INFECTION AND IMMUNITY, Mar. 1986, p. 909–918
0019-9567/86/030909-10$02.00/0

Vol. 51, No. 3

Human and Animal Serotypes of *Bacteroides gingivalis* Defined by Crossed Immunoelectrophoresis

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Received 3 September 1985/Accepted 15 November 1985

The antigenic complexity of three strains of *Bacteroides gingivalis* and four strains resembling *B. gingivalis* isolated from animals was analyzed and compared by crossed immunoelectrophoresis. Thirteen antigens of the human biotype were present in all human strains and six antigens of the animal biotype were present in all animal strains, indicating a marked serological homogeneity within each biotype. Four antigens cross-reacting between the human *B. gingivalis* and the animal strains were identified. This antigenic relatedness defined the serological homogeneity of the two biotypes within the species and allowed recognition of four species-specific antigens. Two antigens specific to the human strains and two antigens specific to the animal strains were identified, indicating that serotype-specific antigens can distinguish each biotype. It is thus proposed that the oral, black-pigmented asaccharolytic *Bacteroides* strains of animal origin be classified as catalase-positive variants of *B. gingivalis*. It is also proposed that two serotypes be recognized within the species *B. gingivalis*. Serotype 1 includes the catalase-negative human biotype, and serotype 2 includes the catalase-positive animal biotype.

Many significant human and animal pathogens are found in the genus *Bacteroides* (11). Although *Bacteroides asaccharolyticus* is almost identical biochemically to *B. gingivalis*, the two species are isolated from different sources. *B. asaccharolyticus* is rarely isolated from the human mouth, whereas *B. gingivalis* is often isolated from the gingival area of chronic periodontitis patients. Strains now called *B. gingivalis* (6, 39) were first named "oral *B. melaninogenicus* subsp. *asaccharolyticus*" according to the classification of Holdeman and Moore (12) and then "oral *B. asaccharolyticus*" according to Finegold and Barnes (9). The recognition of the species *B. gingivalis* was justified by studies performed on human strains. Such studies included DNA homology and base composition (6, 32, 39), biochemical properties including production of phenylacetic acid (23, 42), hemagglutination (35), enzymatic properties (18, 33), and polypeptide analysis (37). A study by Laliberté and Mayrand (16) reported the isolation of 104 strains of black-pigmented, nonsaccharolytic *Bacteroides* spp. from the gingival sulcus of four animal species (cat, dog, jaguar, and raccoon) which only differed from human strains of *B. gingivalis* by a catalase-positive reaction. These animal strains showed some degree of similarity with catalase-positive strains previously isolated from beagle dogs and characterized by Syed (38).

It is now recognized that *B. gingivalis* may play a central role in the etiology of certain forms of periodontal disease (34, 36). It thus appears necessary to determine the antigenic complexity of the species and to evaluate the potential contribution of serology to the taxonomy of this species within the group of nonsaccharolytic *Bacteroides* spp. that produce pigment. Furthermore, considering the difficulty of isolating the bacterium from clinical specimens, it can be anticipated that serological techniques will be helpful in developing rapid methods of identification. Serological techniques will also be helpful in analyzing the virulence factors of *B. gingivalis*. The literature on the serology of *B. gingivalis* is, however, relatively scarce (10, 17, 21, 25, 26, 30). In particular, the antigenic profile of the species has not yet been studied.

The purpose of the present study was to examine and compare the antigenic complexity of several strains of *B. gingivalis* of human origin and of *Bacteroides* sp. strains resembling *B. gingivalis* isolated from animals by the technique of crossed immunoelectrophoresis (CIE). In this paper we describe serogrouping of the human and animal strains within the species *B. gingivalis*. Common and biotype-specific antigens were also identified.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. gingivalis* strain ATCC 33277 was obtained from the American Type Culture Collection. All other strains were kindly provided by Denis Mayrand. Strain 17A3 was isolated in 1979 from the pocket flora of a human female patient with chronic periodontitis. Strain W83 presumably was isolated as early as 1967 (41) from a human "clinical specimen" (32) and was later characterized as *B. gingivalis* (39). The animal strains resembling *B. gingivalis* were isolated from the subgingival plaque of four different species, cat (strain Cat-1), dog (strain Dog-5B), jaguar (strain Jaguar-2), and raccoon (strain Raccoon-1), by Laliberté and Mayrand (16). The strains were anaerobically maintained on human blood agar plates–modified M10 (20) enriched with hemin (10 μg/ml) and vitamin K_1 (5 μg/ml) and grown in enriched Trypticase (BBL Microbiology Systems)-yeast extract broth. They were checked for purity by Gram reaction, inoculation of a duplicate set of blood agar plates for aerobic and anaerobic incubation, and by the API-ZYM enzymatic tests (18, 33).

Preparation of antigens. Cells were harvested by centrifugation (10,000 × *g* for 20 min at 4°C). The wet weight yield of cells was about 5.0 g/liter. Cells were washed twice in sterile 10 mM phosphate-buffered 0.15 M NaCl solution, pH 7.6. The final pellet was suspended in 50 mM Tris hydrochloride buffer, pH 8.6, containing 5 mM EDTA at a

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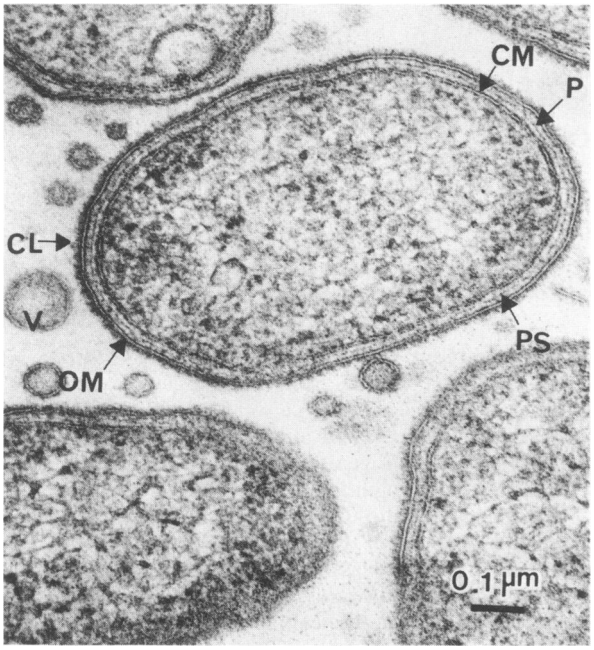

FIG. 1. Electron micrograph of ultrathin section of *B. gingivalis* ATCC 33277 intact cells stained with uranyl acetate and lead citrate. CL, Capsule-like layer; CM, cytoplasmic membrane; OM, outer membrane; P, peptidoglycan; PS, periplasmic space, V, vesicle.

concentration of 3 g (wet weight) of cells in 35 ml of buffer. To this suspension, in a 250-ml Erlenmeyer flask, was added 5 ml of washed and sterilized glass beads (Sigma Chemical Co.) with an average diameter of 0.15 to 0.20 mm. The mixture was agitated for 48 h at 4°C on a horizontal rotator at 80 oscillations per min and was then centrifuged at 10,000 × *g* for 20 min at 4°C.

