Antigens and Enzymes of *Bacteroides* of the *Bacteroides fragilis* Group Compared by Crossed Immunoelectrophoresis

VÉRONIQUE ROGEMOND AND ROLAND M. F. GUINET*

Institut Pasteur de Lyon et du Sud-Est, Centre d'Immunochimie Microbienne, 69365 Lyon Cedex 7, France

In this work we were primarily concerned with antigens of *Bacteroides* strains belonging to the *B. fragilis* group. The following two antisera were produced: anti-*Bacteroides fragilis* E1^T (T = type strain) and anti-*Bacteroides distasonis*. Reference patterns for both of these species were established by crossed immunoelectrophoresis, and these patterns yielded 62 and 70 precipitates, respectively. The crossed reactions of *B. fragilis* E1^T and *B. distasonis* with *Bacteroides fragilis* E2, *Bacteroides thetaiotaomicron*, *Bacteroides ovatus*, and *Bacteroides vulgatus* were studied to test both for total antigens and for certain enzymes (esterase, phosphatase, malate dehydrogenase, and glucose-6-phosphate dehydrogenase). Our results showed that *B. thetaiotaomicron* is the species which is closest to *B. fragilis*, demonstrating more than 70% common antigens, whereas none of the species examined was very closely related to *B. distasonis*. Species-specific antigens, such as the phosphatase of *B. fragilis*, as well as antigens common to the group, such as malate dehydrogenase, were also demonstrated in crossed immunoelectrophoresis. This may allow immunological identification of each species.

Anaerobic bacteria are responsible for at least 10% of all bacterial infections, and *Bacteroides* species alone represent 27% of the anaerobes isolated from pathological materials (22). *Bacteroides* strains belonging to the *B. fragilis* group are most often responsible for intestinal infections (6, 27). The most frequently isolated species is *Bacteroides fragilis*, although this species is a minor component of the fecal flora, in which *Bacteroides vulgatus* and *Bacteroides thetaiotaomicron* are the dominant species (18). This observation has led some authors to seek virulence factors in *B. fragilis* (notably in the capsule) that are lacking in the other species of the group (13, 19).

Classification of the *B. fragilis* group has been modified often. The numerous phenotypic properties which members of this group have in common account for the proposal of subspecies of *B. fragilis* (17). However, deoxyribonucleic acid-deoxyribonucleic acid homology studies showed a diversity (3) which led to reclassification of *B. fragilis*, *Bacteroides ovatus*, *B. vulgatus*, *B. thetaiotaomicron*, and *Bacteroides distasonis* as distinct species (12).

In 1971, Beerens et al. (1) proposed a serological classification for the *B. fragilis* group, which was based on agglutination or gel double-diffusion tests in the presence of a specific antiserum. Lambe and Moroz (15) tested for the presence of thermostable antigens that permitted classification of all *B. fragilis* strains without cross-reactions with *B. distasonis*, *B. ovatus*, and *B. thetaiotaomicron*. Other authors have studied the lipopolysaccharide fractions or outer membrane complex of *B. fragilis* by using either sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4, 14, 21) or immunochemical techniques (5, 10).

All of these studies demonstrated the heterogeneity which exists within the *B. fragilis* group, as well as the heterogeneity among the *B. fragilis* strains.

Shah and Collins (23) have shown that the presence of the enzyme glucose-6-phosphate dehydrogenase (G6PDH) is specific for the *B. fragilis* group. The electrophoretic mobilities of certain enzymes were found to be species specific (24).

This study had the following purposes: to obtain crossed immunoelectrophoretic patterns for soluble antigens of two species of the *B. fragilis* group with the corresponding polyvalent antisera; to compare reactions of other species belonging to the group with these antisera and to estimate their immunological proximity; and to identify certain antigens with enzymes, to study the cross-reactions of these antigens with other species, and to seek antigens specific for the group or species.

