
6	329-1	Human	Periodontitis	Subgingival	Umeå, Sweden
7	OMG 137	Human	Periodontitis	Subgingival	Gothenburg, Sweden
8	19A4	Human	Periodontitis	Subgingival	Quebec City, Canada
9	A 7436	Human	Refractory periodontitis	Subgingival	Atlanta, Ga.
10	W12	Human	Periodontitis	Subgingival	Birmingham, Ala.
11	DCR 2006	Human[c]	None	Submarginal	London, United Kingdom
	A9A2-17	Human[d]	Periodontitis	Subgingival	Sacaton, Ariz.
12	OMG 406	Human	Periodontitis	Subgingival	Kenya
	Y8-1	Human	Periodontitis	Subgingival	Buffalo, N.Y.
13	Bg 18	Human	Periodontitis	Subgingival	Osaka, Japan
14	Bg 4	Human	Periodontitis	Subgingival	Osaka, Japan
15	BE-c	Human	Root canal	Root canal	Umeå, Sweden
16	14 Q	Human	Periodontitis	Subgingival	Malmö, Sweden
17	Y6-1	Human	Periodontitis	Subgingival	Buffalo, N.Y.
	AJW5	Human	Periodontitis	Subgingival	Buffalo, N.Y.
	OMG 1426	Human	Periapical abscess	Fistulous track	Gothenburg, Sweden
18	3079.03	Monkey[e]	Experimental periodontitis[f]	Subgingival	San Antonio, Tex.
19	376F	Human	Periodontitis	Subgingival	Great Lakes, Ill.
20	OMZ 470	Human	Periodontitis	Subgingival	Zürich, Switzerland
21	102	Human	Root canal	Root canal	Umeå, Sweden
	81	Human	Root canal	Root canal	Umeå, Sweden
22	THUR28BM-2	Human	Periodontitis	Subgingival	Buffalo, N.Y.
23	BH 6/26	Human	Periodontitis	Subgingival	Winnipeg, Canada
24	HG 760	Human	Periodontitis	Subgingival	Amsterdam, The Netherlands
25	W50[g]	Human	Unknown	Unknown	West Germany
	W83	Human	Unknown	Unknown	West Germany
	13JC	Human[c]	None	Submarginal	Rennes, France
26	ESO 132	Human	Periodontitis	Subgingival	Okayama, Japan
27	OMZ 481	Human	Periodontitis	Subgingival	Zürich, Switzerland
28	B 129	Human	Odontogenic abscess	Drainage incision	Hofu, Japan
29	JKG 7	Human	Periodontitis	Subgingival	Ann Arbor, Mich.
B					
30	1076 B	Human	Periodontitis	Subgingival	Malmö, Sweden
31	IW-1	Human	Root canal	Root canal	Buffalo, N.Y.
	HW24D-2	Human	Periodontitis	Subgingival	Quebec City, Canada
32	OMG 268	Human	Periodontitis	Subgingival	Gothenburg, Sweden
33	AJW 4	Human	Periodontitis	Subgingival	Buffalo, N.Y.
34	AJW 3	Human	Periodontitis	Saliva	Buffalo, N.Y.
35	BH 18/10	Human	Periodontitis	Subgingival	Winnipeg, Manitoba, Canada
36	873 C	Human	Periodontitis	Subgingival	Malmö, Sweden
37	A7A1-28[h]	Human[d]	Periodontitis	Subgingival	Sacaton, Ariz.
	CLN17-6-1	Human[d]	Periodontitis	Subgingival	Sacaton, Ariz.
	CLN16-6-4	Human[d]	Periodontitis	Subgingival	Sacaton, Ariz.
38	OMG 522	Human	Periodontitis	Subgingival	Gothenburg, Sweden
39	JBB-c	Human	Root canal	Root canal	Umeå, Sweden
40	ESO 75	Human	Periodontitis	Subgingival	Okayama, Japan
41	RB22D-1[i]	Human	Periodontitis	Subgingival	Quebec City, Canada
C					
42	AJW 2	Human	Periodontitis	Subgingival	Buffalo, N.Y.
43	332-2	Human[j]	Juvenile periodontitis	Subgingival	Umeå, Sweden
D					
44	7B5	Human	Periodontitis	Submarginal[k]	Quebec City, Canada
45	34-4	Human	Periodontitis	Subgingival	Minneapolis, Minn.
46	ESO 127	Human	Periodontitis	Subgingival	Okayama, Japan