The protein concentration of the resulting supernatant, designated crude glass bead-EDTA extract (GBE), was measured by the method of Bradford (3). The GBE was divided into two portions: the first portion was used for immunization of rabbits, and the second portion was treated with Triton X-100 (Calbiochem). To 3 volumes of crude GBE was added 1 volume of 20% (vol/vol) Triton X-100 in distilled water. The solution, chilled on ice, was sonicated for 10 s, using a Sonifier cell disruptor W-350 (Heat Systems Ultrasonics Inc.) equipped with a microprobe and set at energy level 5 and at 50% duty cycle, and was then further agitated for 48 h at 4°C. The Triton X-100-insoluble material was pelleted by centrifugation at 100,000 × *g* for 2 h at 4°C and the supernatant fraction containing the solubilized antigens was designated GBE-T.

The nucleic acid content of the extract of strain *B. gingivalis* ATCC 33277 was estimated by the ratio of absorption at 280 nm/260 nm. A sample of cells of strain ATCC 33277 was also disintegrated by vortexing in the presence of glass beads according to Moore et al. (24).

Production of antisera. Antisera against whole cells of strain ATCC 33277 were produced in rabbits, using a suspension (approximately 1.2×10^9 cells per ml) of Formalin-fixed bacteria. Two New Zealand white female rabbits (3.0 to 4.0 kg) received daily intravenous injections with increasing doses of bacterial suspension ranging from 0.3 to 1.5 ml for a total of 10 injections. A 1-week rest period was followed by a booster series of three daily injections with 1.5 ml of antigen suspension. On day 27, 50 ml of blood was drawn. A series of booster injections was repeated 2 weeks after the bleeding. The sera obtained after the second, third, and fourth booster series of both animals were pooled to prepare the antiserum against whole cells of strain ATCC 33277.

Two rabbits each were used for production of antisera against the crude GBE of each strain, using 2 ml of antigen emulsified in 2 ml of Freund incomplete adjuvant. Two milliliters was injected intramuscularly and 0.4 ml was injected subcutaneously at five sites. Twenty-five days later the rabbits were boosted by injecting 0.4 ml of crude GBE subcutaneously at five sites. A 50-ml amount of blood was drawn 1 week later and the animals were rested for 2 weeks before another booster was given. The antiserum against the crude extract of strain ATCC 33277 consisted of a pool of serum obtained after the third, fourth, fifth, and eighth booster injections of both animals. The other antisera used in this study were obtained from a single animal. This animal was selected after preliminary CIE experiments to determine the individual rabbit yielding best results. The antiserum produced against strain W83 came from the bleeding following the third booster injection, while all other antisera came from the bleeding following the first booster injection.

CIE. CIE, CIE with intermediate gel, and tandem CIE were performed according to established procedures (1, 2, 15). Agarose 1% (type 1; low Electro Endo Osmosis [EEO]; Sigma) in Tris hydrochloride-barbital-sodium barbital buffer, pH 8.6 (ionic strength, 0.02), containing 1% (vol/vol) Triton X-100, was used in both first- and second-dimension electrophoreses.

Gels for electrophoresis in the first dimension were cast by layering 15 ml of agarose on glass plates (10 by 10 cm). Three antigen wells (4 mm in diameter) were punched out 1.5 cm from the cathodal end and 2 cm apart in the vertical axis of the glass plate. For tandem experiments, duplicate wells were punched out 0.8 cm apart. Samples applied to the wells were subjected to electrophoresis at 10 V/cm for 60 min in a water-cooled electrophoresis chamber (Multiphor 2117; LKB).

After the first-dimensional electrophoresis, agarose strips (2 by 8 cm) containing the separated antigens were cut and each was transferred to one side of a glass plate (8 by 10 cm). The remaining space on the plate was then covered with agarose containing antiserum at a concentration of 7.8 $\mu l/cm^2$. For CIE with an intermediate gel, a space 2 cm wide above the first-dimension antigen-containing strip was filled with agarose containing another antiserum. In control plates an intermediate gel containing no antibodies was cast. The agarose volume/surface area ratio in the second-dimension gel and in the intermediate gel was 0.15 ml/cm^2. Electrophoresis in the second dimension was performed at 2 V/cm for 18 to 20 h. The gels were pressed, washed twice in 0.15 M NaCl and once with distilled water, pressed again, stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories), and destained by the method of Weeke (40).

Electron microscopy. Intact cells harvested from blood agar plates, or freshly extracted cells, were washed once in 0.15 M sodium cacodylate buffer, pH 7.4, and pelleted by centrifugation at 3,000 × *g* for 5 min. The pellets were suspended in 0.1% (vol/vol) glutaraldehyde plus 2% (wt/vol) paraformaldehyde, buffered with 0.15 M sodium cacodylate, pH 7.4, and incubated at 37°C for 2 h. Cells were then washed twice with cacodylate buffer, postfixed with 1% (wt/vol) osmium tetroxide in buffer for 60 min at room temperature, washed again with buffer, dehydrated through a graded ethanol series and then propylene oxide, and