MATERIALS AND METHODS

Antigens. The antigens were prepared from six strains which represented five species belonging to the *B. fragilis* group; these strains were supplied by C. Romond (Table 1). The bacteria, which were grown in anaerobic jars for 72 h at 37°C in Wilkins-West medium (26), were collected by centrifugation for 15 min at 10,000 × g and then washed three times in physiological saline.

The antigens were extracted by shaking in a Braun ho-

TABLE 1. Cross-reactions between *Bacteroides* antigens as determined by crossed immunoelectrophoresis

Antigen	Protein concn (g/liter)	<i>B. fragilis</i> E1 ^T antiserum		<i>B. distasonis</i> antiserum	
		No. of peaks	% Of common antigens	No. of peaks	% Of common antigens
<i>B. fragilis</i> E1 ^T (=NCTC 9343 ^T)	10	62		9	12.5
<i>B. fragilis</i> E2 (E323)	10	54	87	10	14
<i>B. thetaiotaomicron</i> NCTC 10582 ^T	10	44	71	12	17
<i>B. ovatus</i> ATCC 8483 ^T	2	25	40	35	49
<i>B. vulgatus</i> ATCC 8482 ^T	8	17	27	22	31
<i>B. distasonis</i> ATCC 8503 ^T	10	21	34	70	

* Corresponding author.

TABLE 2. Enzymes detected by crossed immunoelectrophoresis

Antigen	<i>B. fragilis</i> antiserum				<i>B. distasonis</i> antiserum			
	Esterase	Phosphatase	MDH	G6PDH	Esterase	Phosphatase	MDH	G6PDH
<i>B. fragilis</i> E1 ^T	+	+	+	+	—	—	+	—
<i>B. fragilis</i> E2	+	+	+	+	—	—	+	—
<i>B. thetaiotaomicron</i>	—	—	+	+	—	—	+	—
<i>B. ovatus</i>	—	—	+	+	—	+	+	—
<i>B. vulgatus</i>	—	—	+	—	—	+	+	—
<i>B. distasonis</i>	—	—	+	—	+	+	+	—

mogenizer; the bacteria were suspended in saline and were placed into a 70-ml flask (Braun Melsungen) containing 50 g of fine beads (diameter, 0.17 to 0.18 mm) and humidified with 10 ml of saline. The antigens were extracted by homogenization for 2 min at 2,800 rpm, using CO₂ as the cooling agent. The mixture was filtered to discard the beads and then centrifuged for 1 h at 20,000 × g. The supernatant was

lyophilized, and the protein concentration was determined by the method of Lowry et al. (16).

The presence of enzymes in the antigenic extracts was tested for either by zone electrophoresis in agarose, using the enzymic staining technique, or by the API-ZYM strip system (API Système, Montalieu Vercieu, France).

Antisera. Antisera were obtained by immunizing rabbits

FIG. 1. Crossed-immunoelectrophoresis pattern of *B. fragilis* NCTC 9343^T. (A) Coomassie brilliant blue-stained plate. The antigen used was 10 µl of *B. fragilis* extract in the cathodic well, and the antibody was 10 µl of the corresponding rabbit antiserum per cm². (B) Drawing of the 62 precipitates.

FIG. 2. Crossed-immunoelectrophoresis pattern of *B. distasonis* ATCC 8503^T. (A) Coomassie brilliant blue-stained plate. The antigen used was 10 µl of *B. distasonis* extract in the cathodic well, and the antibody was 15 µl of the corresponding rabbit antiserum per cm². (B) Drawing of the 70 precipitates.

FIG. 3. Crossed immunoelectrophoresis in the heterologous system. The antibody used was 10 μ l of rabbit antiserum against *B. fragilis* NCTC 9343^T extract per cm². The antigens used were 10 μ l of *B. thetaiotaomicron* soluble extract (A), 10 μ l of *B. ovatus* soluble extract (B), 10 μ l of *B. vulgatus* soluble extract (C), 10 μ l of *B. distasonis* soluble extract (D), and 10 μ l of *B. fragilis* E323 soluble extract (E)

with soluble extracts of *B. fragilis* E1 (T = type strain) and *B. distasonis*.

