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# Extrachromosomal Elements in a Variety of Strains Representing the *Bacteroides fragilis* Group of Organisms

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Previous nucleic acid association studies have identified at least nine deoxyribonucleic acid (DNA) homology classes of the *Bacteroides fragilis* group of organisms. Using these classes as a taxonomic framework, we have screened representative strains of the *B. fragilis* group for the presence of extrachromosomal (plasmid) DNA. [$^3$H]thymidine-labeled cell lysates were subjected to sodium dodecyl sulfate-salt precipitation, and supernatant fractions from such preparations were analyzed using cesium chloride-ethidium bromide equilibrium centrifugation. One strain from each group was examined in this fashion. Five of the strains were judged to contain no detectable plasmid DNA; however, four strains were observed to yield satellite bands corresponding to covalently closed circular plasmid DNA. Plasmid DNA from such gradients was subjected to velocity sedimentation through both neutral and alkaline sucrose gradients to determine molecular size. A $23 \times 10^6$-molecular-weight plasmid was found in a *B. fragilis* strain representing one DNA homology group of this species, whereas a $3 \times 10^6$-molecular-weight plasmid was found in a *B. fragilis* strain representing a second homology group. Similarly, a $31 \times 10^6$-molecular-weight plasmid was found in a *Bacteroides thetaiotaomicron* strain representing one DNA homology group of this species, whereas a $3 \times 10^6$-molecular-weight plasmid was found in a *B. thetaiotaomicron* strain representing a second homology group. In all instances, the small-molecular weight plasmids were present to the extent of about 15 copies per chromosomal equivalent, whereas the large plasmids were present to the extent of approximately 1 copy per chromosomal equivalent. The biological function of these plasmids is unknown.

Bacterial extrachromosomal elements (plasmids) are known to confer a variety of phenotypic properties of both clinical and ecological significance. Such traits include resistance to antibiotics and heavy metals (11, 20, 32), toxin production including enterotoxins (19), exfoliative toxins (37) and hemolysins (14, 23), surface antigens (2), bacteriocins (8, 32), and hydrocarbon catabolism (6). Many examples of these plasmid-conferred phenotypes occur in both gram-positive and gram-negative bacteria. Additionally, it is quite common for plasmids carrying such genetic information to confer conjugal donor ability on the cell in which they reside. Such conjugative plasmids may be transferred either intra- or intergenerically in many cases.

Plasmid deoxyribonucleic acid (DNA) exists intracellularly as covalently closed (supertwisted) circular DNA, which is physically separate from the host chromosomal DNA (8, 20, 32). This unique configuration, coupled with a small molecular size, makes the isolation and purification of plasmid DNA relatively easy, thus making these genetic elements readily amenable to physical and biochemical study.

We recently have turned our attention to the study of occurrence and function of plasmids in bacteria indigenous to man, in particular the intestinal anaerobes comprising the *Bacteroides fragilis* group of organisms. This quite heterogenous taxon is comprised of a number of species (5, 22, 24) and has been shown to be the most frequently isolated intestinal anaerobe, many strains being present in the range of $1 \times 10^{10}$ to $5 \times 10^{10}$ organisms per g of feces (30). The significance of *Bacteroides* as an opportunistic pathogen has been well established (15, 16, 27), and treatment of *Bacteroides* infections is often difficult since many strains are resistant to a diversity of antibiotics (29, 34). Another line of health-related interest in *Bacteroides* lies in the possibility of its role(s) in human bowel physiology, but scant information is available in this regard (12). Recently, however, consid-

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erable attention has been focused on the occurrence and function of certain bile salt catabolic enzymes in the *B. fragilis* group. These enzymes are known to catalyze reactions that generate a variety of compounds that have been implicated in the etiology of cancer of the large intestine in man (21, 31).

In this study we have taken advantage of the large number of well-characterized human *B. fragilis* isolates collected by, and studied at, the Virginia Polytechnic Institute Anaerobe Laboratory. One strain from each of nine DNA homology groups has been screened for the presence of extrachromosomal DNA. Four of the nine strains examined were found to contain plasmid DNA. The plasmid-containing strains fell exclusively into two species of this genus.