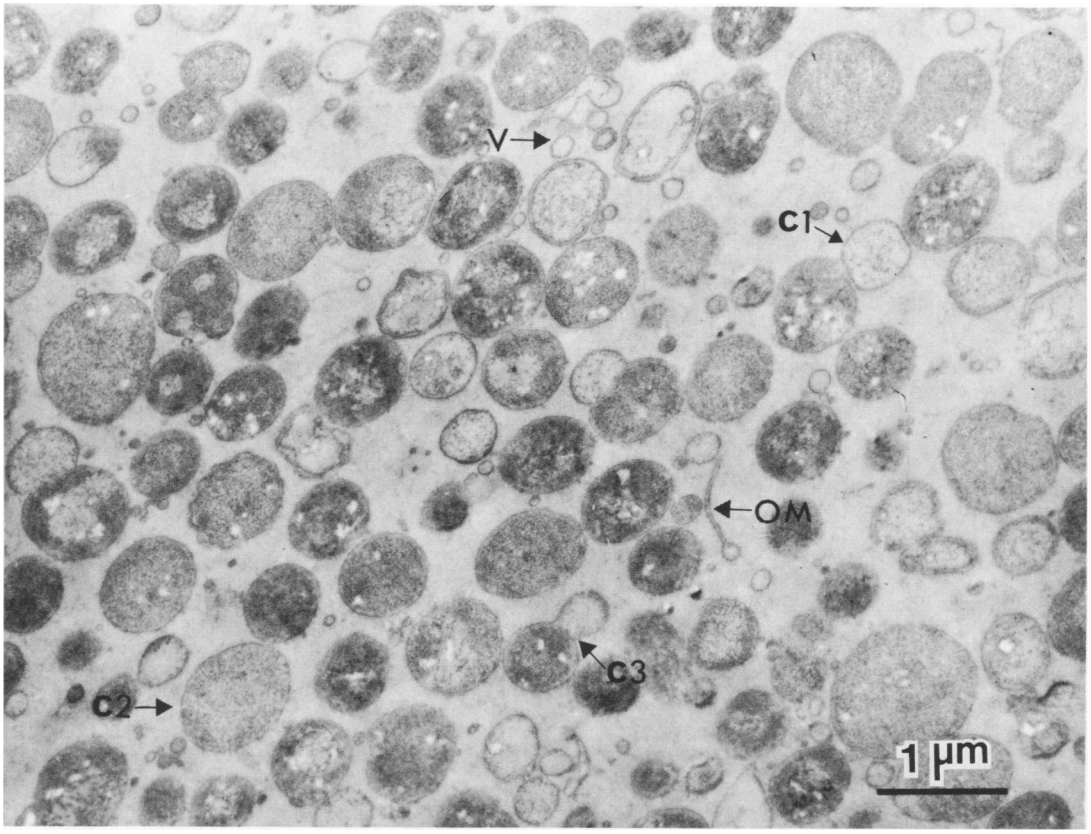

FIG. 2. Electron micrograph of ultrathin section of *B. gingivalis* ATCC 33277 after extraction, stained with uranyl acetate and lead citrate. C1, Ghost cell; C2; cell showing a totally exfoliated outer membrane; C3, cell showing a partially exfoliated outer membrane; F, outer membrane fragment; V, vesicle.

embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate.

Freshly extracted cells, as well as the crude GBE extract, also were negatively stained. One drop of a cell suspension (about 1.2×10^9 cells per ml) or of the crude GBE extract was spotted on a Formvar-coated, carbon-reinforced copper grid. One drop of a 2% (wt/vol) water solution of phosphotungstic acid, pH 6.3, containing approximately 1 U of bacitracin per ml, was then added on the grids. Excess liquid was removed with filter paper and the grids were air dried prior to examination.

Specimens were examined with a Philips EM 300 electron microscope operating at 60 kV.

RESULTS

Extraction of antigens. The nucleic acid content of the crude extract of strain ATCC 33277 was estimated at 0.86 mg per g (wet weight) of cells, while that of the supernatant of the disintegrated cells was 16.40 mg per g (wet weight) of cells. This indicated a minor contribution of cytoplasmic material in the crude extracts.

Transmission electron microscopy of ultrathin sections of intact cells of *B. gingivalis* ATCC 33277 (Fig. 1) revealed a typical ultrastructure of the cell envelope consistent with previous descriptions (21, 22, 27, 43). As reported by Listgarten and Lai (19), numerous intercellular membrane vesicles were observed. The proportion of ghost cells in the preparations of extracted cells (Fig. 2) did not differ from that in the preparations of freshly harvested cells. An increased number of outer membrane fragments and of vesicles were observed, however.

Negative staining of extracted cells (Fig. 3A) revealed the presence of thin fimbria-like fibrils radiating from the periphery of the cells. Partially or totally exfoliated outer membrane fragments were observed in close proximity to the otherwise intact cells. The negatively stained crude GBE (Fig. 3B) revealed the presence of fibrillar material, 4 to 6 nm in diameter and up to 700 nm in length, reminiscent of fimbriae.

Homologous antigenic profiles and reference CIE pattern of human strains. To enumerate and quantitate the antigens of each bacterial strain, the antigenic preparations were subjected to homologous CIE, using the crude extract antisera. An optimal antigen/antibody ratio was determined in preliminary experiments. With 60 μl of antigenic preparation and 7.8 μl of antiserum per cm^2 in the gels, the following working protein concentrations of antigen were retained: strain ATCC 33277, 0.9 mg/ml; strain 17A3, 0.6 mg/ml; and strain W83, 1.0 mg/ml. Twenty-five anodic migrating precipitates were detected and enumerated in the type strain of *B. gingivalis* strain ATCC 33227. The homologous CIE patterns of strains W83 and 17A3 were characterized by 19 and 17 immunoprecipitates, respectively. The CIE pattern of strain ATCC 33277 was thus selected as the reference pattern in the experiments that follow.

CIE reference pattern for analysis of human strains. The immunoprecipitates in the reference pattern are visible in Fig. 4. The most prominent immunoprecipitates stained by Coomassie brilliant blue were antigens numbered 2, 7, 8, 13,

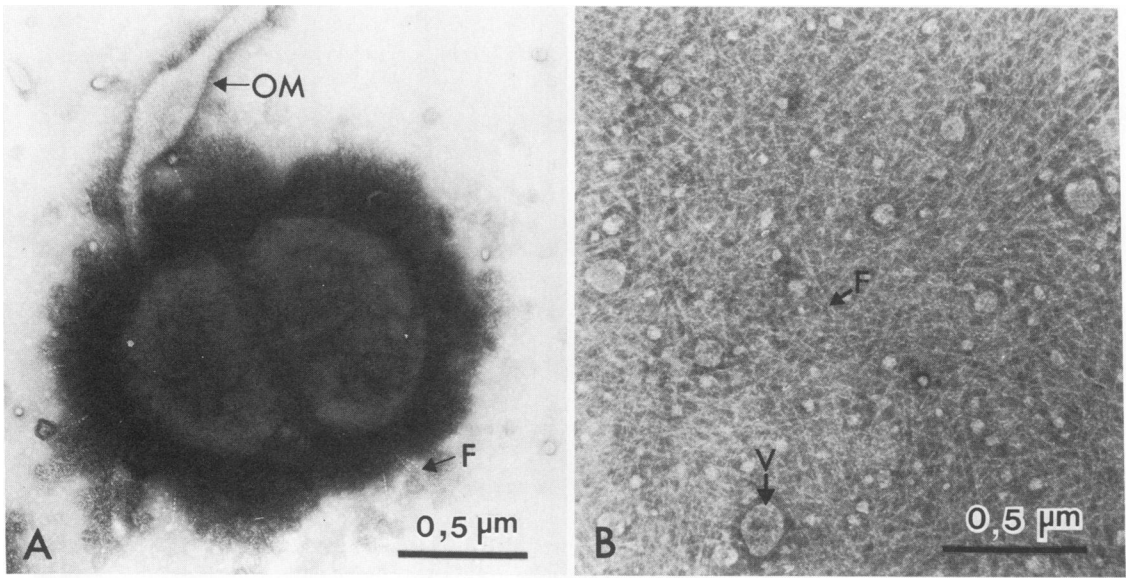

FIG. 3. Electron micrograph of phosphotungstic acid negatively stained preparation of (A) an extracted cell of *B. gingivalis* ATCC 33277 and (B) the crude extract obtained from the same strain. F, Fibrillar material reminiscent of fimbriae; OM, outer membrane fragment; V, vesicle.