Immunizations, as well as evaluations of the titers of serum antibodies, were performed by previously described methods (7).

Crossed immunoelectrophoresis. Crossed immunoelectrophoresis was performed in 1% (wt/vol) agarose medium that was dissolved in 0.025 M tris(hydroxymethyl)aminomethane-barbital buffer (pH 8.6) by using the method of Weeke (25), as described previously (7). Briefly, 10 μ l of antigenic mixture was used for first-dimension electrophoresis, and the antigens thus separated migrated into a gel containing 10 to 15% antibodies during the second-dimension electrophoresis.

Staining of plates. The plates were either stained with Coomassie blue, as previously described (7), or subjected to enzyme staining. In the latter case, the gels were pressed but not dried.

Esterases and phosphatases. The gels were incubated at room temperature in phosphate-buffered saline (pH 7.4) containing the substrate dissolved in the appropriate solvent and a diazonium salt (Fast Red TR) (2).

The substrates used were α -naphthol acetate dissolved in acetone (for esterases) and naphthol phosphate dissolved in distilled water (for phosphatases).

Dehydrogenases. The dehydrogenase (20) test mixtures were incubated at room temperature in tris(hydroxymethyl)aminomethane hydrochloride buffer (pH 7.5).

For malate dehydrogenase (MDH) the test mixture contained (per 100 ml of buffer) 40 mg of potassium cyanide, 2.16 g of sodium malate dissolved in 12 ml of distilled water, 60 mg of nicotinamide adenine dinucleotide, 36 mg of tetranitro blue tetrazolium, and 3 mg of phenazine methosulfate.

For G6PDH the test mixture contained (per 100 ml of buffer) 400 mg of magnesium chloride, 37 mg of nicotinamide

FIG. 4. Crossed immunoelectrophoresis in the heterologous system. The antibody used was 10 μ l of rabbit antiserum against *B. distasonis* ATCC 8503^T extract per cm². The antigens used were 10 μ l of *B. thetaiotaomicron* soluble extract (A), 10 μ l of *B. ovatus* soluble extract (B), 10 μ l of *B. vulgatus* soluble extract (C), 10 μ l of *B. fragilis* NCTC 9343^T soluble extract (D), and 10 μ l of *B. fragilis* E323 soluble extract (E).

adenine dinucleotide phosphate, 17 mg of glucose 6-phosphate, 36 mg of tetranitro blue tetrazolium, and 3 mg of phenazine methosulfate.

RESULTS

The concentrations of soluble extracts ranged from 2 to 10 g/liter (Table 2). Anti-*B. fragilis* and anti-*B. distasonis* sera were obtained by pooling for each rabbit the serum samples that produced the most precipitates during line immunoelectrophoresis (8).

In the crossed-immunoelectrophoresis pattern developed with the *B. fragilis* system, 62 antigenic precipitates were detected. A number was attributed to each peak, starting from the anode side of the first-dimension electrophoresis gel (Fig. 1).

In the same way, 70 precipitates were identified with the *B. distasonis* system (Fig. 2).

Cross-immunoelectrophoresis with heterologous systems permitted us to study cross-reactions of the antigens with the two antisera (Fig. 3 and 4). For each system, the number of precipitates obtained was counted, and the percentage of common antigens was evaluated (Table 1).

Detection of enzymes. The presence of enzymes in the antigenic extracts was verified either by using API-ZYM strips (esterases and phosphatases) or by using zone electrophoresis (G6PDH and MDH).

The four enzymes were also found in the crossed immunoelectrophoresis performed with the *B. fragilis* system; α -esterase corresponded to peak 53 of the reference pattern, phosphatase corresponded to peak 60, MDH corresponded to peak 47, and G6PDH corresponded to peak 48 (Fig. 5).