## MATERIALS AND METHODS

**Bacterial strains.** All of the *Bacteroides* strains employed in this study were obtained from the Virginia Polytechnic Institute Anaerobe Laboratory, Blacksburg, via Phillip B. Hylemon, Department of Microbiology, Virginia Commonwealth University. Working stock cultures were maintained in 15 ml of chopped meat medium (22) and stored at room temperature (20 to 23°C) under 10% carbon dioxide and 90% nitrogen. These stocks were transferred at approximately 6-week intervals. Permanent stocks were prepared by the addition of glycerol (final concentration of 30%) to stationary-phase broth cultures. These cell suspensions were stored at −20°C under 10% carbon dioxide and 90% nitrogen. The bacterial strains employed in the study are presented in Table 1.

**Media and culturing techniques.** Penassay broth (Difco Laboratories, Detroit, Mich.), supplemented with 2 mg of hemin/liter and 1 g of cysteine/liter, was used as a complex medium. Agar (Difco) was added to give a final concentration of 1.5% when solid medium was desired. The defined medium of Varel and Bryant (36) was employed in all work involving the radioactive labeling of cells. This medium always was adjusted to a final pH of 7 to 7.1 before use. Culture plates were incubated anaerobically using the GasPak system (BBL, Cockeysville, Md.). Liquid cultures were grown under an atmosphere of oxygen-free nitrogen using standard anaerobic culturing methodology (22). Cultures were always grown at 37°C.

**Biochemicals and enzymes.** Hemin (type I, bovine), lysozyme (grade I, crystallized three times), crystalline sucrose (grade I, for sucrose gradients), sodium dodecyl sulfate, and ethidium bromide (EB) were purchased from the Sigma Chemical Co. (St. Louis). Technical grade cesium chloride was obtained from Kawecki Berylo Industries, Inc., New York.

Sarkosyl NL97 (sodium dodecyl sarcosinate) was kindly supplied by the Ciba-Geigy Corp., Greensboro, N.C.

[*Methyl*-$^3$H]thymidine (20 Ci/mmol) and [2-$^{14}$C]thymidine (>50 Ci/mmol) were purchased from New England Nuclear Corp., Boston.

TABLE 1. *Bacterial strains*

| *Bacteroides* species or group designation[a] | VPI no.[b] | Nucleotide sequence homology to VPI 2553 (%)[c] | Designation in this laboratory |
|---|---|---|---|
| *B. fragilis*-1[d] | 2553 | 100 | V214 |
| *B. fragilis*-2[d] | 2393 | 68 | V217 |
| "3452-A" group[e] | 3452-A | 23 | V216 |
| *B. distasonis* | 4243 | 18 | V289 |
| *B. vulgatus* | 4245 | 6 | V213 |
| "High-theta" group[e] | 0061-1 | 17 | V218 |
| *B. thetaiotaomicron*-1[d] | 5482 | 28 | V212 |
| *B. thetaiotaomicron*-2[d] | 2302 | 21 | V210 |
| *B. ovatus* | 0038-1 | 24 | V211 |

[a] See references 5 and 24; also J. Johnson (personal communication).

[b] Numerical designation assigned by Virginia Polytechnic Institute Anaerobe Laboratory, Blacksburg, Va.

[c] Data from Johnson (24, and personal communication); measured by DNA-DNA reassociation studies.

[d] The numbers after the species names designate the unique DNA homology group that this strain represents.

[e] Not yet given species names.

**Radioactive labeling of *Bacteroides*.** Cells were labeled with [$^3$H]thymidine as follows. An overnight defined medium broth culture of the strain was used to inoculate fresh defined broth contained in a 50-ml side arm Erlenmeyer flask. The inoculum size was adjusted to give a starting optical density at 660 nm of 0.1. Final culture volume was always 30 ml.

As soon as cells were observed to enter the log phase of growth (absorbance at 660 nm, ~0.2) [$^3$H]thymidine was added to give a final concentration of 10 μCi/ml. Cultures were then allowed to grow to mid-log phase (absorbance at 660 nm, ~0.6 to 0.8). Under such conditions, $^3$H label was readily incorporated into acid-insoluble material that was resistant to both alkali hydrolysis (7) and ribonuclease digestion. We conclude that radioactive precursor is incorporated preferentially, if not exclusively, into DNA. Furthermore, the addition of a variety of deoxyribonucleotides, including deoxyadenosine, deoxycytidine, and deoxyguanosine, failed to enhance [$^3$H]thymidine incorporation as has been reported for *Escherichia coli* and other enteric microorganisms (4). In fact, inhibition of [$^3$H]thymidine incorporation into DNA was mediated by some deoxyribonucleotides in certain strains (unpublished observations).

