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Serological Studies of *Bacteroides gracilis*, *Campylobacter concisus*, *Wolinella recta*, and *Eikenella corrodens*, All from Humans with Periodontal Disease

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Gram-negative, asaccharolytic, agar-corroding organisms were isolated from lesions of advanced destructive periodontal disease. We grouped 78 oral isolates and reference strains of *Eikenella corrodens*, *Bacteroides ureolyticus*, *Wolinella succinogenes* (*Vibrio succinogenes*), and *Campylobacter* spp. serologically by using the microagglutination technique. The four species of oral organisms (*E. corrodens*, *Bacteroides gracilis*, *Wolinella recta*, and *Campylobacter concisus*) were clearly differentiated from each other on the basis of agglutination with rabbit antisera produced against representative live organisms. Agglutination patterns showed both a lack of intergroup relatedness among the oral isolates and a lack of serological identity with the biochemically similar organisms *W. succinogenes*, *B. ureolyticus*, and *Campylobacter* species.

The involvement of microorganisms in the destruction of periodontal tissues has been well established (1-4, 7, 8). Gram-negative, anaerobic bacteria are predominant in the deep pockets of periodontitis (6, 10). The identification of these gram-negative organisms is an obligatory first step toward an understanding of their role in disease.

Asaccharolytic, gram-negative, rod-shaped bacteria that caused pitting or corrosion of the surface of blood agar plates were isolated from lesions in humans with advanced destructive periodontal disease by Tanner et al. (10). In some lesions these bacteria were numerically dominant. Such organisms most closely resembled *Eikenella corrodens*, *Bacteroides ureolyticus*, *Vibrio succinogenes*, and *Campylobacter sputorum*. Reference strains of these four species and 46 oral isolates were studied on the basis of their phenotypic characteristics and the guanine-plus-cytosine contents of their deoxyribonucleic acids (DNAs) and by DNA-DNA hybridization (9). One group of organisms was taxonomically similar to the type strain of *E. corrodens*, and the strains in this group were identified as members of *E. corrodens*. The remaining isolates did not fit existing species descriptions and were thus assigned to newly named species. The nonmotile anaerobic agar corroders were placed in the species *Bacteroides gracilis*. The motile anaerobic rod-shaped organisms had biochemical characteristics and guanine-plus-cytosine contents (42 to 46 mol%) similar to those of *V. succinogenes* as described by Wolin et al. (11); therefore, *V. succinogenes* Wolin et al. 1961 was transferred to a new genus, *Wolinella*, and the anaerobic, motile, rod-shaped bacteria that corroded agar were named *Wolinella recta*. Motile, microaerophilic organisms which had guanine-plus-cytosine contents of 34 to 38 mol% differed from reference *Campylobacter* strains and were named *Campylobacter concisus*.

In this paper we describe serogrouping by microagglutination of the organisms studied by Tanner et al. (9) and additional strains which were isolated at Case Western Reserve University.

MATERIALS AND METHODS

Bacterial strains. The isolates from humans with periodontal lesions were purified and characterized as described by Tanner et al. (9). The nine strains from the Case Western Reserve Dental Clinic were isolated from lesions in humans with advanced periodontitis, and they were identified with a B preceding the strain number. Strain numbers with the suffix R indicate the raised, noncorroding colony type. The reference strains of *E. corrodens* used were ATCC 23834, 597, 598, and MG-7905; the last strain was isolated from a human bite wound by M. Wolinsky, Cleveland Metropolitan General Hospital. Details concerning the sources of the strains from the Forsyth Dental Center and their identification are described by Tanner et al. (9). In addition, strains 375, 444A, 461, 462, 463, 465, 477R, 480, 525, and 530 were isolated from lesions in humans with periodontitis, strains 558, 569R, and 1076 were isolated from pockets associated with gingivitis, and strain 1085 was isolated from a human with a periodontosis lesion.

Media for growth of live vaccines and agglutination antigens. Medium CS3 was used to grow *E. corrodens*; this medium contained (per liter) 17 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 3 g of Phytone (BBL), 2 g of potassium nitrate, 1 g of sodium formate, and 3 mg of hemin. The first four ingredients were dissolved in distilled water at a fivefold concentration, and this preparation was vacuum dialyzed to remove potentially antigenic substances present in the high-molecular-weight fraction. After the effusate was diluted and autoclaved, 1 ml of a sterile solution of 0.3% hemin in 1 M sodium carbonate and 10 ml of filter-sterilized 1 M potassium bicarbonate were added aseptically.