With the *B. distasonis* system, α -esterase corresponded to antigen 51, phosphatase corresponded to antigen 58, and MDH corresponded to antigens 30 and 35; none of the precipitates showed any G6PDH activity.

Table 2 shows the enzyme activities obtained from crossed immunoelectrophoresis done with heterologous systems.

DISCUSSION

Crossed immunoelectrophoresis with the homologous system. Crossed immunoelectrophoresis performed with soluble extracts of *B. fragilis* E1^T and *B. distasonis* and with their respective antisera demonstrated the antigenic complexity of these species, since 62 and 70 precipitation peaks, respectively, were demonstrated.

We emphasize the superiority of such a technique, which is already used for many microorganisms (notably for yeasts [8]) over such techniques as double immunodiffusion (Ouchterlony technique), in which only two or three precipitation arcs are revealed for an antigen of closely related preparations (1).

Thus, we obtained crossed-immunoelectrophoretic patterns that were reliable enough to serve as reference systems for the study of immunological communities of different *Bacteroides* species belonging to the *B. fragilis* group.

Crossed immunoelectrophoresis with the heterologous system. The reactions of antigens from different *Bacteroides* species with the two reference antisera showed species-related differences.

In the *B. fragilis* system, the strain with the largest number of antigens in common with *B. fragilis* E1^T was *B. fragilis* E2, which is logical since these two strains belong to the same species. The species closest to *B. fragilis* was *B. thetaiotaomicron*; the species most distant was *B. vulgatus* (Table 1). The other species showed intermediate percentages of common antigens.

By using the method described above, no species appeared to be immunologically close to *B. distasonis* since the closest species, *B. ovatus*, did not show one-half the number of antigens of *B. distasonis* (Table 1). However, the absence of cross-reactive antigens may have been due to the low concentration of the *B. ovatus* extract (11).

Therefore, our findings confirmed the validity of different species within the *B. fragilis* group, although we could not draw final conclusions as to the relative proximity of each

TABLE 3. Specific identification of *Bacteroides* strains belonging to the *B. fragilis* group with antigenic enzyme patterns determined by crossed immunoelectrophoresis

Antigen	<i>B. fragilis</i> antiserum			<i>B. distasonis</i> antiserum	
	MDH	G6PDH	Phosphatase	Esterase	Phosphatase
<i>B. fragilis</i> E1 ^T or E2	+	+	+	—	—
<i>B. thetaiotaomicron</i>	+	+	—	—	—
<i>B. ovatus</i>	+	+	—	—	+
<i>B. vulgatus</i>	+	—	—	—	+
<i>B. distasonis</i>	+	—	—	+	+

species to the others. Therefore, it will be necessary to use antisera obtained from these other species and to study several strains of each species.

Enzyme activity revelations on antigenic precipitates. Our method enabled us to identify some antigens of the two reference patterns to enzymes. The value of this finding is twofold; (i) the identification of group- or species-specific antigens is facilitated, as it is based on a small number of antigens that are easily identifiable, compared with the complexity of the patterns observed after staining with Coomassie blue, and (ii) there is taxonomic value since antigenic communities of enzymes are studied between species.

The phosphatase of *B. fragilis* seems to be species specific; it is identical for the two subspecies of *B. fragilis* (strains E1^T and E2) and does not share antigens with any other species when it is tested by crossed immunoelectrophoresis. This also applies to the esterases of *B. fragilis* and *B. distasonis*, but the results are more difficult to analyze since certain precipitates occur nonspecifically (2).

The MDH is common to, and shared by, every system; therefore, it is an antigen which is common to all members of the *B. fragilis* group.

The alkaline phosphatase of *B. distasonis* is identical to the alkaline phosphatases of *B. ovatus* and *B. vulgatus*. However, the latter two species can be distinguished by their G6PDHs; that of *B. ovatus* is actually identical to the G6PDH of *B. fragilis* E1^T, just like the G6PDH of *B. fragilis* E2 and the G6PDH of *B. thetaiotaomicron*.

