JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1985, p. 24–28
0095-1137/85/010024-05$02.00/0

Vol. 21, No. 1

Bacterial Chromosomal Restriction Endonuclease Analysis of the Homology of *Bacteroides* Species

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Received 25 May 1984/Accepted 21 September 1984

Chromosomal DNAs of selected *Bacteroides* organisms whose relatedness had been previously determined by "conventional" filter-annealing studies (J. L. Johnson, Int. J. Syst. Bacteriol. 28:245, 1978) were further analyzed by restriction endonuclease analysis coupled with the Southern hybridization procedure (E. M. Southern, J. Mol. Biol. 98:503, 1975). By comparing their *Eco*RI restriction fragment patterns in agarose gel electrophoresis, each *Bacteroides* strain could be clearly differentiated. As a simple and direct means for comparison purposes this method was particularly useful for differentiating genetically similar organisms such as *Bacteroides* strains of the same species which shared >75% homology. In contrast, bacterial chromosomal restriction endonuclease analysis in conjunction with Southern hybridizations was most effectively used to determine the significance of low levels of homology (<24%) as this technique provided additional information on the nature and relative distribution of that homology when the areas of homology were displayed as reproducible bands in autoradiograms.

Bacterial organisms of the genus *Bacteroides* are a group of phenotypically similar gram-negative rods (15), which were formerly classified as a single species *Bacteroides fragilis*, with five subspecies (12). Recent DNA hybridization studies have separated this group at the species level, with 10 major homologous groups now recognized (12). We have chosen representatives of these groups showing a wide range of homology values (<4 to 80%), as determined by Johnson (12), for further examination, using bacterial chromosomal restriction endonuclease analysis (REA) coupled with hybridizations by the Southern blotting technique (23). This method displays DNA fragments in order of size and indicates the distribution and equivalence of these fragments in related genomes by the use of appropriate labeled probes. Therefore, REA data can be used to extend the observations of conventional DNA filter hybridization techniques. In the present communication we find that *Bacteroides* species showing similar homology by "conventional" DNA filter-annealing studies can be further distinguished by REA.

MATERIALS AND METHODS

Bacterial strains and media. *Bacteroides* strains were obtained from J. L. Johnson (Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg). The six strains used in our study were selected from a larger collection of 16 reference strains that Johnson (12) used to subdivide the former "*Bacteroides fragilis* group" into 10 distinct homology groups. These six strains are *B. ovatus* 0038 (neotype strain), *B. ovatus* 3524, "3524-A," *B. vulgatus* 4245, *B. uniformis* 0061, and *B. fragilis* 2553 (neotype strain) (Table 1). The strain identification numbers are those of the Johnson reference collection. They were cultivated in brain heart infusion broth (Difco Laboratories, Detroit, Mich.), supplemented as below, in an anaerobic tent at 37°C. The medium for the 1-liter cultures used for DNA isolation contained 3.7% (wt/vol) dehydrated brain heart infusion broth, 0.5% yeast extract (Difco), 0.05% cysteine-HCl, 1% heme, and 0.02% vitamin K. This medium was seeded with 30 ml of culture grown overnight.

DNA isolation. Cells were washed once with single-strength citrate buffer (SSC; containing 0.15 M NaCl, 0.01 M trisodium citrate, pH 7.0) and suspended in 15 ml of STE buffer (0.1 M NaCl, 0.02 M Tris, 0.01 M EDTA, pH 7.4). DNA samples were prepared according to the method of Cohen et al. (5) with modifications. Samples were treated with pronase (Sigma Chemical Co., St. Louis, Mo.) (self-digested for 2 h at 37°C, 1 mg/ml) and 1% sodium dodecyl sulfate for 1 h or overnight at 37°C. The mixture was then treated three separate times with equal volumes of phenol-chloroform (50:50), and the upper aqueous phases were carefully collected and pooled. Each sample was treated with RNase (bovine pancreatic, type 1A; Worthington Diagnostics, Mississauga, Ontario, Canada) (heat treated at 90°C for 10 min; 50 μg/ml) for 1 h at 37°C. Each sample was dialyzed against TE buffer (5 mM Tris-hydrochloride, 0.1 mM EDTA, pH 7.4), and the DNA concentrations were determined by the diphenylamine assay of Burton (4). The DNA samples were subjected to agarose gel electrophoresis before restriction endonuclease digestion to test for the presence of extrachromosomal plasmid DNA (1, 14), but no plasmid DNA was detected. Only a single 19- to 21-megadalton (Md) band, representative of chromosomal DNA (1), was seen.