**Equilibrium centrifugation and plasmid isolation.** $^3$H-labeled cells, grown as described above, were sedimented by centrifugation at 15,000 × *g* at 4°C for 15 min. Sedimented cells were suspended in 30 ml of TES buffer [0.05 M NaCl, 0.05 M tris(hydroxymethyl)aminomethane (Tris), and 0.005

M EDTA (ethylenediaminetetraacetate), pH 8]. This TES washing step was repeated once. The washed-cell pellet (from a 30-ml culture) then was suspended in 0.9 ml of TES buffer containing 1 mg of lysozyme per ml and 100 mg of sucrose per ml. This cell suspension was incubated for 35 min at 37 C. This incubation resulted in the generation of osmotically fragile forms that were sensitive to lysis (measured spectrophometrically) by both ionic (e.g., Sarkosyl NL97, sodium dodecyl sulfate [SDS]), and nonionic (Brij-58 and Triton X-100) detergents. The cell suspension was then placed on ice for 5 min, and lysis was effected by the addition of 0.9 ml of 2% SDS in 0.01 M Tris, pH 9. The suspension always became clear and viscous after the addition of the SDS. The sodium chloride concentration of this lysate was then adjusted to 1 M using a 5 M NaCl stock solution. This lysate was then stored at 4°C overnight, during which time the large chromosomal molecules precipitate leaving extrachromosomal DNA in the supernatant fluid. This method has been fully described by Guerry et al. (17).

After precipitation at 4°C, the lysate was centrifuged at 0°C for 30 min at ~34,000 × *g*. A 0.8-ml sample of the supernatant fluid obtained in this manner was mixed with 3.1 ml of sterile water, 1.6 ml of EB (2 mg/ml in phosphate buffer, pH 7) and 5.22 g of CsCl. This mixture then was placed in a polyallomer centrifuge tube, overlayed with light mineral oil, and centrifuged in a Beckman 50Ti rotor. Centrifugation was for 40 h at 105,000 × *g* and 20°C.

After centrifugation, gradients were fractionated by puncturing the tube bottom with a 17-gauge needle and collecting 5-drop fractions into sterile glass tubes using a proportioning pump (Technicon Corp., Tarrytown, N.Y.). Samples (50 or 30 μl) from each tube were spotted on Whatman 3MM disks, washed twice in ice-cold 5% trichloroacetic acid and twice in 95% ethanol, and air dried. The disks were placed in 1-dram (ca. 1-g) glass vials containing 3 ml of 0.8% 2,5-bis-[2-(5 tert-butylbenzoxazolyl)]thiophene in toluene and counted in a Beckman LS-350 Liquid Scintillation System.

Analysis of crude lysates of cells was essentially as above except that cells were lysed by the addition of 0.9 ml of 2% Sarkosyl NL97. This lysate was then sheared according to the method of Bazaral and Helinski (3). A sample of this sheared lysate (0.8 ml) was centrifuged and processed as described above.

Fractions representing covalently closed circular (CCC) DNA were pooled and dialyzed against TES to remove CsCl and EB. Such preparations of purified plasmid DNA were stored at −20°C.

**Sedimentation velocity centrifugation.** Neutral sucrose gradient diluent consisted of 1.0 M NaCl, 0.001 M EDTA, 0.01 M Tris, and 0.01 M β-mercaptoethanol. Alkaline sucrose gradient diluent consisted of 1.0 M NaCl, 0.01 M EDTA, and 0.3 M NaOH. Five to twenty percent linear sucrose density gradients (alkaline or neutral) were centrifuged in a Beckman SW50.1 rotor using a Beckman L5-50 or L2-65B preparative ultracentrifuge. Velocity sedimentation gradients were fractionated from top to bottom using a Buchler Auto Densi-Flow II (Buchler Instruments, Fort Lee, N.J.) in connection with the Technicon proportioning pump. About 30 4-drop fractions were collected directly onto Whatman 3MM disks, acid precipitated, washed, and counted as described above. Recovery of input counts on sucrose gradients was always greater than 85%.

