Chromosomal DNA Probes for the Identification of *Bacteroides* Species

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We compared 22 *Bacteroides* species by DNA–DNA homology studies using the S1 endonuclease method. None of the currently defined species shared more than 30% DNA homology with any other species examined with the exception of *B. buccae* and *B. capillus* (which along with *B. pentosaceus* are now considered a single species), which shared 86% of their DNA sequences. Two clusters showed weak genetic relationships, with DNA homology >10%. The first cluster included *B. corporis*, *B. disiens*, *B. bivius*, *B. intermedius* and *B. melaninogenicus*. The second cluster included *B. fragilis*, *B. eggerthii*, *B. ovatus*, *B. thetaiotaomicron* and *B. uniformis*. Five of the oral species, *B. asaccharolyticus*, *B. gingivalis*, *B. loescheii*, *B. intermedius* and *B. melanogenicus*, were chosen for study as whole chromosomal probes in dot blot assays. These were tested against 243 clinical strains biochemically identified as *Bacteroides* species. The DNA probes correctly identified 94% of the clinical strains. DNA probe and biochemical identification was 100% for two of the five species. In contrast, only 86% of the strains biochemically identified as *B. intermedius* were identified by the DNA probe. The DNA probes gave a species identification to seven strains which could not be biochemically identified.

INTRODUCTION

The genus *Bacteroides* consists of a diverse group of Gram-negative, obligately anaerobic, non-spore-forming rods (Johnson, 1973; Holdeman *et al.*, 1982). Some species are pathogenic for humans and animals and many are found as normal flora in a variety of anatomical sites (Williams & Holt, 1985). Separation of *Bacteroides* species by biochemical tests has been difficult (Johnson & Ault, 1978). Many of the recently described new species represent elevations of previous subspecies ranks to the species level based on nucleic acid studies (Cato & Johnson, 1976; Holdeman *et al.*, 1984). In other cases, new species represent the identification of new distinct taxa (Coykendall *et al.*, 1980; Holdeman & Johnson, 1977, 1982; Holdeman *et al.*, 1982; Jackson & Goodman, 1978; Johnson & Holdeman, 1983; Mitsuka *et al.*, 1974; van Steenberg *et al.*, 1981, 1984; Watabe *et al.*, 1983; Werner *et al.*, 1983). No comprehensive study of the DNA homology between the various species has been published. The limited data available are fragmented, having been obtained in different laboratories using different techniques. As a result the DNA–DNA homology between the various species within the genus is not clear. Recently a comprehensive study of rRNA homology values among *Bacteroides* strains was published (Johnson & Harich, 1986). The majority of the *Bacteroides* strains tested fell into one of three clusters. The intracluster rRNA homology values ranged from 35 to 100%, and intercluster homology values were about 25%. However, homology between *Escherichia coli* and the *Bacteroides* rRNA ranged from 4 to 29%, suggesting the conservation of rRNA sequences between genetically (at the DNA level) dissimilar genera.

Recently we found that DNA dot blots could be used to identify clinical strains of the new anaerobic genus *Mobiluncus* (Roberts *et al.*, 1984, 1985). The two species of *Mobiluncus* share 9–25% of their DNA sequences and show no DNA homology with other established genera

(Spiegel & Roberts, 1984). The DNA dot blot provided a simple method of identifying and confirming the identity of the strains as *Mobiluncus*, and was more reproducible than any of the currently available biochemical tests. We were interested in determining whether a similar type of assay could be developed for identifying oral *Bacteroides* species, some of which are thought to play a role in periodontal disease (Holdeman *et al.*, 1982; Loesche *et al.*, 1982; Tanner *et al.*, 1979; White & Mayrand, 1981).