The G6PDH of *B. distasonis* differs from that of *B. fragilis*, but the absence of the corresponding antibody in anti-*B. distasonis* antiserum prevented us from determining community between the G6PDHs of *B. distasonis* and *B. vulgatus*. Therefore, the availability of an anti-*B. vulgatus* antiserum would be of interest. The method described above enabled us to differentiate most species of the *B. fragilis* group through their enzyme activities; such a demonstration is not possible, however, if one is solely concerned with the presence or absence of an enzyme (9).

Our results are summarized in Table 3. No two of the species have identical profiles. Table 2 shows that with two different antisera and four enzyme activities it is possible to differentiate the five species of the *B. fragilis* group which we studied.

Our results obtained by using Coomassie blue staining are also interesting; the two strains of *B. fragilis* studied are closely related (no difference in antigenic enzymes), and *B. thetaiotaomicron* appears to be the species that is most similar to *B. fragilis*.

Contrary to the results obtained with Coomassie blue-stained crossed-immunoelectrophoresis preparations, the enzymatic staining of precipitates permitted differentiation

FIG. 5. Enzyme staining of a crossed-immunoelectrophoresis preparation. The antigen used was 10 μ l of *B. fragilis* NCTC 9343^T extract. The antibody used was 10 μ l of the corresponding rabbit antiserum per cm². P, Phosphatase; E, esterase.

of *B. ovatus* from *B. vulgatus*, which supports the notion of different species within the *B. fragilis* group.