and 14. The weaker immunoprecipitates, either partially lost on photographic reproduction or difficult to discern on immunoplates, were antigens 1, 3, 4, 10, 12, 15, 17, 21, 22, 23, 24, and 25. Some immunoprecipitates which showed various degrees of heterogeneity were antigens 2, 12, and 19. Of these, however, only antigen 2 showed characteristics suggestive of multiple products originating from endogeneous degradation. The peaks observed were numbered 2a, 2b, 2c, and 2d. Precipitates 2b and 2c were seen to split from precipitate 2a, whereas precipitate 2d resulted from the fusion, after a further anodal migration, of precipitates 2b and 2c. Immunoprecipitates 12 and 19 appeared as double peaks, which were numbered 12a and 12b and 19a and 19b, respectively (Fig. 4).

It is known that low electrophoretic migration of some antigens and electroendosmosis flow resulting in undesired cathodal migration of antibodies may impair resolution of immunoprecipitates. An antibody-free intermediate gel was

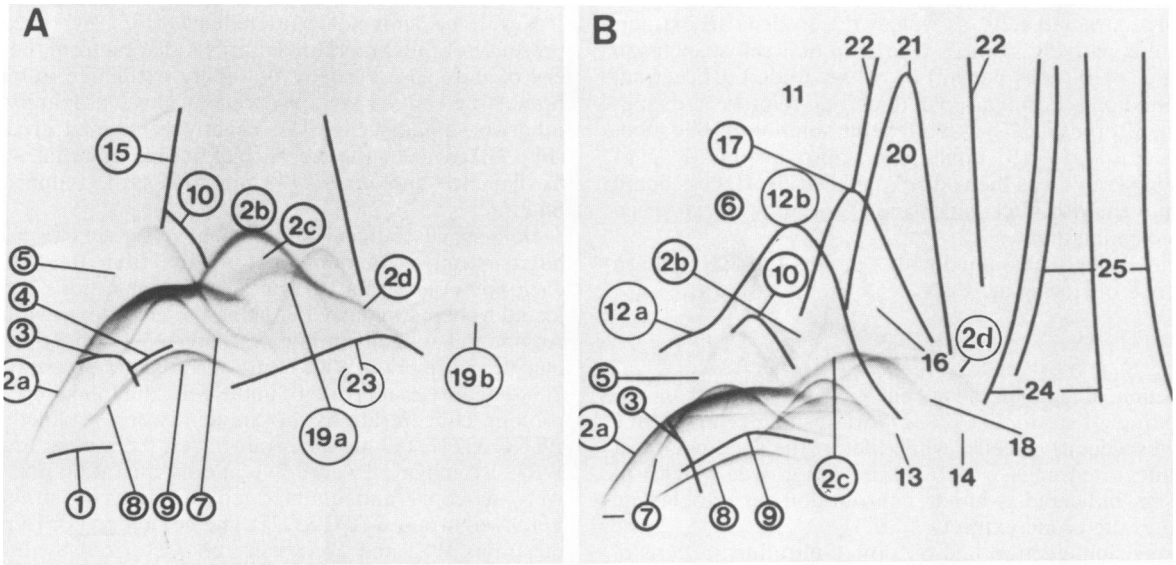

FIG. 4. Homologous CIE of *B. gingivalis* strain ATCC 33277. A volume of 60 µl of GBE-T was electrophoresed in each case. (A) The upper gel contains 370 µl of whole-cell antiserum. (B) The upper gel contains 370 µl of crude extract antiserum. Anode to the right and top. The weaker immunoprecipitates either partially lost on photographic reproduction or difficult to discern on immunoplates appear as superimposed lines. Twelve of 15 antigens revealed by the whole-cell antiserum are seen on (A). The reference pattern for analysis of *B. gingivalis* is seen on (B), which shows 20 of the 21 antigens taken into account in this study (antigen 19 is missing). The antigens identified by numbers in open circles are those which were recognized by both antisera as a result of multiple experiments. They may not all appear simultaneously on a single plate, which explains why several are missing in one or the other photograph.

TABLE 1. Pattern of detection of the 25 antigens present in the GBE-T extract of *B. gingivalis*, using different antisera

Detection with:	Antigen no.
Whole-cell antiserum	1, 2 (a, b, c, d), 3, 4, 5, 6, 7, 8, 9, 10, 12 (a, b), 15, 17, 19 (a, b), 23
Crude GBE extract anti-serum[a]	1, 2, (a, b, c, d), 3, 4, 5, 6, 7, 8, 9, 10, **11**, 12 (a, b), **13**, **14**, 15, **16**, 17, **18**, 19 (a, b), **20**, **21**, **22**, 23, **24**, **25**

[a] Boldface numbers indicate those antigens detected only by the crude extract antiserum.

thus routinely inserted which improved resolution of precipitates 1, 3, 4, 8, and 9.

The reactivity of the antiserum produced against whole cells of strain ATCC 33277 was compared with that of the crude extract antiserum (i) by homologous CIE, (ii) by CIE with intermediate gel, and (iii) by CIE using both antisera simultanously in the upper gel. Those experiments (data not shown) established that the crude extract antiserum, as well as detecting the same 15 antigens as the whole-cell antiserum, revealed 10 additional antigens. Since we were able to detect a greater number of antigens with the crude extract antiserum we elected to use this antiserum for the remainder of the study. Table 1 summarizes the pattern of detection of the 25 immunoprecipitates revealed. Twenty-one of these precipitates could be detected on a reproducible basis and, thus, only these antigens (no. 2, 3, 5 to 14, 16 to 22, 24, and 25) were taken into account in the experiments that follow.