METHODS

Strains. The 23 *Bacteroides* species in the study included the 21 listed by Holdeman *et al.* (1984) for the proposed type strain for each species. Each strain was obtained from the American Type Culture Collection (ATCC) and verified biochemically: *B. asaccharolyticus* ATCC 25260, *B. bivius* ATCC 29303, *B. buccae* ATCC 33574, *B. capillus* ATCC 33690, *B. corporis* ATCC 33547, *B. denticola* ATCC 33185, *B. disiens* ATCC 29426, *B. distasonis* ATCC 8503, *B. eggerthii* ATCC 27754, *B. fragilis* ATCC 25285, *B. gracilis* ATCC 33236, *B. gingivalis* ATCC 33277, *B. intermedius* ATCC 25611, *B. loescheii* ATCC 15930, *B. melaninogenicus* ATCC 25845, *B. multiaacidus* ATCC 27723, *B. oris* ATCC 33573, *B. ovatus* ATCC 8483, *B. splanchnicus* ATCC 29572, *B. thetaiotaomicron* ATCC 29148, *B. uniformis* ATCC 8492, *B. ureolyticus* ATCC 33387, *B. vulgatus* ATCC 8482. Two hundred and forty-two clinical strains isolated from the oral cavity and one genital isolate which had been biochemically identified using the API AnIdent system and VPI system (Holdeman *et al.*, 1977) were tested in the dot blot assay. These included 2 *B. asaccharolyticus*, 70 *B. gingivalis*, 23 *B. loescheii*, 106 *B. intermedius*, 35 *B. melaninogenicus* and 7 *Bacteroides* species. Other strains used included: *Eubacterium lentum* ATCC 2559, *E. saburreum* ATCC 33271, *E. timidum* ATCC 33092, *E. plautii* ATCC 29863, *E. nodatum* ATCC 33099, *Eikenella corrodens* ATCC 23834, *Selenomonas sputigena* ATCC 33150, *Fusobacterium nucleatum* ATCC 23726, *F. gonidiaformans* ATCC 25563, *Haemophilus aphrophilus* ATCC 19415, *Actinomyces naeslundii* ATCC 12104, *A. viscosus* ATCC 15987, *A. odontolyticus* ATCC 17929, *A. israeli* ATCC 12102, *Rothia dentocariosa* ATCC 17931, *Capnocytophaga gingivalis* ATCC 33624, *C. ochracea* ATCC 33596, *Arachnia propionica* ATCC 14157, *Peptostreptococcus micros* ATCC 33270, *Wolinella recta* ATCC 33238, *Veillonella parvula* ATCC 10790, and *Actinobacillus actinomycetemcomitans* ATCC 29522. Field strains of many of these species were also tested. Field strains of *Staphylococcus aureus* and *Bacteroides forsythus* were also used.

Patient selection and sampling. Patients with various forms of periodontal disease were obtained from the periodontal clinic at the University of Washington. Subgingival samples were taken from 5–10 mm periodontal pockets using a Morse scaler. The samples were placed in 2 ml prereduced Hanks' balanced salts solution containing 10% (v/v) heat-inactivated horse serum and transported to an anaerobic glove box within 10 min. The plaque was dispersed by a 5 s pulse on low power from a micro-ultrasonic cell disrupter (Kontes). Samples were spotted onto nitrocellulose and serial dilutions were prepared in the same medium and plated on prereduced blood agar plates for bacterial identification. Plates were incubated for 7–10 d in an anaerobic environment at 35 °C.

Media. Blood agar plates were prepared using Columbia blood agar base (Difco) as described by the manufacturer. Each lot of plates was quality controlled. Other media were prepared as described in the VPI Manual (Holdeman *et al.*, 1977). The liquid medium used to grow large quantities of bacteria for preparation of DNA was a soy-peptone fresh yeast extract dialysate broth (Kenny, 1967), or enriched trypticase soy broth (Syed, 1980).

DNA extraction and purification. Each strain was grown in 700–1000 ml liquid medium for 5–7 d at 37 °C in an anaerobic environment. The DNA extraction method was as previously described for use with *Mobiluncus* spp. (Spiegel & Roberts, 1984).

DNA–DNA hybridization. DNA was isolated from each of the strains. The DNA from 23 strains representing 22 species of *Bacteroides* (two strains, *B. capillus* and *B. buccae*, are now placed in a single species) was labelled by nick translation, using 0.5 µg DNA, [³H]thymine 5'-triphosphate and three unlabelled nucleotide triphosphates (Roberts *et al.*, 1984; Spiegel & Roberts, 1984). The unincorporated radiolabel was separated from the labelled double-stranded DNA by passage through a 15 cm Sephadex G-75 column. The probes were stored at –10 °C and used within 3 weeks. The radiolabelled probes were diluted and concentrations of 5000 total c.p.m. were used as probes. Test DNA (15 µg) and 5000 c.p.m. of radioactive probe DNA were mixed, denatured, hybridized overnight at 65 °C, and the amount of reassociation was assayed with S1 endonuclease (Spiegel & Roberts, 1984). The levels of reassociation of unlabelled homologous DNA and unlabelled heterologous DNA (calf thymus DNA) with each labelled probe DNA served as controls in each experiment. The heterologous control measured the amount of self-annealing of the probe (≤10%) and this value was subtracted from all other values. The homologous control always had ≥75% of the total probe activity. Test results were normalized to 100% for the homologous DNAs and all other values normalized. Duplicate tests were run for each assay on two to four separate occasions. The results in Table 1 are the means of these separate runs. Individual assays varied from each other by ±2%.