Continued on following page

TABLE 1—*Continued*

Cluster or lineage and ET	Isolate[a]	Host	Type of oral infection	Site of isolation	Geographic locality
47	HG 445	Human	Periodontal abscess	Subgingival	Amsterdam, The Netherlands
48	Bg 57	Human	Periodontitis	Subgingival	Osaka, Japan
	OMZ 482	Human	Periodontitis	Subgingival	Zürich, Switzerland
49	16V2K-10	Monkey[e]	Experimental periodontitis[f]	Subgingival	Buffalo, N.Y.
50	19X2K-1	Monkey[e]	Experimental periodontitis[f]	Subgingival	Buffalo, N.Y.
	18Y2K-10	Monkey[e]	Experimental periodontitis[f]	Subgingival	Buffalo, N.Y.
51	4ZR6-13	Human	Root canal	Root canal	Buffalo, N.Y.
F					
52	EM-3	Human	Root canal	Root canal	Buffalo, N.Y.
G					
53	E20-1	Human	Root canal	Root canal	Buffalo, N.Y.
H					
54	HG 405	Human	Periodontitis	Subgingival	Amsterdam, The Netherlands
55	2561[l]	Human	Unknown	Subgingival	Buffalo, N.Y.
	W	Human	Periodontitis	Subgingival	Ann Arbor, Mich.
	381	Human	Periodontitis	Subgingival	Boston, Mass.
56	H965	Human	Periodontitis	Subgingival	Amsterdam, The Netherlands
I					
57	Po1.1	Human	Periodontitis	Subgingival	Amsterdam, The Netherlands
58	816 B	Human	Periodontitis	Subgingival	Malmö, Sweden
59	HG 863	Human	Periodontitis	Subgingival	Amsterdam, The Netherlands
60	FAY19M-1	Human	Periodontitis	Subgingival	Buffalo, N.Y.
61	9-14K-1	Human[b]	Periodontitis	Subgingival	Buffalo, N.Y.
62	JKG 6	Human[b]	Periodontitis	Subgingival	Ann Arbor, Mich.
63	817 H	Human	Periodontitis	Subgingival	Malmö, Sweden
	Y12-1	Human	Periodontitis	Subgingival	Buffalo, N.Y.
64	OMZ 479	Human	Periodontitis	Subgingival	Zürich, Switzerland
65	B220-2	Human	Periodontal abscess	Subgingival	Umeå, Sweden
	23A4	Human	Periodontitis	Subgingival	Quebec City, Canada
J					
66	JKG 5	Human	Periodontitis	Subgingival	Ann Arbor, Mich.
K					
67	17-5	Human	Periodontitis	Subgingival	Minneapolis, Minn.
68	HG 826	Human	Periodontitis	Subgingival	Amsterdam, The Netherlands
	HG 184	Human	Periodontitis	Subgingival	Amsterdam, The Netherlands
L					
69	OMZ 409	Human	Periodontitis	Subgingival	Zürich, Switzerland
M					
70	AJW1	Human	Periodontitis	Subgingival	Buffalo, N.Y.
N					
71	DCR 2011	Human[c]	None	Submarginal	London, United Kingdom
	DCR 2015[m]	Human	Periodontitis	Subgingival	London, United Kingdom
Division II					
72	I-372*	Monkey[n]	Experimental periodontitis[f]	Subgingival	Gainesville, Fla.
73	I-433*	Monkey[n]	Experimental periodontitis[f]	Subgingival	Gainesville, Fla.
74	G-251*	Monkey[n]	Experimental periodontitis[f]	Subgingival	Gainesville, Fla.
75	Chien 5B*	Dog	None	Submarginal	Quebec City, Canada
	Chat 2*	Cat	None	Submarginal	Quebec City, Canada

Continued on following page

TABLE 1—*Continued*

Cluster or lineage and ET	Isolate[a]	Host	Type of oral infection	Site of isolation	Dunedin, New Zealand
Division III					
76	TT 1	Sheep	Broken-mouth periodontitis	Subgingival	Dunedin, New Zealand
77	BTS 50	Sheep	Broken-mouth periodontitis	Subgingival	Dunedin, New Zealand
78	TG 1	Sheep	Broken-mouth periodontitis	Subginbival	Dunedin, New Zealand

[a] All isolates were biotype 1 (catalase activity not detected) except for those marked with an asterisk, which were biotype 2 (catalase activity detected).
[b] Patient with insulin-dependent diabetes mellitus.
[c] Healthy adolescent without any oral infections.
[d] Patient with non-insulin-dependent diabetes mellitus. Native American on the Pima Indian Reservation, Sacaton, Ariz.
[e] Cynomolgus monkey (*Macaca fascicularis*).
[f] Periodontitis induced by ligating teeth subgingivally.
[g] ATCC 53978.
[h] ATCC 53977.
[i] ATCC 49417.
[j] Adolescent.
[k] Isolate recovered from a nonaffected site in a patient with periodontitis.
[l] ATCC 33277^T (type strain).
[m] Isolate DCR 2015 was recovered from the same site in the same subject as was DCR 2011 (ET 71), but 2 years later, at which time the site had converted to periodontitis.
[n] Squirrel monkey (*Saimiri scuireus*).

genotypes), 17 of which were represented by more than one isolate (Table 1). Mean genetic diversity per locus (*H*) among the 100 isolates was 0.384 (Table 2).

A dendrogram generated by clustering all ETs on the basis of pairwise estimates of genetic distance revealed three major divisions (Fig. 1). The average genetic distances between ETs of division I and those of division II and division III were 0.70 and 0.87, respectively, and the comparable value for division II versus division III was 0.87.