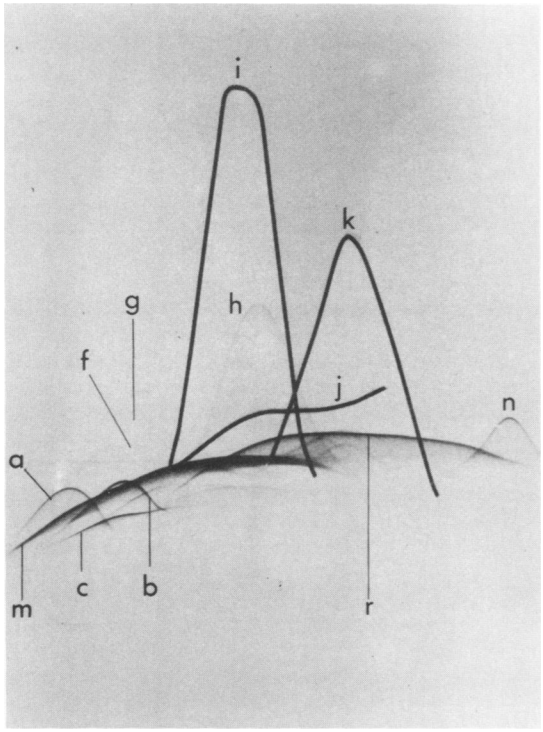

FIG. 5. Reference pattern for animal strains resembling *B. gingivalis*, established by homologous CIE. Antigen: 60 μl of GBE-T of strain Jaguar-2. Antibodies: 370 μl of Jaguar-2 antiserum in the upper gel. Anode to the top and right. The weaker immunoprecipitates either partially lost on photographic reproduction or difficult to discern on immunoplates appear as superimposed lines. Of the 21 antigens observed in this pattern, 12 were reproducibly detected as prominent immunoprecipitates (identified by letters).

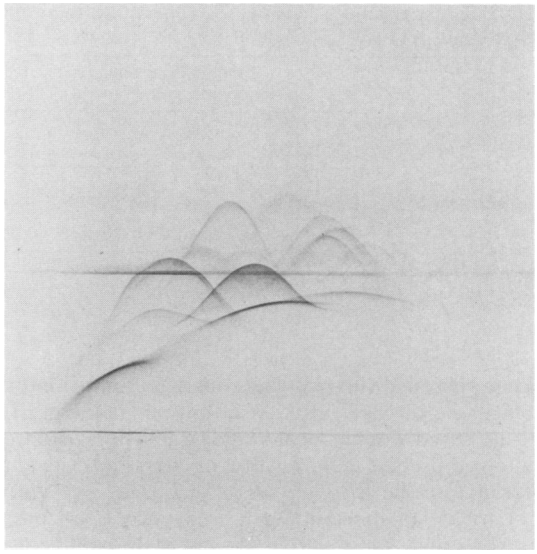

FIG. 6. Heterologous CIE used to demonstrate cross-reactions between the human strains of *B. gingivalis* W83 and ATCC 33277. Antigen: 60 μl of GBE-T of strain ATCC 33277. Antibodies: 370 μl of W83 antiserum. Anode to the top and right. The 17 immunoprecipitates revealed indicate that at least 17 antigens are common to both strains.

Homologous antigenic profiles of the animal strains resembling *B. gingivalis*. The following working protein concentrations of antigens were used: strain Jaguar-2, 1.0 mg/ml; strain Raccoon-1, 0.6 mg/ml; strain Dog-5B, 0.7 mg/ml; and strain Cat-1, 1.2 mg/ml. Homologous CIE allowed detection of a maximal number of 21 anodic migrating immunoprecipitates in strain Jaguar-2, whereas 20, 17, and 16 antigens were respectively demonstrated in strains Raccoon-1, Cat-1, and Dog-5B. The 21 antigens of strain Jaguar-2 were identified by letter (a to u) and were designated as the reference system of the animal strains resembling *B. gingivalis*. Twelve of these precipitates were reproducibly detectable (antigens a, b, c, f, g, h, i, j, k, m, n, and r; Fig. 5). The remaining nine antigens could only be detected when the antigen/antibody ratio was changed, presumably for reasons of both low antigenic concentration in the extract and low antibody concentration in the antiserum. Therefore, only the 12 reproducibly detectable immunoprecipitates were included in the serological comparisons that follow.

Demonstration of cross-reacting antigens in *B. gingivalis* strains. Antigenic preparations of, and antisera against, strains 17A3 and W83 were compared with the *B. gingivalis* ATCC 33277 reference system by means of heterologous CIE, tandem CIE, and CIE with intermediate gel. Immunoprecipitates detected in each heterologous CIE test (Fig. 6) and its reciprocal were recorded as cross-reactions. The heterologous CIE experiments revealed that 16 to 20 antigens of the three *B. gingivalis* strains cross-reacted (Table 2).

In the tandem CIE experiments each antiserum was tested twice against the set of two antigenic preparations to be compared. In a first setup the antigenic samples were each placed in one of the application wells, while in a second setup the samples were reversed in the wells. In each experiment, the appropriate homologous and heterologous

TABLE 2. Cross-reactions between *B. gingivalis* strains and the type strain ATCC 33277 detected by heterologous CIE

Antiserum to:	No. of immunoprecipitates detected in antigenic prepn of:		
	ATCC 33277	17A3	W83
ATCC 33277	21[a]	16	16
17A3	16	17[a]	NT[b]
W83	20	NT	19[a]

[a] Homologous reaction.
[b] NT, Not tested.

reactions were also run simultaneously to control interassay variability. Serological cross-reactions in tandem CIE appeared as fused double peaks and the fusion of precipitin lines allowed precise identification of cross-reacting antigens by reference to the *B. gingivalis* ATCC 33277 system numbered 1 to 25. In the tandem CIE experiment, comparing strains 17A3 and ATCC 33277, a total of 14 confluent peaks were revealed. Antigens 2, 3, 5, 6, 8, 9, 10, 11, 13, 14, 16, 20, 24, and 25 of strain ATCC 33277 were thus identified as cross-reacting antigens with strain 17A3. In addition, two antigens not previously identified in the *B. gingivalis* ATCC 33277 reference system were clearly revealed as confluent peaks by the 17A3 antiserum and were numbered 26 and 27. In the tandem CIE experiments comparing strain W83 to the reference ATCC 33277, a total of 17 confluent peaks were revealed and included antigens 2, 5, 6, 7, 8, 11, 12b, 13, 14, 16, 17, 19, 20, 21, 22, 24, and 25. In addition, two antigens not previously identified were clearly revealed by the W83 antiserum as fused precipitin lines. They were considered to be additional antigens and were numbered 28 and 29. Antigen 8 occasionally had a changeable cross-reaction pattern, suggesting that variations in antigenic determinants are responsible for partial identity only.

In the experiments that used CIE with intermediate gel, the reference antigenic preparation of strain ATCC 33277 was separated in the first-dimension electrophoresis to compare the reactivity of each antiserum against strains 17A3 and W83 with that of the reference antiserum. Each comparison consisted of two experiments. In the first setup the reference antiserum was in the upper gel and the antiserum to be compared was in the intermediate gel. In the second setup the antisera in the upper and the intermediate gels were reversed. For each experiment, control plates of the appropriate homologous and heterologous reactions were produced simultaneously. Figure 7 illustrates typical results obtainable with the technique of CIE with intermediate gel.