Dot blots. DNA (0.1 µg) was nick translated as previously described (Roberts *et al.*, 1984) using two ^{32}P -labelled nucleotide triphosphates and two unlabelled nucleotide triphosphates. The unincorporated radiolabel was separated from the labelled double-stranded DNA by passage through a 15 cm Sephadex G-75 column. The probes were stored at -10°C and used within a week. Organisms were grown on blood agar plates at 35°C for 2 d, then growth was removed with a sterile cotton applicator and transferred to sterile saline. The density of the suspensions were adjusted to 0.5 MacFarland units and 5 µl of organisms was then spotted onto nitrocellulose paper. Filters were treated to release and fix the DNA to the nitrocellulose paper and stored until needed at room temperature (Roberts *et al.*, 1984). Routinely, 20–30 clinical strains plus controls were spotted on one piece of nitrocellulose and five replicates were prepared of each filter so that one could be tested with each probe. The filters were pretreated at 42°C for ≥ 1 h with a solution containing 50% (w/v) formamide, 1% (w/v) glycine, 50 mM-sodium phosphate, 0.02% (w/v) polyvinylpyrrolidone, 0.1% (w/v) albumin, 0.1% (w/v) Ficoll, 0.9 M-NaCl, 0.05 M-monobasic sodium phosphate, 0.005 M-EDTA, 0.055 M-NaOH and 100 µg boiled calf thymus DNA ml $^{-1}$. They were then drained and incubated overnight at 42°C in a solution containing 50% (v/v) formamide, 1% (w/v) glycine, 20 mM-sodium phosphate buffer (pH 6.5), 100 µg boiled calf thymus DNA ml $^{-1}$, 0.9 M-NaCl, 0.05 M-monobasic sodium phosphate, 0.005 M-EDTA, 0.02% (w/v) polyvinylpyrrolidone, 0.02% (w/v) albumin, 0.02% (w/v) Ficoll, 0.055 M-NaOH, 10% (w/v) sodium dextran sulphate (M_r 500 000), 100 µg boiled calf thymus DNA ml $^{-1}$, and boiled ^{32}P -labelled probe (10^8 c.p.m. per µg DNA). The filters were then removed and washed three times at room temperature with 250 ml of a solution containing 0.002 M-EDTA, 0.02 M-monobasic sodium phosphate, 0.36 M-NaCl and 0.1% (w/v) SDS, followed by two washes at 50°C in 500 ml of a solution containing 0.001 M-EDTA, 0.001 M-monobasic sodium phosphate, 0.18 M-NaCl and 0.1% (w/v) SDS. The filters were dried and exposed overnight to Kodak X-omat AR film at -70°C and then developed (Roberts *et al.*, 1984).

RESULTS AND DISCUSSION

In order to determine the genetic relationships between the organisms classified in the genus *Bacteroides*, we carried out a reciprocal cross-comparison using 20 probes representing 20 species, by the S1 endonuclease method (Table 1). We also included *B. capillus* to test its relatedness to *B. buccae*. These two strains have recently been placed into a single species along with the strain originally named *B. pentosaceus* (Johnson & Holdeman, 1985). Two additional probes, *B. gracilis* and *B. denticola*, were tested against the panel of 20 species, *B. capillus* and themselves in a one-way cross. This is the first time that a single laboratory has examined this number of strains using a single method and at a single time. Overall, the *Bacteroides* species were remarkably distinct. Only 50 of the 180 cross-hybridizations (excluding the 20 homologous reactions) in the reciprocal cross-comparisons showed homology of 10% or greater (Table 1). *B. gracilis*, *B. denticola* and *B. capillus* were tested in one-way cross-comparisons and only 6 of the 43 hybridizations (excluding the 3 homologous reactions) showed homology of 10% or greater. The *B. capillus* vs *B. buccae* reaction was considered a homologous one, since they are now considered a single species. Two clusters of species showed weak reciprocal homology and were determined by the reciprocal cross-hybridizations having a value greater than 10%. The first cluster included *B. corporis*, *B. disiens*, *B. bivius*, *B. intermedius* and *B. melaninogenicus*. *B. denticola* may also be linked to this cluster but since only one-way crosses were examined this is not clear. The second cluster comprised *B. fragilis*, *B. eggerthii*, *B. ovatus*, *B. thetaiotaomicron* and *B. uniformis*. These two groups are very similar to the clusters reported using rRNA probes (Johnson & Harich, 1986) but the homology using rRNA probes was significantly higher than what we found using chromosomal DNA as the probe. The largest homology observed was with the two strains labelled *B. buccae* and *B. capillus* (86%). Similar values (82–87%) have been reported in other studies (Johnson & Holdeman, 1985). In our study the DNA homologies between *B. buccae* and the other species was very similar to the results obtained between *B. capillus* and the other species. This would be predicted and provides further evidence of their genetic similarity.