Genetic diversity within phylogenetic divisions. Division I included ETs 1 to 71, which were represented by 88 human and 4 monkey isolates, all of which were of biotype 1 (catalase negative). Table 3 shows that, among these ETs, 14 of the 17 enzymes (82%) were polymorphic; the mean number of alleles per locus was 3.2 (range, 1 to 8) (Tables 2 and 3). The enzyme loci PGD and PGI were monomorphic, while the absence of CAT activity was regarded as one allele

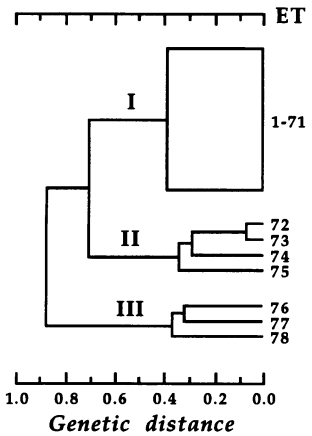

FIG. 1. Genetic relationships among 78 ETs of *Porphyromonas* species, numbered sequentially from top to bottom. Division I consists of a cluster of 71 ETs of *P. gingivalis*, for which genetic relationships are summarized separately in Fig. 2; division II contains ETs 72 to 75, which are represented by five isolates recovered from animals (a cat, a dog, and monkeys [Table 1]); and division III includes ETs 76 to 78, which are represented by three isolates from sheep with broken-mouth periodontitis (Table 1).

(CAT^1) just for the purposes of this study; for ADA, ES1, GDA, GP3, and LGG, genetic diversity was relatively high (Table 3). The mean genetic diversity per locus among the 92 isolates was 0.321 (Tables 2 and 3).

Division II consisted of four ETs (ETs 72 to 75), which were represented by five isolates recovered from a cat, a dog, and three monkeys; all five strains were of biotype 2 (catalase positive, regarded as a single allele [CAT^2]). Eight of the 17 loci (47%) were polymorphic, the mean number of alleles was 1.5, and genetic diversity among the five strains was 0.275 (Table 2).

Division III included three ETs (ETs 76 to 78), which were represented by three isolates from sheep with broken-mouth periodontitis. These isolates resembled those of division I in that they were catalase negative (biotype 1). Because they lacked activity for ES1 and GP3, assessments of genetic diversity and distance for these isolates were based on only 15 loci. Seven of 15 loci (47%) were polymorphic, the mean number of alleles was 1.5, and genetic diversity was 0.356 (Table 2).

Genetic relationships among ETs of division I. The dendrogram in Fig. 2 summarizes the genetic relationships among the 71 ETs in division I. The smallest observed genetic distance (0.06) corresponds to a single-locus difference between ETs, and the largest distance (0.38) reflects an average difference at 8 of the 17 loci assayed. Clusters of three or more ETs diverging from one another at a genetic distance of 0.12 or less were truncated at the deepest level of divergence of the lineages. At a genetic distance of 0.28, there were

TABLE 2. Measures of genetic diversity among isolates of *P. gingivalis* in divisions I, II, and III

Division	No. of isolates	No. of ETs	% Polymorphic loci	Mean no. of alleles	H^a
I	92	71	82	3.2	0.321
II	5	4	47	1.5	0.275
III[b]	3	3	47	1.5	0.356
I, II, and III	100	78	100	4.5	0.384

[a] *H*, mean genetic diversity per locus.
[b] Assessment of genetic diversity was on the basis of 15 loci.

TABLE 3. Number of alleles and genetic diversity per enzyme locus for 92 isolates of *P. gingivalis*[a]

Locus[b]	No. of alleles	h^c
ADA	4	0.527
CAT	1[d]	0.000
ES1	7	0.801
ES2	5	0.446
GDA	3	0.561
GLU	2	0.084
GP1	3	0.382
GP2	3	0.369
GP3	5	0.722
IP1	2	0.089
IP2	2	0.064
LGG	8	0.836
MDH	3	0.124
NSP	3	0.043
PGD	1	0.000
PGI	1	0.000
PGM	2	0.402
Mean	3.2	0.321

[a] All isolates were in division I.

[b] Abbreviations: ADA, adenosine deaminase; CAT, catalase; ES1, esterase 1; ES2, esterase 2; GDA, guanine deaminase; GLU, glutamate dehydrogenase; GP1, glycine-phenylalanine peptidase 1; GP2, glycine-phenylalanine peptidase 2; GP3, glycine-phenylalanine peptidase 3; IP1, indophenol oxidase 1; IP2, indophenol oxidase 2; LGG, leucine-glycine-glycine peptidase; MDH, malate dehydrogenase; NSP, nucleoside phosphorylase; PGD, phosphoglycerate dehydrogenase; PGI, phosphoglucose isomerase; PGM, phosphoglucomutase.

[c] *h*, genetic diversity per locus.

[d] For the purposes of this study, we have assigned the absence of CAT activity to one allele (CAT^1).

eight clusters of ETs and six lineages represented by single ETs; these are labelled by letters (A to N) in Fig. 2.