Preparation of ^{32}P-labeled DNA probes. The DNA samples were labeled by nick translation (13, 21) with minor modifications. Standard reactions used approximately 0.5 μg of duplex DNA, 180 pmol each of [α-^{32}P]dATP and [α-^{32}P]dTTP (400 Ci/mmol; Amersham Radiochemicals, Oakville, Ontario, Canada), and 180 and 400 pmol of unlabeled dCTP and dGTP, respectively. The reaction was initiated with 5 to 10 U of DNA polymerase I (New England Nuclear, Beverly, Mass.) and incubated for 1 h at 15°C in the presence of DNase (5 ng/ml). The addition of 0.5 M EDTA, pH 7.8, terminated the reaction, and the labeled DNA was extracted two separate times with an equal volume of chloroform-isoamyl alcohol (24:1). The aqueous phases were pooled and loaded on a G-75 Sephadex column (10 ml) in 1 mM Tris-hydrochloride (pH 8.0)–0.25 mM EDTA and eluted.

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TABLE 1. Reciprocal interstrain homology values among DNA reference organisms used in this study[a]

Probe	% Homology					
	B. fragilis 2553	*B. ovatus*			*B. uniformis* 0061	*B. vulgatus* 4245
		0038	3524	"3452-A"		
B. fragilis 2553	100	15	13	10	21	10
(neotype strain)	100	24	9	23	17	20
B. ovatus 0038	24	100	80	31	17	8
(neotype strain)	15	100	75	37	9	8
B. vulgatus 4245	20	8	0	4	19	100
	10	8	8	0	14	100

[a] Data are reproduced from reference 12. *B. fragilis* 2553, *B. ovatus* 0038, and *B. vulgatus* 4245 were used for the nick-translated, ^{32}P-labeled DNA probes in our study.

Peak fractions were collected and precipitated overnight at −20°C. The specific activities obtained were routinely 10^8 cpm/μg of DNA. The labeled probes were used within 1 month of preparation.

REA. Approximately 5 to 10 μg of high-molecular-weight bacterial DNA was completely digested in a reaction mixture containing a fivefold excess of *Eco*RI enzyme (Boehringer Mannheim, St. Laurent, Quebec, Canada; or Bethesda Research Laboratories, Bethesda, Md.) in *Eco*RI buffer (100 mM Tris-hydrochloride, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$) at 37°C for 16 to 18 h. Identical results were obtained with *Eco*RI from both commercial sources. The reaction volumes were approximately 100 μl and the reactions were monitored for complete digestion, using bacteriophage λ DNA (Miles Laboratories, Elkhart, Ind.). All reactions were terminated by the addition of 10 μl of DGE buffer (0.12% bromophenol blue, 25% glycerol, 0.2 M EDTA) and heated for 10 min at 68°C. The DNA fragments were separated by electrophoresis in 0.7% agarose gels in EB buffer (40 mM Tris-hydrochloride, pH 8.0, 2 mM EDTA, 20 mM sodium acetate) (22). Gels were stained with ethidium bromide (1 μg/ml) for 1 h and photographed under UV illumination on Kodak High Contrast Copy 35-mm film, using a no. 25 red Kodak Wratten filter. Banding patterns in all digests were reproducible.