A summary of the results generated during the analysis of human strains, using heterologous CIE, tandem CIE, and CIE with intermediate gel, appears in Table 3. The degree of cross-reactivity of the antigenic components of strains 17A3 and W83 with the type strain ATCC 33277 was also calculated as a matching coefficient (5, 8, 13). This coefficient expresses the ratio between the number of cross-reactive antigens detected by heterologous CIE, tandem CIE, CIE with intermediate gel, and their combination, and the total number of antigens demonstrated in the ATCC 33277 refer-

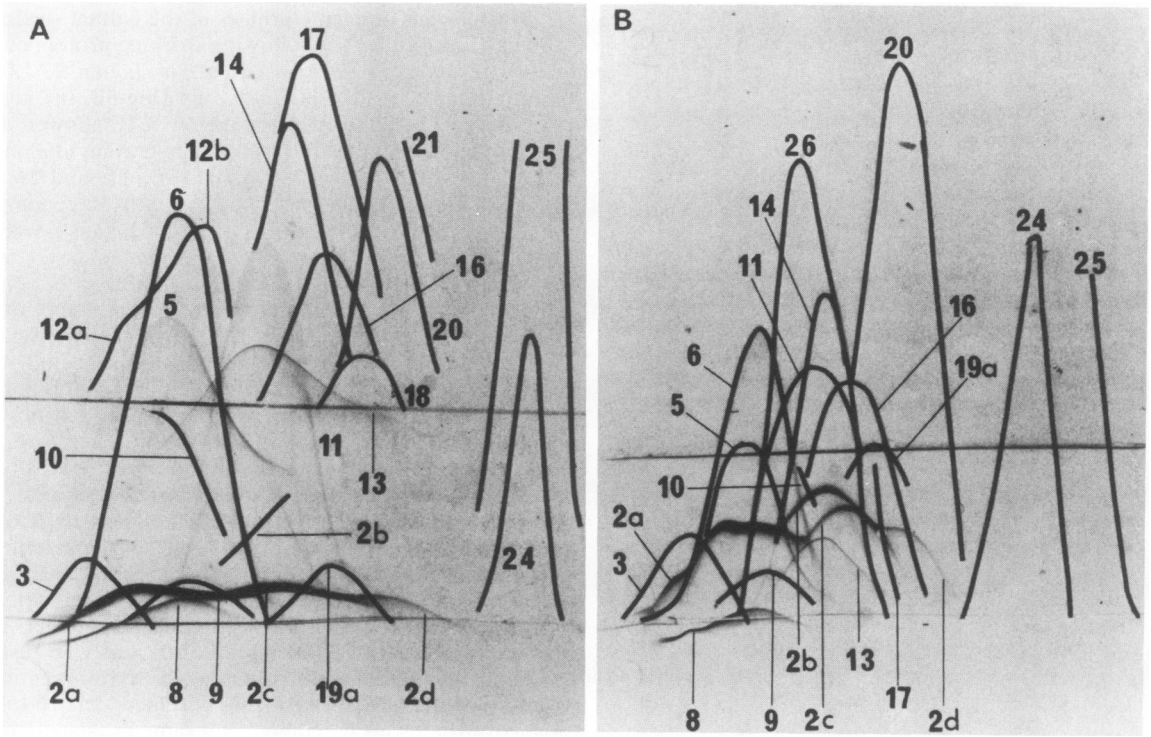

FIG. 7. Intermediate gel CIE used to compare human strains of *B. gingivalis*. Antigen: 60 μl of GBE-T of strain ATCC 33277. Antibodies in (A): (upper gel) 370 μl of ATCC 33277 antiserum; (intermediate gel) 130 μl of 17A3 antiserum. Antibodies in (B): (upper gel) 370 μl of 17A3 antiserum; (intermediate gel) 130 μl of ATCC 33277 antiserum. Anode to the top and right. The weaker immunoprecipitates either partially lost on photographic reproduction or difficult to discern on immunoplates appear as superimposed lines. Immunoprecipitate 24 is typical of an antigen recognized by antibodies present in each antiserum used in the upper and in the intermediate gels of both (A) and (B). Immunoprecipitate 17 is typical of an antigen recognized by antibodies present in only one antiserum and revealed in the upper gel only of (A) and in the intermediate gel only of (B). Immunoprecipitate 21 is a typical example of several antigens whose presence can be recognized (A, upper gel) but cannot be further characterized.

TABLE 3. Cross-reactions between *B. gingivalis* strains and the type strain ATCC 33277 detected by heterologous, tandem, and intermediate gel CIE

Strain	No. of cross-reacting antigens	Identity of cross-reacting antigens	Matching coefficient	Revised matching coefficient[a]
ATCC 33277	21	All 21 antigens	1.00	1.00
17A3	14	2, 3, 5, 6, 8–11, 13, 14, 16, 20, 24, 25	0.67	
	16	Additional 26 and 27 revealed by 17A3 antiserum	0.70	0.64
W83	17	2, 5–8, 11–14, 16, 17, 19–22, 24, 25	0.81	
	19	Additional 28 and 29 revealed by W83 antiserum	0.83	0.72
	21	Two additional unidentified antigens revealed by W83 antiserum in heterologous reaction	0.84	

[a] Using a total number of 25 antigens identified in the ATCC 33277 reference system; see text for explanation.

ence system. Sixty-seven percent of the antigens of strain 17A3 were cross-reactive with the 21 reference antigens of strain ATCC 33277 (matching coefficient = 0.67). The matching coefficient of strain 17A3 was, however, raised to 0.70 by taking into account the two additional cross-reacting antigens (no. 27 and 27) revealed in the antigenic preparation of strain ATCC 33277 by the 17A3 antiserum. Similarly, 17 antigens of strain W83 cross-reacting with strain ATCC 33277 (matching coefficient = 0.81) were identified, to which two additional antigens, 28 and 29 (matching coefficient = 0.83), revealed by the W83 antiserum, and two unidentified cross-reacting antigens seen in heterologous reactions could be added for a total of 21 (matching coefficient = 0.84). The total number of antigens identified in the ATCC 33277 reference system was thus raised from 21 to 25. Accordingly, a revised matching coefficient was calculated to include the additional antigens (Table 3).

A total of 13 antigens cross-reacted with all three *B. gingivalis* strains studied: those were antigens numbered 2, 5, 6, 8, 11, 13, 14, 16, 20, and 24 to 27, among which 2, 8, 13, and 14 were particularly conspicuous.