LITERATURE CITED

- Beerens, H., P. Wattre, T. Shinjo, and C. Romond. 1971. Premiers résultats d'un essai de classification sérologique de 131 souches de *Bacteroides* du groupe *fragilis*. Ann. Inst. Pasteur Paris 121:187-198.
- Brogren, C. H., and T. C. Bog-Hansen. 1973. Enzyme characterization in quantitative immunoelectrophoresis. An enzymological study of human serum esterases, p. 37-51. In N. H. Axelsen et al. (ed.), Quantitative immunoelectrophoresis. Blackwell Scientific Publications, Oslo.
- Cato, E. P., and S. L. Johnson. 1976. Reinstatement of species rank for *Bacteroides fragilis*, *Bacteroides ovatus*, *Bacteroides distasonis*, *Bacteroides thetaiotaomicron*, and *Bacteroides vulgatus*: designation of neotype strains for *Bacteroides fragilis* (Veillon and Zuber) Castellani and Chalmers and *Bacteroides thetaiotaomicron* (Distaso) Castellani and Zuber. Int. J. Syst. Bacteriol. 26:230-237.
- Cousland, G., and I. R. Poxton. 1983. Analysis of lipopolysaccharide of *Bacteroides fragilis* by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroblot transfer. FEMS Microbiol. Lett. 20:461-465.
- Cousland, G., and I. R. Poxton. 1984. Crossed immunoelectrophoresis and ELISA of the cell surface antigens of *Bacteroides fragilis*. J. Gen. Microbiol. 130:645-665.
- Finegold, S. M. 1977. Anaerobic bacteria in human disease. Academic Press, Inc., New York.
- Gabriel-Bruneau, S. M., and R. M. F. Guinet. 1984. Antigenic relationships among some *Candida* species studied by crossed-line immunoelectrophoresis: taxonomic significance. Int. J. Syst. Bacteriol. 34:227-236.
- Guinet, R. M. F., and S. M. Gabriel. 1980. *Candida albicans* group A-specific soluble antigens by quantitative immunoelectrophoresis. Infect. Immun. 29:853-858.
- Hofstad, T. 1980. Evaluation of the API ZYM system for identification of *Bacteroides* and *Fusobacterium* species. Med. Microbiol. Immunol. 168:173-177.
- Hofstad, T. 1982. Immunochemical studies of partially degraded LPS from *Bacteroides fragilis* IPL E 323. Acta. Pathol. Microbiol. Immunol. Scand. Sect. B 90:281-287.
- Holby, N. 1975. Cross-reactions between *Pseudomonas aeruginosa* and thirty-six other bacterial species, p. 187-196. In N. H. Axelsen (ed.), Quantitative immunoelectrophoresis. New developments and applications. Universittforlaget, Oslo.
- Holdeman, L. V., R. W. Kelley, and W. E. C. Moore. 1984. Genus 1. *Bacteroides* Castellani and Chalmers 1919, 959^{AL}, p. 604-631. In N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
- Kasper, D. L., M. E. Hayes, B. G. Reinap, F. O. Craft, A. B. Onderdonk, and B. F. Polk. 1977. Isolation and identification of encapsulated strains of *Bacteroides fragilis*. J. Infect. Dis. 136:75-81.
- Kasper, D. L., and M. W. Seiler. 1975. Immunochemical characterization of the outer membrane complex of *Bacteroides fragilis* subspecies *fragilis*. J. Infect. Dis. 132:440-450.
- Lambe, D. W., Jr., and D. A. Moroz. 1976. Serogrouping of *Bacteroides fragilis* subspecies *fragilis* by the agglutination test. J. Clin. Microbiol. 3:586-592.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
- Moore, W. E. C., and L. V. Holdeman. 1973. New names and combinations in the genera *Bacteroides* Castellani and Chalmers, *Fusobacterium* Knorr, *Eubacterium* Prvt, *Propionibacterium* Delwiche, and *Lactobacillus* Orla-Jensen. Int. J. Syst. Bacteriol. 23:69-74.
- Moore, W. E. C., and L. V. Holdeman. 1974. Human fecal flora: the normal flora of 20 Japanese-Hawaiians. Appl. Microbiol. 27:961-979.
- Onderdonk, A. B., D. L. Kasper, R. L. Cisneros, and J. G. Bartlett. 1977. The capsular polysaccharide of *Bacteroides fragilis* as a virulence factor: comparison of the pathogenic potential of encapsulated and unencapsulated strains. J. Infect. Dis. 136:82-89.
- Owen, P., and M. R. J. Salton. 1975. Antigenic and enzymatic architecture of *Micrococcus lysodeikticus* membranes established by crossed immunoelectrophoresis. Proc. Natl. Acad. Sci. U.S.A. 72:160-171.
- Poxton, I., and R. Brown. 1979. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cell-surface proteins as an aid to the identification of the *Bacteroides fragilis* group. J. Gen. Microbiol. 112:211-217.
- Sedallian, A., and C. Romond. 1982. p. 494-508. In L. Le Minor and M. Vron (ed.), Bactriologie mdicale. Flammarion, Paris.
- Shah, H. N., and M. D. Collins. 1983. A review. Genus *Bacteroides*: a chemotaxonomical perspective. J. Appl. Bacteriol. 55:403-416.
- Shah, H. N., and R. A. D. Williams. 1982. Dehydrogenase pattern in the taxonomy of *Bacteroides*. J. Gen. Microbiol. 128:2955-2965.
- Weeke, B. 1973. General remarks on principles, equipment, reagents and procedures, p. 15-35. In N. H. Axelsen, J. Kroll, and B. Weeke (ed.), A manual of quantitative immunoelectrophoresis. Methods and applications. Blackwell Scientific Publications, Oslo.
- West, S. E. H., and T. D. Wilkins. 1980. Vaspar broth-disk procedure for antibiotic susceptibility testing of anaerobic bacteria. Antimicrob. Agents Chemother. 17:288-291.
- Wren, M. W. D., A. W. F. Baldwin, C. P. Eldon, and P. J. Sanderson. 1977. The anaerobic culture of clinical specimens: a 14 month study. J. Med. Microbiol. 10:49-61.