For seven of the species, we compared our data with those from several other DNA–DNA homology studies in which a variety of methods and strains were used (Bradbury *et al.*, 1985; Cato *et al.*, 1976; Cooper *et al.*, 1984; Johnson, 1978; Johnson & Ault, 1978) (Table 2). In many cases, the values obtained in other laboratories are compatible and within $\pm 3\%$ of the data presented in this paper. One pair which is significantly different is *B. ovatus* vs *B. thetaiotaomicron* which, in our study, had 18–20% homology while other laboratories report 22–

Table 2. Comparison of DNA-DNA homologies obtained in this study with those of the literature

The actual extent of binding of ^3H -labelled DNA with the unlabelled chromosomal DNA of the parent strain was 75–85%; however, all were set at 100% and all other values were normalized to give relative homology. Values in parenthesis were taken from the literature (Cato & Johnson, 1976; Johnson, 1973, 1978; Johnson & Ault, 1978).

Unlabelled DNA	Labelled probe:						
	<i>B. dist.</i>	<i>B. frag.</i>	<i>B. egg.</i>	<i>B. ovatus</i>	<i>B. thet.</i>	<i>B. unif.</i>	<i>B. vulg.</i>
<i>B. distasonis</i>		(8–21)	(3)	(0–3)	(4–7)	(5–10)	(0–7)
	100	7	4	3	6	15	4
<i>B. fragilis</i>	(5)			(9–24)	(21–28)	(17–21)	(10–20)
	8	100	16	15	11	11	11
<i>B. eggerthii</i>	(8)	(25–28)		(12–18)	(17–23)	(25–58)	(20–26)
	5	10	100	13	17	20	13
<i>B. ovatus</i>	(0–4)	(9–24)			(39–40)	(9–21)	(0–8)
	6	19	13	100	20	16	4
<i>B. thetaiotaomicron</i>	(5–7)	(21–30)	(21–23)	(22–49)		(3–28)	(4–28)
	8	25	19	18	100	17	8
<i>B. uniformis</i>	(6–14)	(9–28)	(29–62)	(9–100)	(14–40)		(9–29)
	10	9	29	11	11	100	8
<i>B. vulgatus</i>	(6–24)	(0–9)	(6–14)	(0–16)	(9–17)	(0–20)	
	12	8	17	8	8	9	100

49% and 39–40% depending on which strain was used as the probe and which as the test. The reciprocal values within our testing conditions are identical, suggesting that the difference seen when *B. thetaiotaomicron* (39–40%) was used as a probe may be due to differences in the strains tested or in the method for assaying homology. Other differences between our data and previous work were seen between *B. uniformis* vs *B. ovatus*, for which homology ranged from 9–100% in other studies while it was 11% in this study. Similarly, homology between *B. uniformis* vs *B. thetaiotaomicron* ranged from 14 to 40% and between *B. uniformis* vs *B. eggerthii* from 25 to 58% in other studies, whereas values of 11% and 20% respectively were obtained in this study.

Many of the *Bacteroides* spp. are difficult to differentiate from each other by classical phenotypic and biochemical tests, yet appear to share very little genetic homology. This is similar to the problem described for the new anaerobic genus *Mobiluncus* (Roberts *et al.*, 1984, 1985). *Mobiluncus* strains are difficult to isolate, maintain and identify to species level; however, we have found identification by chromosomal DNA dot blot to be a convenient and reliable way of confirming an identification of species within the genus even though two species share 9–25% homology (Spiegel & Roberts, 1984). Most of the *Bacteroides* species share <30% DNA homology with other species in the genus. This suggests that the dot blot assay could be used to speciate strains of *Bacteroides*.