Demonstration of cross-reacting antigens in animal strains resembling *B. gingivalis*. Antigenic preparations of, and antisera against, the animal strains resembling *B. gingivalis* were compared with the strain Jaguar-2 reference system by means of heterologous and tandem CIE. For each experiment the appropriate homologous and heterologous plates were produced simultaneously to control interassay variability. Figure 8 illustrates typical results achievable with the technique of tandem CIE. Immunoplates showing homologous and heterologous CIE reactions are not shown. The results obtained in these experiments are summarized in Table 4. It was found that strain Dog-5B was most similar to the reference strain Jaguar-2, sharing all 12 studied antigens (matching coefficient = 1.0), strain Cat-1 was next with 11 cross-reacting antigens (matching coefficient = 0.92), and strain Raccoon-1 was least similar with 7 cross-reacting antigens (matching coefficient = 0.58). Six antigens cross-reacted with all four animal strains studied, namely, antigens h, i, k, m, n, and r.

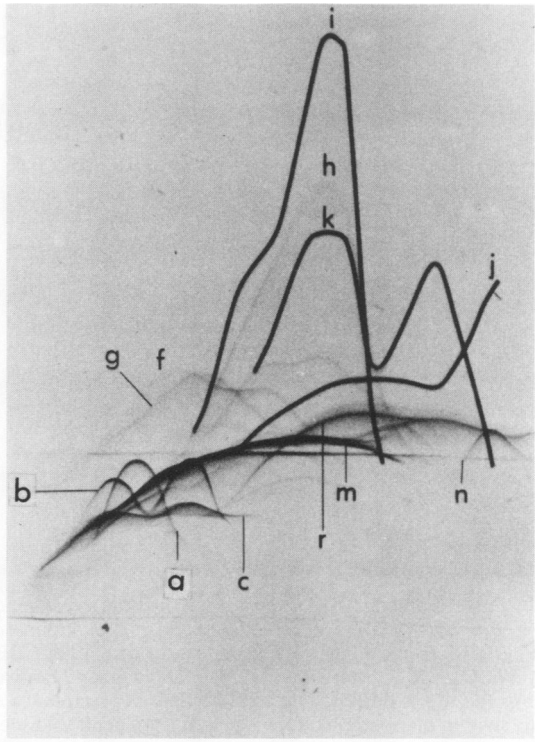

FIG. 8. Tandem CIE used to compare animal strains resembling *B. gingivalis*. Antigen: (left well) 60 μl of GBE-T of strain Cat-1; (right well) 60 μl of GBE-T of strain Jaguar-2. Antibodies: 370 μl of Jaguar-2 antiserum. Anode to the top and right. The weaker immunoprecipitates either partially lost on photographic reproduction or difficult to discern on immunoplates appear as superimposed lines. The typical double-peak appearances of immunoprecipitates b, c, f, g, h, i, j, k, and m are each indicative of a common antigen present in the antigenic extracts. Immunoprecipitate a is typical of an antigen present in only one antigenic extract since it is not revealed as a double peak. Because of it's exceptionally high electrophoretic mobility, antigen n does not appear as a double peak on this plate.

Demonstration of cross-reacting antigens between *B. gingivalis* and animal strains resembling *B. gingivalis*. Once the cross-reactivities within the group of strains of *B. gingivalis* and within the group of animal strains resembling *B. gingivalis* were established, the reference CIE patterns of each group were compared by heterologous and tandem CIE. For each experiment the appropriate homologous and heterologous reactions were produced to keep the interassay variability under control. Figure 9 illustrates typical results obtainable with the technique of heterologous CIE. Immunoplates showing homologous and tandem CIE reactions

TABLE 4. Cross-reactions between strain Jaguar-2 and three other animal strains resembling *B. gingivalis* detected by heterologous and tandem CIE

Strain	No. of cross-reacting antigens	Identity of cross-reacting antigens	Matching coefficient
Jaguar-2	12	All 12 antigens	1.00
Dog-5B	12	All 12 antigens	1.00
Cat-1	11	All but No a	0.92
Raccoon-1	7	a, h, i, k, m, n, r	0.58

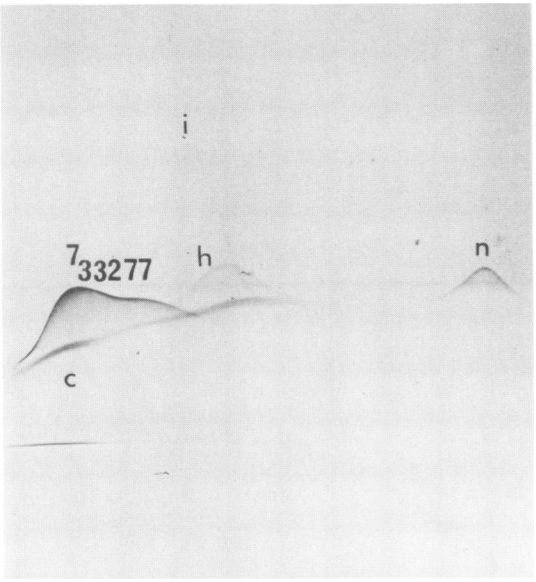

FIG. 9. Heterologous CIE used to demonstrate cross-reactions between *B. gingivalis* (reference antigen) and animal strains resembling *B. gingivalis* (reference antiserum). Antigen: 60 μl of GBE-T of strain ATCC 33277. Antibodies: 370 μl of Jaguar-2 antiserum. Anode to the top and right. The weaker immunoprecipitates either partially lost on photographic reproduction or difficult to discern on immunoplates appear as superimposed lines. The presence of five immunoprecipitates indicates that at least five antigens are common to both strains.

are not shown. On Fig. 9 it can be seen that the Jaguar-2 antiserum detected five cross-reacting antigens in the extract of strain ATCC 33277. Of these, it was later established that antigens 8, 11, 13, and 25 of strain ATCC 33277 cross-reacted with antigens c, i, h, and n of strain Jaguar-2, respectively. The fifth antigen, not previously identified in the Jaguar-2 reference system, presumably because of a low antigen concentration in the Jaguar-2 extract, was clearly revealed by the Jaguar-2 antiserum in the extract of strain ATCC 33277. It was later shown to be identical to antigen 7 in the *B. gingivalis* ATCC 33277 reference system. Tandem CIE experiments also showed that antigens 20 and k were cross-reactive. A total of six cross-reacting antigens were thus found. Of these, antigens 11, 13, 20, and 25 were cross-reactive in all seven strains included in the present study and thus qualify as common antigens. Antigen 7, which did not cross-react with *B. gingivalis* 17A3 (although it cross-reacted with strain Jaguar-2), and antigen 8, which cross-reacted with all animal strains except strain Raccoon-1, cannot be considered as common antigens.

The remaining seven antigens of the Jaguar-2 reference system, namely, antigens a, b, f, g, j, m, and r, did not cross-react with the ATCC 33277 reference system. Of these, antigens m and r cross-reacted with all four animal strains resembling *B. gingivalis* and thus qualify as potential animal biotype-specific antigens.

A summary of the cross-reactions demonstrated in this study appears in Table 5.