Evidence of strong genetic differentiation among 72 isolates in the major clusters A, B, E, and I is provided by highly significant values of G_{ST} for six of the enzyme loci (Table 4). For example, a large proportion of the total genetic diversity at the ADA locus among these isolates was apportioned among the four clusters ($G_{ST} = 0.442$). This is in part the result of the fixation of one ADA allele in the isolates of cluster A, while another ADA allele was predominant (frequency, 0.889) among strains of cluster I. The mean G_{ST} among all 17 loci for isolates of the four clusters was 0.253 (Table 4), which indicates that 25% of the total single-locus diversity is accounted for by differences among the clusters.

Genetic variation in relation to clinical disease. Sixty-nine of the 92 isolates in division I were recovered from subgingival lesions in adult patients with periodontitis (Table 1). These isolates were of 63 ETs, one or more of which was represented in each of the 14 clusters or lineages, except F and G (Table 1 and Fig. 2). Eight isolates were recovered from infected root canals (Table 1), and the seven ETs they represented (indicated by squares in Fig. 2) were widely distributed in the dendrogram. ET 31 was represented by one periodontal isolate and one root canal isolate. There was little or no evidence of genetic differentiation between the strains recovered from periodontal disease and those from root canal infections, as reflected by a low value for mean G_{ST} among loci (0.077). Similarly, the isolates recovered from patients with other oral infections (Table 1) did not represent the same ET or a group of related ETs; neither did strains recovered from patients with diabetes (ETs 4, 11, 37, 61, and 62).

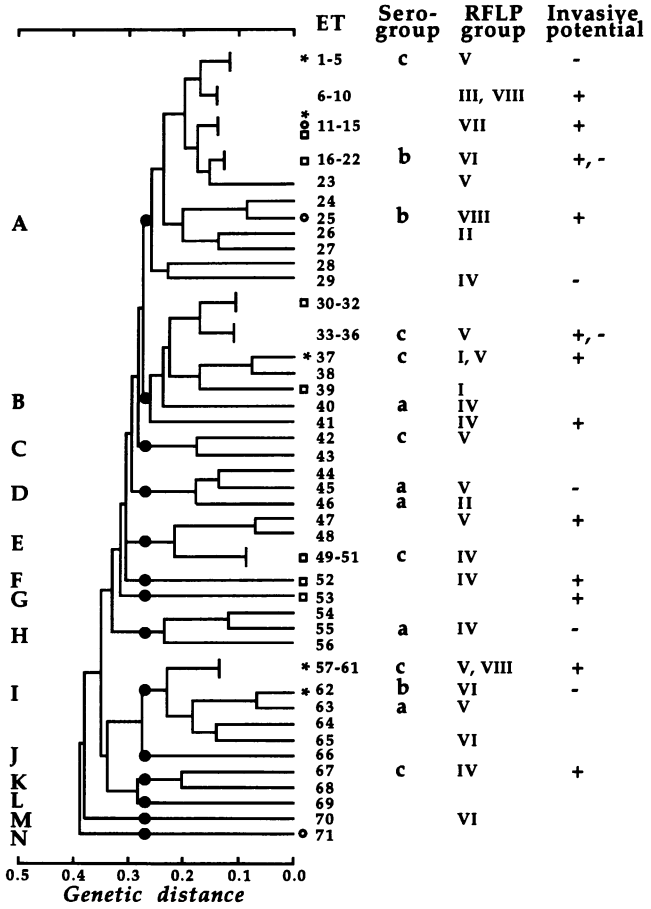

FIG. 2. Genetic relationships among the 71 ETs of *P. gingivalis* of division I. ETs are numbered sequentially from top to bottom. Each cluster that was represented by multiple ETs diverging from one another at a genetic distance of 0.12 or less was truncated at the deepest level of divergence of the ETs. Fourteen major clusters or lineages are indicated by a letter code (A to N). ETs of isolates from infected root canals are indicated by a square, ETs of isolates from periodontally healthy adolescents are indicated by a circle, and ETs of isolates from patients with diabetes are indicated by an asterisk (also Table 1). Available information regarding serogroup (a, b, or c) (7, 19), fimbrial RFLP group (roman numerals) (17), and invasive potential (+ or −) (4, 23) of the isolates is indicated in the columns to the right of the figure.

Two isolates (DCR 2011 and DCR 2015) of ET 71, which is separated from all other ETs by an average genetic distance of 0.38, were recovered 2 years apart from the same subgingival site in the same patient. At the time of the first sampling, the site had been diagnosed as healthy, but 2 years later, the site had developed periodontal disease. The two other isolates recovered from healthy subjects (DCR 2006 and 13JC) represented ET 11 and ET 25, respectively, both of which are in cluster A and which have no particular genetic relationship to ET 71. The widespread distribution of these three ETs in the dendrogram (Fig. 2) suggests that strains recovered from healthy subjects do not form a single, genetically distinct group.