We prepared whole-cell DNA probes from five oral *Bacteroides* species: *B. asaccharolyticus*, *B. gingivalis*, *B. loescheii*, *B. intermedius* and *B. melaninogenicus*. Some of these bacteria are thought to play a role in periodontal disease. When each probe was tested against the other species in dot blot assays, using purified DNA, no hybridization was seen between each of the probes and any of the other species. In contrast, a good reaction occurred with the homologous probe and homologous test DNA. Similar results were seen when whole bacteria were spotted instead of pure DNA. The bacterial cell density of whole organisms was not critical as long as a minimum of 10^4 bacteria were spotted. Other bacteria often found in the oral cavity were also tested and included: *Eubacterium lentum*, *E. saburreum*, *E. timidum*, *E. plautii*, *E. nodatum*, *Campylobacter gingivalis*, *C. ochracea*, *Eikenella corrodens*, *Selenomonas sputigena*, *Fusobacterium nucleatum*, *F. gonidiaformans*, *Haemophilus aphrophilus*, *Actinomyces naeslundii*, *A. viscosus*, *A. odontolyticus*, *A. israelii*, *Rothia dentocariosa*, *Arachnia propionica*, *Peptostreptococcus micros*, *Staphylococcus aureus*, *Wolinella recta*, *Veillonella parvula*, *Bacteroides forsythus* and *Actinobacillus actinomycetemcomitans*. These included biochemically identified field and reference strains. None of these species cross-reacted with the five *Bacteroides* probes.

Table 3. *Identification of clinical strains by DNA probes*

Clinical strains identified biochemically	Positive by DNA probe				
	<i>B. asacch.</i>	<i>B. inter.</i>	<i>B. ging.</i>	<i>B. loescheii</i>	<i>B. melan.</i>
<i>B. asaccharolyticus</i> (n = 2)	2	0	0	0	0
<i>B. intermedius</i> (n = 106)	0	93	0	0	0
<i>B. gingivalis</i> (n = 70)	0	0	70	0	0
<i>B. loescheii</i> (n = 23)	0	0	0	22	0
<i>B. melaninogenicus</i> (n = 35)	0	0	0	0	33
<i>B. melaninogenicus</i> or <i>B. denticola</i> (n = 3)	0	1	0	1	1
<i>Bacteroides</i> sp. (n = 4)	0	1	0	2	1

We tested 236 clinical isolates biochemically identified as: *B. asaccharolyticus* (1 genital and 1 oral), 70 oral *B. gingivalis*, 23 oral *B. loescheii*, 106 oral *B. intermedius* and 35 oral *B. melaninogenicus* (Table 3). Two of the five DNA probes (*B. asaccharolyticus* and *B. gingivalis*) identified all of the strains identified by biochemical tests as the same species. The *B. intermedius* probe missed 12% of the biochemically identified strains. These missed strains may represent the *B. intermedius* type II strains which share only 40% homology with the *B. intermedius* type I strains (Johnson & Harich, 1986). Our probe strain was a type I strain. A type II strain is being prepared and will be used to examine these non-reactive strains. It is also possible that these strains biochemically type as *B. intermedius* but genetically resemble one or more other *Bacteroides* species. We are at present determining whether these strains react with other members of the 22 species examined in this study. However, it is possible that these bacteria represent one or more new species. Twenty-two out of 23 (96%) *B. loescheii* strains, and 33 out of 35 (94%) *B. melaninogenicus* strains, were confirmed by DNA dot blot as the species identified by biochemical tests.

Seven strains could not be biochemically assigned to species (Table 3), but all were identified when tested against the five probes. The three strains identified as either *B. melaninogenicus* or *B. denticola* were identified by DNA probes as one strain each of *B. intermedius*, *B. loescheii* and *B. melaninogenicus*, while the four strains identified as *Bacteroides* sp. were identified as one each of *B. intermedius* and *B. melaninogenicus* and two of *B. loescheii*. Successful identification of this small group of strains suggests that the dot blot assay could help identify strains which are ambiguous by biochemical identification schemes.

The dot blot assay required on the average 36 h to complete, while the biochemical testing required 7–21 d. The DNA probes will provide for more definitive identification of the species because small genetic changes leading to phenotypic variation will not be detected by the whole genomic probe assay.

Our results indicate that the nitrocellulose blot is a quick method of identifying *Bacteroides* isolates to species, and from our DNA–DNA homology data it is apparent that whole chromosomal probes prepared from any of the species examined should provide a specific probe for identifying the majority of the strains isolated in the laboratory. The advantage of this system is that cloning is not required, the genetic organization of the species need not be known and the probability of the probe recognizing all members of the species is very high. In contrast, with cloned probes a number of different fragments need to be tested for specificity. In the study reported by Salyers *et al.* (1983), only two of the five cloned fragments which were screened hybridized specifically with *B. thetaiotaomicron* and no other *Bacteroides* species. The other problem with cloned fragments is that they may not react with all members of the species, as was reported by Kuritza *et al.* (1986). In that report, some of the isolated fragments were specific for the type I or type II subgroup of *B. fragilis* and reacted only with strains from the same subgroup. Finally, if single-copy genes are used as the probe fragment, the sensitivity can be as much as 100-fold less as compared to multicopy genes or whole chromosomal DNA probes (McLafferty *et al.*, 1986).

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