Southern transfer and hybridization to ^{32}P-labeled probe DNA. Agarose gels were treated for 1 to 2 h in 1 liter of 1.5 M NaCl–0.5 M NaOH at room temperature and then for 3 to 4 h in 1 liter of 0.5 M Tris-hydrochloride (pH 7.0)–3 M NaCl at room temperature. The DNA was blotted onto nitrocellulose filters according to the method of Southern (23) as adapted by Shank et al. (22). The blots were dried in vacuo at 80°C for 1 to 2 h and stored at room temperature in vacuo until required for the hybridization experiments.

Before hybridization, each of the ^{32}P-labeled DNA probes was boiled for 10 min in 3 M NaOH (16). Filters were hybridized as previously described (6, 17, 22). The filters were first preannealed at 41°C for 24 h with annealing buffer (3 × SSC, 50% formamide, 200 μg of yeast RNA per ml, 20 μg of sheared denatured salmon sperm DNA per ml, 1× Denhardt buffer). Each labeled DNA probe was hybridized separately to a single filter at approximately 8,500 cpm/cm^2 of filter and incubated at 41°C for 48 h in annealing buffer. Filters were then washed in successive baths of (i) 2× SSC (1 liter) for 1 h at room temperature, (ii) 0.1× SSC (2 liters; 1:10)–0.1% sodium dodecyl sulfate at 50°C for 1 h and then 30 min at room temperature, and (iii) a 30-min wash in 0.1× SSC (1:10). Filters were then air dried and exposed at −70°C to Kodak RP-Royal X-Omat film in the presence of Dupont Cronex Lightening Plus intensifying screens (24). Each hybridization was performed at least twice.

RESULTS

The patterns of the bacterial restriction endonuclease *Eco*RI digests of chromosomal DNA extracted and purified from the six *Bacteroides* strains may be compared in Fig. 1A. Clearly, each strain can be differentiated on the basis of its characteristic restriction pattern in agarose gel electrophoresis. This was not surprising as it was known that these *Bacteroides* species were very heterogeneous by DNA homology, ranging from <4 to 80%, as determined by a conventional filter competition method of hybridization (12) (Table 1).

To determine the specific nature of this homology, we compared the patterns generated by the annealing of *B. fragilis* 2553 labeled DNA probe with Southern blots of *Eco*RI-digested DNA from *B. uniformis* 0061 and *B. ovatus* 0038. It was previously established that *B. fragilis* 2553 shared only 15 to 24% homology with the latter two organisms (12) (Table 1). In Fig. 2 it is clearly evident that the areas of homology visible by REA are drastically different in their *Eco*RI sites and thus do not represent identical areas in each of the *Bacteroides* genomes. Although their restriction patterns appeared similar in agarose gel electrophoresis, few *Eco*RI fragments were homologous (Fig. 2, arrows). An interesting observation on these data was that the only visible area of homology between *B. fragilis* 2553 and *B. uniformis* 0061 was in the region of a 17.5-Md *Eco*RI fragment.

Similar data were obtained when we annealed a *B. vulgatus* 4245 labeled DNA probe with *Eco*RI-digested DNA from *B. uniformis* 0061, "3452-A," and *B. ovatus* strains 0038 and 3524. It was previously shown that *B. vulgatus* 4245 shared very limited homology with either of the *B. ovatus* strains (0 to 8%) and <19% homology with *B. uniformis* 0061 (12) (Table 1). Again, analysis with *Eco*RI indicated that areas of homology were quite different in their *Eco*RI sites and could be clearly distinguished, although in some instances it was apparent that the same *Eco*RI site was common to different strains (Fig. 3). This was observed for a 14.6-Md *Eco*RI fragment present in both the 0061 and 3524 genomes and a 13.8-Md *Eco*RI fragment present in both the 0038 and "3452-A" genomes (Fig. 3, arrows).

In the above cases, the use of REA coupled with hybridization and the Southern blotting procedure (23), using total chromosomal nick-translated DNA probes, permitted further differentiation of *Bacteroides* species known to share limited homology (<24% [12]) (Table 1).