Genetic structure of populations. Of the 71 ETs in division I, 17 were represented by two or three isolates. The isolates of 10 of these 17 ETs were from patients in different localities and even on different continents. For example, isolates of

TABLE 4. Genetic diversity and coefficients of differentiation for 17 enzyme loci in 72 *P. gingivalis* isolates[a]

Locus	H_S[b]	H_T[c]	G_{ST}[d]
ADA	0.300	0.538	0.442*
CAT	0.000	0.000	
ES1	0.570	0.744	0.234*
ES2	0.038	0.326	0.883*
GDA	0.391	0.549	0.289
GLU	0.143	0.081	0.000
GP1	0.236	0.230	0.000
GP2	0.185	0.278	0.334
GP3	0.605	0.689	0.121*
IP1	0.048	0.058	0.178
IP2	0.086	0.081	0.000
LGG	0.658	0.806	0.183*
MDH	0.119	0.131	0.092*
NSP	0.013	0.028	0.538
PGD	0.000	0.000	
PGI	0.000	0.000	
PGM	0.293	0.390	0.249
Mean	0.217	0.290	0.253

[a] Isolates were across clusters A, B, E, and I. See footnote *b* in Table 3 for enzyme abbreviations.
[b] Mean diversity of isolates within clusters A, B, E, and I.
[c] Diversity in the combined sample of 72 isolates in clusters A, B, E, and I.
[d] Coefficients of differentiation were calculated as explained in the text. Significance was determined from contingency tables: *, $P \leq 0.005$.

ET 12 were recovered from patients in North America (Buffalo, N.Y.) and Africa (Kenya), and isolates of ET 48 were from patients in Switzerland and Japan (Table 1). Each of seven ETs was represented by isolates recovered at the same locality, either from different host individuals or, in one case (ET 71), from the same individual 2 years apart. ET 37 was represented by isolates from three patients on the Pima Indian Reservation, whose subgingival samples were taken over a 2-year period. Collectively, these findings suggest that the population structure of *P. gingivalis* is basically clonal, with chromosomal genotypes persisting long enough to achieve widespread, if not global, distributions.

For several localities, multiple isolates were available; strains from any one locality appeared to be of a wide variety of genotypes, corresponding with the observation above that there is, in general, a worldwide distribution of clones. To confirm this observation, G_{ST} was calculated as a measure of subdivision for samples of isolates from five localities (Buffalo, N.Y.; Amsterdam, the Netherlands; Umeå and Gothenburg, Sweden; and Ann Arbor, Mich.). In this analysis, the G_{ST} value was 0.007, indicating that virtually none of the diversity is apportioned among localities.

Genetic variation in relation to phenotypic properties. For some isolates, we have information on their serogroups, fimbrial RFLP groups, and invasive potentials (Fig. 2). Seventeen isolates were assigned to one of three serogroups, a, b, and c (7, 19); for 32 isolates, the fimbrial RFLP group was known (17); and for 22 isolates, we had information on whether they were invasive (+) or noninvasive (−) in the mouse model (4, 23). None of these three properties showed a clear pattern of association with particular ETs or a group of related ETs (Fig. 2). For example, isolates in cluster A were represented by strains of both serogroups b and c and fimbrial RFLP groups II, IV, V, VI, and VII, and both invasive and noninvasive phenotypes were represented in cluster A.

Laboratory strains. We noted that five commonly used laboratory strains belonged to only two ETs. Strains W50 and W83 were both of ET 25, and strains 2561 (ATCC 33277^T), 381, and W represented ET 55.

DISCUSSION

Unclassified (cryptic) porphyromonad species. The estimates of genetic relatedness based on multilocus enzyme electrophoresis for species of the *Streptococcus mutans* complex (10) and several other bacteria (24, 28, 30) have been shown to be strongly correlated with measures of similarity in total nucleotide sequence derived from DNA hybridization experiments. Thus, there is reason to believe that the observed allelic variation at 17 enzyme loci in *Porphyromonas* strains usefully indexes the overall relationships of their chromosomal genomes. Some isolates in our collection were sufficiently different in multilocus genotype as to suggest a degree of nucleotide sequence similarity well below the level now widely accepted as the criterion of species limits in bacteria (13). Because the genetic distances between the *P. gingivalis* ETs of division I and ETs of divisions II and III are large (0.70 or 0.87) (Fig. 1), it appears that we have uncovered two cryptic species of *Porphyromonas*, both of which may be exclusively associated with animal hosts. Porphyromonads of division II (ETs 72 to 75) (Fig. 1; Table 1) (catalase activity present) were recovered from a cat, a dog, and three squirrel monkeys, whereas those of division III (ETs 76 to 78) (Fig. 1 and Table 1) (catalase activity absent) were isolated from sheep with broken-mouth periodontitis. We propose that the species *P. gingivalis* (strict sense) is limited to the human host, as independently suggested by Fournier and Mouton (8) on the basis of serological and biochemical characteristics. Four isolates in division I (ETs 18, 49, and 50) (Fig. 2 and Table 1) form an apparent exception, since they were recovered from cynomolgus monkeys, but inasmuch as these isolates were closely related to human isolates, it is possible that these monkeys, while living in laboratory animal facilities, were infected with strains originating from their handlers. However, the squirrel monkey isolates in division II may represent indigenous porphyromonads limited to animal hosts (8).