The supplemented Todd-Hewitt broth used to grow *Wolinella*, *Campylobacter*, and *Bacteroides* strains contained 2 g of potassium formate per liter, 3 g of potassium fumarate per liter, and 3 mg of hemin per liter, which were added as described above.

Antigen preparation. Single colonies were streaked onto sheep blood agar plates, which were incubated under 80% N_2–10% CO_2–10% H_2 in a gas evacuation replacement jar with a palladium catalyst; 10 to 20 colonies were removed from each plate with a Pasteur pipette and inoculated into 10 ml of dialyzed medium CS3 or supplemented Todd-Hewitt broth. After 24 h of anaerobic incubation, 5 ml of each culture was used to inoculate 100 ml of broth. After 24 h, the cells were harvested by centrifugation at 12,000 × *g* for 10 min and then washed once with 10 ml of phosphate-buffered saline (pH 7.2) (if they were used for agglutination tests), or twice with phosphate-buffered saline (if they were used for vaccines).

For agglutination tests, the cell paste was suspended in phosphate-buffered saline at an optical density at 540 nm of 1.2, or it was matched to a McFarland no. 9 standard. Cells were suspended in phosphate-buffered saline at a concentration of 2×10^9 cells per ml (optical density at 540 nm, 0.82) for vaccines.

Antiserum production in rabbits. Two regimens were used. Anti-*Eikenella* sera were produced in New Zealand white rabbits (5 to 6 lb [2.27 to 2.72 kg]) by injecting them intravenously with 10^9 live organisms in 1.0 ml of phosphate-buffered saline. The rabbits were injected every other day with 1.5×10^9, 3×10^9, and 5×10^9 cells for a total of four injections, bled after 17 to 21 days, rested for 1 week, injected with 5×10^9 live cells, and sacrificed 5 days later by exsanguination from the heart.

Antisera were prepared against the remaining strains by injecting rabbits subcutaneously at four sites with 1×10^9 to 4×10^9 live bacterial cells which had been dispersed by mixing in incomplete Freund adjuvant. Injections were repeated at 1-week intervals for a total of four injections. The rabbits were exsanguinated by cardiac puncture 1 week after the last vaccination.

Sera were sterilized by filtration, dispensed in 1-ml volumes into sterile plastic tubes (12 by 75 mm), and frozen at −70°C. Working tubes of sera were thawed rapidly and stored at 4°C in 1 mM sodium azide.

Microagglutination test procedure. Initially, all sera were diluted 1:10. A total of 12 twofold serial dilutions were made in phosphate-buffered saline containing 5% sucrose by using microtiter plates and 0.025-ml diluters (Dynatech Laboratories, Inc., Alexandria, Va.). The addition of sucrose prevented autoagglutination of the whole-cell antigen preparation. One drop (0.025 ml) of bacterial suspension (optical density at 540 nm, 1.2) was added to each well. Control wells contained diluted preimmune serum. The plates were rocked for 10 min on a platform shaker (Eberbach and Son Co., Ann Arbor, Mich.) which moved 5 cm at 120 oscillations per min; then they were incubated at 37°C for 20 min and read under a dissecting microscope. Positive agglutination patterns varied from perceptible small granules to large floccules which settled quickly into a pellet. Homologous titers and duplicate samples were within one dilution of the expected titer.

RESULTS

The four species of oral strains could be distinguished serologically, since microagglutination between strains of different species did not occur.

Antisera were prepared against *E. corrodens* strains 1073, 530, 479, and 373 from patients with periodontitis and two reference strains, strains ATCC 23834 and 598. All of these immunizing strains except strain 479 were of the corroding-spreading colony type. Table 1 shows the microagglutination titers of these antisera with 50 isolates of *E. corrodens*. Many strains displayed multiple antigens and cross-reactivity, as has been reported by Schroter (5) for clinical isolates of *E. corrodens*. The strains were grouped according to the serum or sera with which the highest agglutination titer was observed. Strains that were most similar to strain 1073 were placed in group A (Table 1). The organisms in group A-1 were also identified by anti-1073 serum, but in addition they contained antigens in common with reference strains ATCC 23834 and 598. Strains in group A-1 were agglutinated by anti-1073 serum at titers which were greater than or equal to the titers obtained when anti-ATCC 23834 serum was used; this was in contrast to group B strains, which all showed lower titers when they were tested with anti-1073 serum than when they were tested with anti-ATCC 23834 serum. Strains in groups B and C most closely resembled the reference strains ATCC 23834 and 598, respectively. Approximately 60% of the strains could be identified as *E. corrodens* by the antiserum to strain 1073. Groups E and F had unique antigenic determinants and showed little cross-reactivity with other strains.