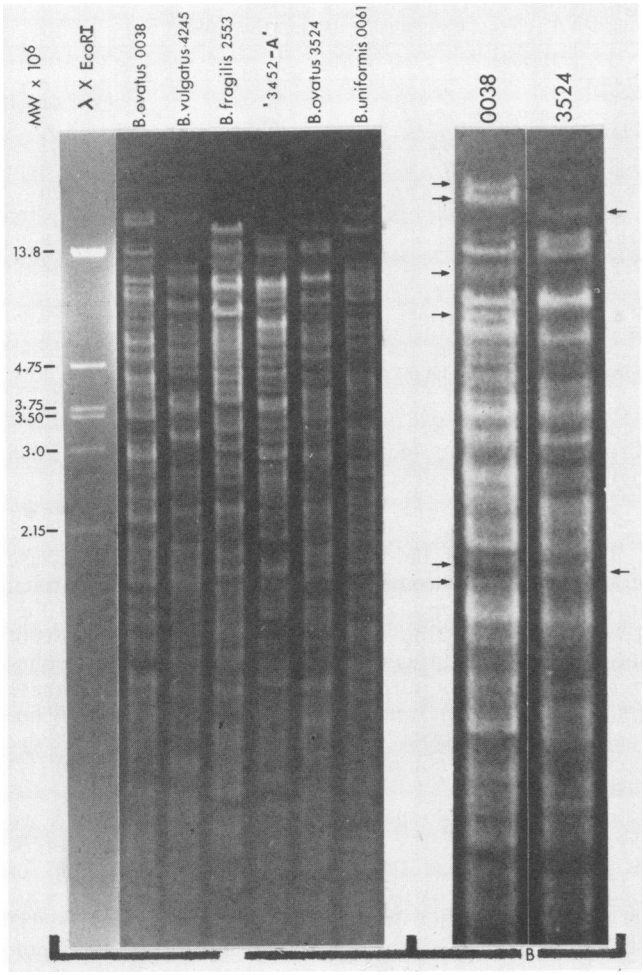

FIG. 1. Agarose gel electrophoresis of *Eco*RI digests of *Bacteroides* chromosomal DNA. (A) Approximately 1 to 2 μg of DNA was used in each lane for illustration. *Eco*RI digests were subjected to electrophoresis through 0.7% agarose and photographed by UV illumination after ethidium bromide staining as described in the text. In each electrophoresis run the molecular weights of the *Bacteroides* DNA fragments were determined by using *Eco*RI fragments of bacteriophage λ DNA of known molecular weights (MW) ranging from 2.15×10^6 to 13.8×10^6. (B) Enlargement of *B. ovatus* 0038 and *B. ovatus* 3524 as in (A) above. Although these closely related strains shared >90% homology (12), they could be differentiated by their patterns of *Eco*RI-digested DNAs (arrows).

On the other hand, the extent of *Eco*RI homology between *Bacteroides* strains of the same species which shared >75% homology (e.g., *B. ovatus* 0038 and 3524, Table 1) resulted in the hybridization of multiple restriction fragments which yielded smeared tracks in autoradiograms when *B. ovatus* 0038 nick-translated DNA was used as probe (shorter exposure periods yielded similar results; data not shown). In this case discernible differences in their *Eco*RI restriction patterns in agarose gel electrophoresis made it possible to differentiate these closely related *B. ovatus* strains (Fig. 1B, arrows).

DISCUSSION

The patterns of bacterial chromosomal DNA fragmentation or genome "fingerprints" produced by *Eco*RI restriction endonuclease in agarose gel electrophoresis clearly differentiated each *Bacteroides* organism examined. *Bacteroides* strains of the same species (e.g., *B. ovatus* 0038 and 3524) that were genetically very similar by hybridization analysis (>75% homology [12]) could also be distinguished by their characteristic restriction patterns in gels. Thus, when the genetic relationships between bacterial organisms has been established by analytical hybridization techniques, REA may be used directly as a simple and rapid method for strain and isolate differentiation. Furthermore, this techique offers considerable potential for use as an epidemiological tool for identifying sources and tracing routes of transmission of infectious bacteria, since it permits a direct approach to differentiating organisms regardless of whether or not alternative typing methods such as serotyping, bacteriophage typing, and bacteriocin typing are available. For instance, we have recently demonstrated the ability of REA to establish the epidemiological relationships between isolates of *Campylobacter jejuni* involved in cases of enteritis in a laboratory worker (20) and residents of a boarding school