The two cryptic *Porphyromonas* species are not to be confused with *Bacteroides macacae* and *Bacteroides salivosus*, which are also gram-negative, anaerobic, black-pigmented coccobacilli (20, 33). These *Bacteroides* species have API ZYM profiles distinct from those of all 100 isolates examined in the present study. Moreover, the type strains of these two *Bacteroides* species showed no homology to the fimbrial gene probe derived from strain 381 (ET 55, division I), whereas moderate or weak homology was shown by strains of division II (ET 75) and a strain of division III (ET 78) (17). Additionally, multilocus enzyme electrophoresis analysis demonstrated that *B. macacae* does not share any alleles with the isolates of *P. gingivalis* of division I or with those of divisions II and III (data not shown).

Genetic diversity in *P. gingivalis* (strict sense). The present multilocus enzyme electrophoresis analysis demonstrated extensive genetic heterogeneity among *P. gingivalis* (division I) isolates, which was earlier indicated by DNA fingerprinting (19). Among the 92 isolates, we identified 71 ETs, and the observed degree of genetic diversity ($H = 0.321$) is within the range reported for many other human pathogenic bacteria, including *Legionella pneumophila* (30), *Haemophilus influenzae* serotype b (21), and the periodontal pathogen *Actinobacillus actinomycetemcomitans* (3).

Lack of relationship between phenotypic characteristics and phylogenetic structure. We were unable to discern any pattern of association between serogroups and the distribution of *P. gingivalis* isolates in the dendrogram (Fig. 2). This result was not surprising, because it has previously been shown for *Neisseria meningitidis*, *Escherichia coli*, and several other bacteria that strains of the same serotype are not necessarily genetically related and that, conversely, genetically related strains may have different serotypes (1, 2, 31). Cell-surface antigens may be adaptive in response to host defense mechanisms and other environmental pressures and are, therefore, subject to both convergence as a result of natural selection and to rapid divergence through diversifying selection, as well as recombination (31).

Previously, we identified nine distinct fimbrial RFLP groups among 39 strains of *P. gingivalis* (17). The fact that we found no association between ETs and RFLP groups was again not unexpected, since fimbriae are surface appendages. And finally, we found no relationship between the invasive potential of various strains of *P. gingivalis* and the genetic structure of the natural population, which means that strains of a wide variety of clonal genotypes can cause invasive infections in the mouse model.

A wide variety of *P. gingivalis* genotypes is associated with disease. The isolates from periodontal disease in adults, representing 63 ETs, as well as those from infected root canals (7 ETs) were widely distributed in the dendrogram (Fig. 2). The few available isolates from periodontally healthy individuals showed no particular relationship to one another (Fig. 2). Thus, with respect to the distribution of pathogenic potential among strains, *P. gingivalis* differs from many other pathogenic bacteria, such as *H. influenzae* serotype b and *N. meningitidis*, in which most strains causing disease represent one or a few clones (31). Instead, the genetic structure of *P. gingivalis* is reminiscent of that of nontypeable *H. influenzae* (21), which is a commensal in the oral and nasopharyngeal floras of healthy children and adults and acts as an opportunistic pathogen.

In sum, there is no evidence that specific genetic lineages or clusters of clones of *P. gingivalis* (strict sense) are associated with distinct types of oral infections; dental patients can be infected by a wide variety of chromosomal genotypes, which suggests that interclonal variation in pathogenicity is relatively small. This supports the hypothesis, previously suggested by epidemiological evidence (18), that the role of this microorganism in the pathogenesis of periodontitis and other oral infections is largely opportunistic. However, the available data do not exclude the alternative possibility that the pathogenic strains possess virulence factors that are generally not found in strains colonizing healthy individuals. These hypotheses can be tested by further comparative analyses of the characteristics of large numbers of *P. gingivalis* strains recovered from both healthy subjects and patients with periodontitis or other oral infections.

ACKNOWLEDGMENTS

We thank D. P. Dickinson for his instigation of this study and M. N. Gilmour and P. Beltran for technical advice on the selection of enzymes for analysis. We also acknowledge the following investigators for their courteous participation in providing strains: G. H. W. Bowden, J. Carlsson, G. Dahlén, J. L. Dzink, S. Edwardsson, K. W. Frisken, C. A. Genco, R. D. Gmür, J. A. Herweijer, S. C. Holt, M. Lantz, W. J. Loesche, D. Mayrand, C. A. Mouton, A. M. Nilius, R. J. Nisengard, H. S. Reynolds, T. J. M. van Steenbergen, G. K. Sundqvist, C. Walker, R. F. Wilson, A. J. van Winkelhoff, L. F. Wolff, F. Yoshimura, and J. J. Zambon. Finally, we thank the staff in the laboratory of R.K.S. for technical assistance.