Table 2 shows the serological relationships among strains of *W. recta*. Antisera were prepared against strains 285 and 371 and the atypical strain 286. *W. recta* isolates formed two serogroups which showed no cross-reactions with strains of *C. concisus*, *E. corrodens*, and *B.*

TABLE 1. *Groupings of E. corrodens strains as determined by microagglutination titer tests*

Group	Strain that was the source of whole-cell antigen[a]	Titer with antiserum from:[b]					
		Strain 1073	Strain ATCC 23834	Strain 598	Strain 530	Strain 373	Strain 479R
A	1073	10,240[c]	160	320	640	80	—[d]
	1073R	5,120	640	640	1,280	40	—
	1079	10,240	640	640	320	80	80
	1079R	10,240	320	640	640	80	80
	1085	5,120	320	320	640	80	—
	B-122	5,120	640	640	320	320	80
	558	1,280	160	—	160	—	80
	558R	1,280	80	—	80	—	160
	1008R	1,280	80	—	80	—	80
	1006R	1,280	80	—	80	—	80
	464	1,280	640	160	160	640	160
	1081	1,280	40	—	80	—	80
A-1	525	2,560	640	2,560	640	80	—
	B-120	2,560	640	2,560	640	40	—
	B-126	2,560	160	2,560	320	—	80
	MG-7905[e]	5,120	1,280	5,120	1,280	640	160
	1009	5,120	5,120	640	640	160	80
	1009R	2,560	160	320	320	—	—
	1010	2,560	2,560	640	320	—	—
	467	1,280	1,280	640	80	320	—
B	ATCC 23834	1,280	5,120[c]	1,280	640	160	40
	B-121	160	2,560	320	320	—	—
	569R	160	640	320	160	80	40
C	598	160	320	10,240[c]	320	160	160
	597	160	640	1,280	320	80	80
	1078	1,280	320	5,120	320	80	160
	1078R	1,280	320	1,280	320	80	80
	B-124	320	320	1,280	160	—	—
	477R	320	40	1,280	640	160	40
	469	—	320	640	80	320	—
D	530	2,560	160	640	10,240[c]	40	—
E	373	80	40	160	40	5,120[c]	80
	374	80	160	—	—	5,120	80
	1075	—	40	—	—	5,120	—
	1075R	—	—	—	—	2,560	—
	384	80	160	160	—	5,120	—
	1074	40	—	—	—	2,560	—
	1076	—	320	80	80	2,560	80
	1076R	—	40	80	—	80	320
	375	160	40	—	—	2,560	—
	470	80	80	80	80	640	—
	470R	—	—	40	—	1,280	40
F	479R	—	80	160	—	—	2,560[c]
	444A	—	80	40	—	—	1,280
	461	—	160	40	—	160	1,280
	462	—	80	80	—	—	640
	463	—	80	80	—	40	1,280
	465	—	80	40	—	—	640
	466	—	—	160	—	—	640
	480	—	80	40	—	80	640
	468	—	80	80	—	—	320
	469R	—	—	80	—	40	640

[a] The suffix R indicates raised, noncorroding colony types.
[b] Titer is expressed as the reciprocal of the highest serum dilution that caused agglutination.
[c] Homologous titer.
[d] —, No agglutination detected.
[e] Strain MG-7905 is a hospital isolate from a human bite wound.

gracilis or with strain VPI 9584. Strain 286 showed high homologous titers but low cross-reactivities with some *W. recta* isolates.

Table 3 shows the microagglutination titers of *B. gracilis* strains. These isolates formed a single serogroup which showed no cross-reactivity with isolates in any of the other groups. Antiserum to strain 1084 did not react with *B. ureolyticus* strain 7815.

The microagglutination titers of the *C. concisus* strains tested are shown in Table 4. These organisms showed no cross-reactivity with any other reference strain tested, including *C. sputorum* and *Campylobacter fetus*. With the exception of strain 288, these strains formed one serological group.

DISCUSSION

E. corrodens, *B. gracilis*, *W. recta*, and *C. concisus* can be differentiated from each other serologically by agglutination (Tables 2 through 4) or by immunofluorescent staining (Badger, unpublished data).