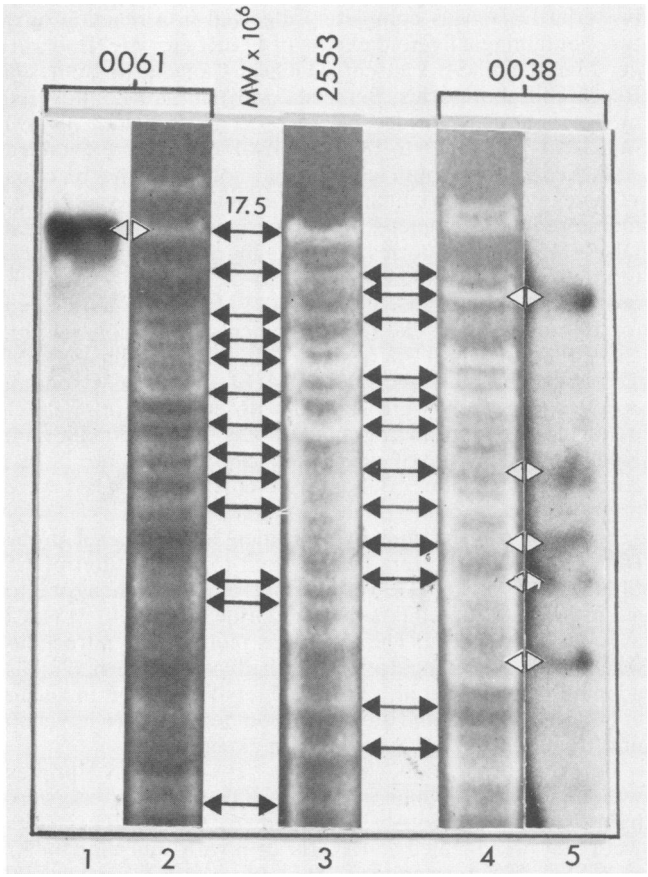

FIG. 2. Hybridization of *B. uniformis* 0061 and *B. ovatus* 0038 *Eco*RI-digested chromosomal DNAs with *B. fragilis* 2553 probe DNA. DNA was prepared from each strain and digested with *Eco*RI, followed by agarose gel electrophoresis. The DNA fragments were then transferred to nitrocellulose filters by the blotting methods of Southern (23) and hybridized with the *B. fragilis* 2553 probe DNA as described in the text. (Lanes 2 to 4) *Eco*RI digests in agarose gel electrophoresis; (lanes 1 and 5) hybridization profiles generated by the 0038 probe DNA annealed with 0061 and 0038 chromosomal DNAs. Many of the *Eco*RI DNA restriction fragments of 2553, 0061, and 0038 had very similar mobilities in agarose gel electrophoresis (arrows) but few of them were homologous (open arrowheads). MW, Molecular weight.