This study was supported by Public Health Service grants DE07034, DE08240, DE04898, AI22144, and AI24566 and by the Mark Diamond Fund from the Graduate Student Association of the State University of New York at Buffalo.

REFERENCES

1. **Caugant, D. A., B. R. Levin, I. Ørskov, F. Ørskov, C. Svanborg Eden, and R. K. Selander.** 1985. Genetic diversity in relation to serotype in *Escherichia coli*. Infect. Immun. **49**:407–413.
2. **Caugant, D. A., L. F. Mocca, C. E. Frasch, L. O. Frøholm, W. D. Zollinger, and R. K. Selander.** 1987. Genetic structure of *Neisseria meningitidis* populations in relation to serogroup, serotype, and outer membrane protein pattern. J. Bacteriol. **169**:2781–2792.
3. **Caugant, D. A., R. K. Selander, and I. Olsen.** 1990. Differentiation between *Actinobacillus* (*Haemophilus*) *actinomycetemcomitans*, *Haemophilus aphrophilus* and *Haemophilus paraphrophilus* by multilocus enzyme electrophoresis. J. Gen. Microbiol. **136**:2135–2141.
4. **Chen, P. B.** 1992. Personal communication.
5. **Clark, W. B., I. Magnusson, C. Abee, B. Collins, J. E. Beem, and W. P. McArthur.** 1988. Natural occurrence of black-pigmented *Bacteroides* species in the gingival crevice of the squirrel monkey. Infect. Immun. **56**:2392–2399.
6. **Dzink, J. L., S. S. Socransky, and A. D. Haffajee.** 1988. The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. J. Clin. Periodontol. **15**: 316–323.
7. **Fisher, J. G., J. J. Zambon, and R. J. Genco.** 1987. Identification of serogroup-specific antigens among *Bacteroides gingivalis* components. J. Dent. Res. **66**:222.
8. **Fournier, D., and C. Mouton.** 1992. The animal reservoir is excluded for transmission to humans of *Bacteroides* (*Porphyromonas*) *gingivalis*. J. Dent. Res. **71**:216.
9. **Frisken, K. W., J. R. Tagg, A. J. Laws, and M. B. Orr.** 1988. Suspected periodontopathic bacteria associated with broken-mouth periodontitis in sheep. J. Periodontal Res. **23**:18–21.
10. **Gilmour, M. N., T. S. Whittam, M. Kilian, and R. K. Selander.** 1987. Genetic relationships among the oral streptococci. J. Bacteriol. **169**:5247–5257.
11. **Hjelmeland, L. M., and A. Chrambach.** 1984. Solubilization of functional membrane proteins. Methods Enzymol. **104**:305–328.
12. **Holt, S. C., J. Ebersole, J. Felton, M. Brunsvold, and K. S. Kornman.** 1988. Implantation of *Bacteroides gingivalis* in nonhuman primates initiates progression of periodontitis. Science **239**:55–57.
13. **Johnson, J. L.** 1984. Nucleic acids in bacterial classification, p. 8–11. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology. Williams & Wilkins, Baltimore, Md.
14. **Klausen, B., R. T. Evans, N. S. Ramamurthy, L. M. Golub, C. Sfintescu, J.-Y. Lee, G. Bedi, J. J. Zambon, and R. J. Genco.** 1991. Periodontal bone level and gingival proteinase activity in gnotobiotic rats immunized with *Bacteroides gingivalis*. Oral Microbiol. Immunol. **6**:193–201.
15. **Kornman, K. S., S. C. Holt, and P. B. Robertson.** 1981. The microbiology of ligature-induced periodontitis in the cynomolgus monkey. J. Periodontal Res. **16**:363–371.
16. **Laliberté, M., and D. Mayrand.** 1983. Characterization of black-pigmented *Bacteroides* strains isolated from animals. J. Appl. Bacteriol. **55**:247–252.
17. **Loos, B. G., and D. W. Dyer.** 1992. Restriction fragment length polymorphism analysis of the fimbrillin locus, *fimA*, of *Porphyromonas gingivalis*. J. Dent. Res. **71**:1173–1181.
18. **Loos, B. G., D. W. Dyer, R. J. Genco, R. K. Selander, and D. P. Dickinson.** Natural history and epidemiology of *Porphyromonas gingivalis*. *In* H. N. Shah, D. Mayrand, and R. J. Genco (ed.), *Porphyromonas gingivalis*, in press. CRC Press, Inc., Boca Raton, Fla.
19. **Loos, B. G., D. Mayrand, R. J. Genco, and D. P. Dickinson.** 1990. Genetic heterogeneity of *Porphyromonas* (*Bacteroides*)

gingivalis by genomic DNA fingerprinting. J. Dent. Res. **69:** 1488–1493.