We observed two serogroups of *W. recta*. Strain 286, which represented a third serogroup, showed a Jaccard similarity coefficient of 80% and a similarity as determined by DNA hybridization of 50% with respect to *W. recta* strains

TABLE 2. *Serological reactions of oral strains of W. recta*

Strain that was the source of whole-cell antigen	Guanine-plus-cytosine content of DNA (mol%)	Titer with antiserum from:[a]					
		Strain 285	Strain 371	Strain 286	*C. concisus* 484	*B. gracilis* 1084	*E. corrodens* 1073
W. recta							
285	42	640[b]	40	—[c]	—	—	—
1082V	45	320	40	—	—	—	—
285R	42	320	40	—	—	—	—
267	43	640	40	—	—	—	—
B-16		320	—	80	—	—	—
B-8		320	80	40	—	—	—
371	43	80	320[b]	—	—	—	—
603		80	320	—	—	—	—
302	43	—	320	—	—	—	—
302R	43	—	320	—	—	—	—
B-136		—	320	—	—	—	—
372	43	—	160	40	—	—	—
303	45	—	160	—	—	—	—
557		—	160	—	—	—	—
B-98		—	160	—	—	—	—
286	45	—	—	1,280[b]	—	—	—
VPI 9584	46	—	—	—	—	—	—

[a] Titer is expressed as the reciprocal of the highest antiserum dilution that caused agglutination.
[b] Homologous titer.
[c] —, No agglutination detected.

TABLE 3. *Serological reactions of B. gracilis strains*

Strain that was the source of whole-cell antigen	Guanine-plus-cytosine content of DNA (mol%)	Titer with antiserum from:[a]				
		Strain 1084	*W. recta* 285	*W. recta* 371	*C. concisus* 484	*E. corrodens* 1073
B. gracilis						
1084	45	640[b]	—[c]	—	—	—
1083	45	320	—	—	—	—
1082	45	160	—	—	—	—
401	45	640	—	—	—	—
401R	45	640	—	—	—	—
402	46	640	—	—	—	—
406	46	320	—	—	—	—
B. ureolyticus VPI 7815	28	—	—	—	—	—

[a] Titer is expressed as the reciprocal of the highest dilution of antiserum that caused agglutination.
[b] Homologous titer.
[c] —, No agglutination detected.

TABLE 4. *Serological reactions of C. concisus isolates*

Strain that was the source of whole-cell antigen	Guanine-plus-cytosine content of DNA (mol%)	Titer with antiserum from:					
		Strain 484	*W. recta* 285	*W. recta* 371	*W. recta* 286	*B. gracilis* 1084	*E. corrodens* 1073
C. concisus							
484	38	1,280[a]	—[b]	—	—	—	—
483	37	640	—	—	—	—	—
522	38	320	—	—	—	—	—
569	38	320	—	—	—	—	—
485	36	320	—	—	—	—	—
288	38	—	—	—	640	—	—
Reference *Campylobacter* strains							
C. sputorum VPI S17	30	—	—	—	—	—	—
C. fetus VPI 1176	31	—	—	—	—	—	—

[a] Homologous titer.
[b] —, No agglutination detected.

(9). A similar strain, which was isolated from a root canal, has been reported recently; this strain was homologous with strain 286 (R. A. Visconti, A. C. R. Tanner, L. V. Holdeman, G. Sundqvist, and S. S. Socransky, J. Dent. Res. **58A:**105, abstr. 976, 1979), and this information suggests that these two organisms represent another *Wolinella* species.

With the exception of strain 288, the motile microaerophilic organisms used in this study were a serologically distinct group. Strain 288 appeared to have an antigen in common with strain 286.

The *B. gracilis* strains formed a single serogroup. The relatively small number of strains examined may have precluded the observation of other serovars (serotypes).

E. corrodens strains were antigenically diverse, with many of the isolates showing strong cross-reactions. Coykendall and Kaczmarek found that strains of *E. corrodens*, including many isolates tested in this investigation, represented a single DNA homology group (A. L. Coykendall and F. S. Kaczmarek, J. Dent. Res. **59A:**512, abstr. 971, 1980), so the serological heterogeneity of these organisms apparently does not indicate the presence of more than one species. Antiserum to strain 1073 reacted with 60% of the isolates. This strain seemed to be more typical of oral *E. corrodens* isolates than the reference strains ATCC 23834 and 598 did. Two serogroups of *E. corrodens*, groups E and F (Table 1), corresponded closely to clusters defined by an analysis of phenotypic features (9), and each appeared to display one predominant antigen. The antisera which reacted with strains in serogroups E and F were prepared against strains 373 and 479, respectively. Both of these strains were isolated from patients with rapidly progressive periodontal disease. No strains belonging to serogroups E and F were found among 40 isolates of *E. corrodens* from the healthy gingiva of young adults, suggesting that some serovars of *E. corrodens* are more pathogenic than others (S. Badger and J. Peterson, J. Dent. Res. **57A:**315, abstr. 962, 1978).

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REPRINT REQUESTS

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