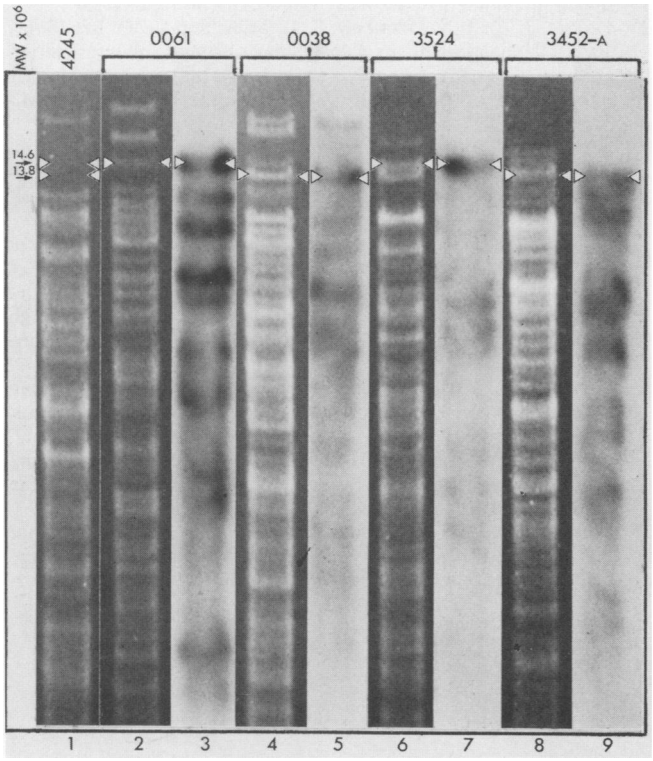

FIG. 3. Hybridization of 0061, 0038, 3524, and "3452-A" *Eco*RI-digested chromosomal DNAs with *B. vulgatus* 4245 probe DNA. (Lanes 1, 2, 4, 6, 8) *Eco*RI digests in agarose gel electrophoresis; (lanes 3, 5, 7, 9) hybridization profiles generated by the 4245 probe DNA annealed with 0061, 0038, 3524, and "3452-A" chromosomal DNAs. (open arrowheads) A 13.8-M fragment was present in 0038 and "3452-A" genomes but not in others, and, similarly, a 14.6-M fragment was present in the 0061 and 3524 genomes but not in others. MW, Molecular weight.

(2). In the latter studies, inconsistencies in serotyping reactions prompted the use of the *Hin*dIII restriction endonuclease, which subsequently clearly distinguished between epidemiologically linked and unlinked *C. jejuni* isolates by agarose gel electrophoresis.

Bacterial chromosomal REA in conjunction with Southern hybridizations (23) can be combined very effectively with conventional hybridization data to determine the significance of low levels of homology detected by conventional hybridization procedures (e.g., <24%; Table 1). The reported homology values of 0 to 4% between *B. vulgatus* 4245 and "3452-A" are nomalized values (12; Table 1), and therefore it is difficult to determine if these low levels of annealing are significant by using conventional hybridization techniques. In contrast, with the additional information on the nature and relative distribution of that homology provided by REA and Southern hybridizations, the significance of the low levels of annealing between distantly related organisms may be determined directly, when the areas of homology are displayed as reproducible bands in autoradiograms.

Nucleic acid hybridization probes and assays offer new possibilities to clinical laboratory diagnosis to identify, locate, characterize, and quantitate the genomic information of any target organism. In this regard, REA makes it possible to investigate the nature of shared DNA sequences. For example, specific DNA fragments may be identified by REA for their homology to a specific group of organisms (7) or their association with a particular disease (18). These fragments may be extracted from gels, purified from other host cell sequences by molecular cloning (13), and labeled for use as a probe.

It is anticipated that DNA probes prepared by nick translation of whole bacterial chromosomal DNA in the manner we have demonstrated have an application in taxonomic studies and assessment of the genetic relationships between bacterial organisms when DNA-DNA homology data indicate separation at the species level. We are presently studying the usefulness of this type of DNA probe in dot-blot assays (25) for rapid identification of clinically important anaerobes such as *Clostridium difficile* (19) and *Bacteroides* species (3, 9).

ACKNOWLEDGMENTS

We are grateful to the Medical Research Council of Canada and to the National Cancer Institute of Canada for the support given to our research. One of us (W.C.B.) was supported as a Post-Doctoral Fellow 1979-80 by funds from the Medical Research Council of Canada.

We are particularly appreciative of the encouragement, growth facilities, and helpful discussions by R. H. Behme of the Microbiology Department, University Hospital, London, Ontario. The technical assistance of E. C. M. LeeChan and M. Hall is gratefully acknowledged.

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