20. **Love, D. N., J. L. Johnson, R. F. Jones, and A. Calverley.** 1987. *Bacteroides salivosus* sp. nov., an asaccharolytic black-pigmented species from cats. Int. J. Syst. Bacteriol. **37:**307–309.
21. **Musser, J. M., S. J. Barenkamp, D. M. Granoff, and R. K. Selander.** 1986. Genetic relationships of serologically nontypeable and serotype b strains of *Haemophilus influenzae*. Infect. Immun. **52:**183–191.
22. **Nagata, A., T. Man-yoshi, M. Sato, and R. Nakamura.** 1991. Serological studies of *Porphyromonas* (*Bacteroides*) *gingivalis* and correlation with enzyme activity. J. Periodontal Res. **26:** 184–190.
23. **Neiders, M. E., P. B. Chen, H. Suido, H. S. Reynolds, J. J. Zambon, M. Shlossman, and R. J. Genco.** 1989. Heterogeneity of virulence among strains of *Bacteroides gingivalis*. J. Periodontal Res. **24:**192–198.
24. **Ochman, H., T. S. Whittam, D. A. Caugant, and R. K. Selander.** 1983. Enzyme polymorphism and genetic population structure in *Escherichia coli* and *Shigella*. J. Gen. Microbiol. **129:**2715–2726.
25. **Parent, R., C. Mouton, L. Lamonde, and D. Bouchard.** 1986. Human and animal serotypes of *Bacteroides gingivalis* defined by crossed immunoelectrophoresis. Infect. Immun. **51:**909–918.
26. **Robert, J. C., and C. Mouton.** 1989. *Bacteroides gingivalis* and other black-pigmented *Bacteroides* in children. J. Dent. Res. **68:**987.
27. **Rodenburg, J. P., A. J. van Winkelhoff, E. G. Winkel, R. J. Goené, F. Abbas, and J. de Graaff.** 1990. Occurrence of *Bacteroides gingivalis*, *Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in severe periodontitis in relation to age and treatment history. J. Clin. Periodontol. **17:**392–399.
28. **Selander, R. K., P. Beltran, and N. H. Smith.** 1991. Evolutionary genetics of *Salmonella*, p. 25–57. *In* R. K. Selander, A. G. Clark, and T. S. Whittam (ed.), Evolution at the molecular level. Sinauer Associates, Inc., Sunderland, Mass.
29. **Selander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. N. Gilmour, and T. S. Whittam.** 1986. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl. Environ. Microbiol. **51:**873–884.
30. **Selander, R. K., R. M. McKinney, T. S. Whittam, W. F. Bibb, D. J. Brenner, F. S. Nolte, and P. E. Pattisson.** 1985. Genetic structure of populations of *Legionella pneumophila*. J. Bacteriol. **163:**1021–1037.
31. **Selander, R. K., and J. M. Musser.** 1990. Population genetics of bacterial pathogenesis, p. 11–36. *In* B. H. Iglewski and V. L. Clark (ed.), The bacteria: a treatise on structure and function, vol. XI. Molecular basis of bacterial pathogenesis. Academic Press, Inc., New York.
32. **Shah, H. N., and M. D. Collins.** 1988. Proposal for reclassification of *Bacteroides asaccharolyticus*, *Bacteroides gingivalis*, and *Bacteroides endodontalis* in a new genus, *Porphyromonas*. Int. J. Syst. Bacteriol. **38:**128–131.
33. **Slots, J., and R. J. Genco.** 1980. *Bacteroides melaninogenicus* subsp. *macacae*, a new subspecies from monkey periodontopathic indigenous microflora. Int. J. Syst. Bacteriol. **30:**82–85.
34. **Sundqvist, G., E. Johansson, and U. Sjögren.** 1989. Prevalence of black-pigmented *Bacteroides* species in root canal infections. J. Endod. **15:**13–19.
35. **Tanner, A. C. R., S. S. Socransky, and J. M. Goodson.** 1984. Microbiota of periodontal pockets losing crestal alveolar bone. J. Periodontal Res. **19:**279–291.
36. **van Steenbergen, T. J. M., F. G. A. Delemarre, F. Namavar, and J. de Graaff.** 1987. Differences in virulence within the species *Bacteroides gingivalis*. Antonie Leeuwenhoek **53:**233–244.
37. **van Winkelhoff, A. J., A. W. Carlee, and J. de Graaff.** 1985. *Bacteroides endodontalis* and other black-pigmented *Bacteroides* species in odontogenic abscesses. Infect. Immun. **49:** 494–497.
38. **van Winkelhoff, A. J., U. van der Velden, M. Clement, and J. de Graaff.** 1988. Intra-oral distribution of black-pigmented *Bacteroides* species in periodontitis patients. Oral Microbiol. Immunol. **3:**83–85.
39. **Watson, M.-R., D. E. Lopatin, W. A. Bretz, I. J. Ertel, and W. J. Loesche.** 1991. Detection of two anaerobic periodontopathogens in children by means of the BANA and ELISA assays. J. Dent. Res. **70:**1052–1056.
40. **Whittam, T. S., M. L. Wolfe, and R. A. Wilson.** 1989. Genetic relationships among *Escherichia coli* isolates causing urinary tract infections in humans and animals. Epidemiol. Infect. **102:**37–46.