DISCUSSION

In the present study, a mild extraction procedure (28) resulting in minimal lysis of the cytoplasmic membrane was used. The presence of nucleic acids in GBE was assessed to be 20 times less than that in a disrupted cell preparation. Also, electron microscopy of the bacteria after extraction revealed that the proportion of ghost cells had not increased as compared with a 5- to 7-day culture. As reported by Poxton and Byrne (29), EDTA inhibits cellular autolysis. It thus appears that the presence of cytoplasmic soluble components in the crude GBE extract was minimal and may be considered similar to that produced by autolysis. Accordingly, our antigen preparation may be considered as consisting of essentially outer membrane and cell surface components. The crude GBE was used as a vaccine to produce antisera which presumably contained antibodies directed against all antigens present in the extract. The multiplicity and the clear individuality of immunoprecipitates in CIE must be considered to be dependent on the relative concentration of antigens and corresponding antibodies in the system. Since it can be assumed that a crude GBE containing a low proportion of cytoplasmic components would only induce a low antibody liter against cytoplasmic components, it is likely that the number and resolution of immunoprecipitates corresponding to cytoplasmic antigens was also low in our CIE patterns.

Laliberté and Mayrand (16), who studied 104 strains of black-pigmented, catalase-positive, asaccharolytic *Bacteroides* spp. isolated from the oral cavity of four animal species, reported that the biochemical and physiological properties of the animal strains, apart from their catalase

TABLE 5. Antigenic distribution and cross-reactive antigens of *B. gingivalis* and animal strains resembling *B. gingivalis*[a]

Strain	Antigen no.[b]																								
	2	3	5	6	7	8	9	10	11	12	13	14	16	17	18	19	20	21	22	24	25	26	27	28	29
B. gingivalis																									
ATCC 33277	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17A3	+	+	+	+	−	+	+	+	+	−	+	+	+	−	−	−	+	−	−	+	+	+	+		
W83	+	−	+	+	+	+	−	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+
Animal strains resembling *B. gingivalis*																									
Jaguar-2	−			−	+	+			+		+						+				+				
Dog-5b						+			+		+						+				+				
Cat-1						+			+		+						+				+				
Raccoon-1						−			+		+						+				+				

[a] Antigens are identified relative to the *B. gingivalis* ATCC 33277 reference system.

[b] +, Presence of a given antigen and a positive cross-reaction with *B. gingivalis* ATCC 33277; −, verified absence of a given antigen identified in the *B. gingivalis* ATCC 33277 reference pattern. Blank indicates antigen that was either not observed or not characterized.

activity, were similar to those of human *B. gingivalis* strains. They suggested that the distinctive catalase production by the animal strains was not sufficient to recognize a separate species. Indeed, examples of variation of catalase production among strains of gram-negative bacteria can be found in the species *B. thetaiotaomicron*, *B. ovatus*, and *B. eggerthii* (11). A recent example involves the current taxonomy of the genus *Veillonella* (31), which recognizes a single species and regards *Veillonella alcalescens* as a synonym of *V. parvula* since the former two species only differed in their ability to produce catalase: *V. parvula* was negative and *V. alcalescens* was positive (12). Pending determination of the guanine-plus-cytosine ratio (in moles percent) and DNA homology to confirm or refute the proposal of a single species, two biotypes can presently be recognized in the species *B. gingivalis*: a human biotype characterized by a catalase-negative reaction, and an animal biotype for which catalase production is a distinctive diagnostic test. Our comparative analysis of the antigens of three human *B. gingivalis* strains and of four animal *Bacteroides* sp. strains isolated by Laliberté and Mayrand (16) revealed (i) a serological homogeneity within each group of strains, (ii) a serological specificity, and (iii) an antigenic relatedness between the two groups. The information derived from such serological studies is considered of value in bacterial taxonomy (14).

The similarity of the antigenic components within strains of the human biotype was high since 16 to 20 antigens cross-reacted in heterologous reactions. It was seen that the human strains shared 64 to 84% of their surface antigens in common with the type strain ATCC 33277, using matching coefficients for analysis of the degree of similarity (Table 3). According to Espersen et al. (8), matching coefficients > 0.92 seem to be characteristic of strains belonging to the same species. It must be noted, however, that these authors and others (4) used sonicated preparations which, in contrast to the cell envelope extract of our study, contained a high proportion of cytoplasmic antigens. Cytoplasmic antigens are most likely responsible for serological cross-reactions even between strains of distant taxonomic relatedness, whereas cell surface and associated antigens are known to impart immunochemical specificity to bacteria (7). Our data are therefore indicative of a marked serological homogeneity within the human biotype of *B. gingivalis* on the basis of 13 common surface antigens. Similarly, our data demonstrated a serological homogeneity within the animal biotype of *B. gingivalis* which shared 58 to 100% surface antigens in common with strain Jaguar-2 (Table 4). A total of six antigens common to the animal strains were identified. Strain Raccoon-1, however, which was least similar (matching coefficient = 0.58), suggests that multiple serovars exist within the biotype.

It was also observed that each biotype had several distinctive antigens, indicating that two serotypes could be recognized within the species *B. gingivalis*. Antigens 2a and 6 were specific to the human strains and may be used to define serotype 1 of *B. gingivalis* which includes the catalase-negative human biotype. Both antigens 2a and 6 can be detected by a whole-cell antiserum, indicating that serological specificity for strains of the human biotype is expressed on the bacterial outer surface. The outer surface location of these antigens renders them likely to stimulate antibody production in *B. gingivalis*-infected patients. The serotype 1-specific antigens may therefore be significant antigens in the serodiagnosis of *B. gingivalis* infections. Antigens m and r were specific to the animal strains and can be used to define serotype 2 of *B. gingivalis* which includes the catalase-positive animal biotype. It can be anticipated that monospecific antisera against the serotype-specific antigens described in this study may provide useful tools for antigenic analysis of *B. gingivalis* and, when needed, for serotyping human and animal clinical isolates.

Four cross-reacting antigens between the human and the animal biotypes were common to all seven strains included in this study. These common antigens may be considered species-specific antigens and may be used to define the serological homogeneity of the two biotypes within the species. It also can be anticipated that production of antibodies against these species-specific antigens has practical value for the identification of the species *B. gingivalis* by serological methods.

Further studies need to be carried out with additional strains to determine the distribution of common and serotype-specific antigens among laboratory and clinical isolates, to verify their presence or absence in other members of the genus *Bacteroides* and unrelated gram-negative bacteria, and thus to assess their relative importance in the taxonomy of *Bacteroidaceae*.

ACKNOWLEDGMENTS

We thank Denis Mayrand for providing the strains and Jean Joly for helpful criticism, as well as Christine Barthe and Eric Morin for skillfully performing several immunoelectrophoreses.

This work was supported by Medical Research Council grants DG-246 and MA-8761.

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