**Preparation of marker DNA.** $^{14}$C-labeled R6K plasmid DNA was used as a sedimentation reference in these studies. *E. coli* K-12 (strain χ925; see reference 28) carrying this plasmid was grown in Penassay broth containing 250 μg of deoxyadenosine per ml and 2.5 μCi of [$^{14}$C]thymidine/ml. Cells were harvested, washed and converted to spheroplasts with lysozyme as has been previously described (28). Spheroplasts prepared from 30 ml of mid-log-phase cells were lysed with SDS and precipitated as described above according to the method of Guerry et al. (17). Supernatant fluid obtained (~2 ml) was subjected to CsCl EB centrifugation (2 ml of supernatant fluid, 1.9 ml of sterile water, 1.6 ml of EB [2 mg/ml solution] in phosphate buffer and 5.22 g of CsCl). After centrifugation, the gradient was examined using a UVL-21 Blak-Rak ultraviolet light source (UV Products, San Gabriel, Calif.), and two fluorescent bands were easily visualized, the lower band corresponding to the CCC R6K plasmid DNA. This lower band was withdrawn by puncturing the side of the tube with an 18-gauge needle attached to a 3-ml syringe. This material was subjected to three isoamyl alcohol extractions and then exhaustively dialyzed against TES. Purified plasmid DNA prepared in this fashion was stored at −20°C. The sedimentation coefficients of the closed and open circular forms of the R6K plasmid are 51*S* and 38*S* respectively (26). In addition, high-molecular-weight forms in the 69*S* range consisting of catenated molecules (interlocked monomeric molecules) are also found in these preparations (25). Sedimentation coefficients of the *Bacteroides* plasmids (CCC DNA) were converted to molecular-weight values using the following formula of Bazaral and Helinski (3): $s_{20,w} = 0.034\ M^{0.428}$.

## RESULTS

**Analysis of strains by equilibrium centrifugation.** Each of the strains listed in Table 1 was grown and labeled with [$^3$H]thymidine as described in Materials and Methods. After SDS precipitation, cleared lysates were subjected to CsCl-EB equilibrium centrifugation and fractionated, and $^3$H radioactivity profiles were obtained for each gradient.

As can be seen in Fig. 1A and B, strains representing both of the DNA homology groups of the species *B. fragilis* (V214 and V217; DNA homology groups 1 and 2, respectively) displayed satellite bands (peak fractions 20 and 16, respectively) corresponding to CCC plasmid DNA. The upper bands (fractions 29 and 24, respectively) seen in such gradient analysis

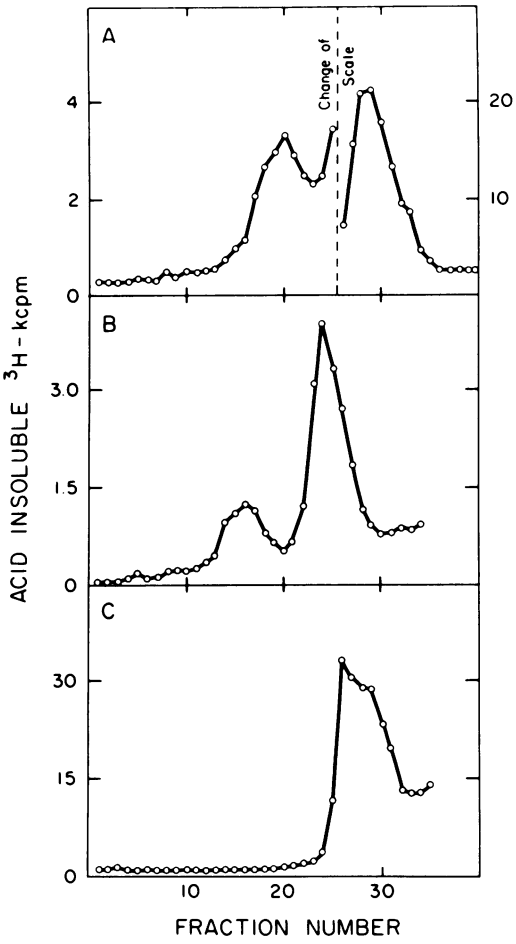


FIG. 1. *$^3$H profiles of CsCl-EB gradients of V214, V217, and V289. [$^3$H]thymidine-labeled lysates were precipitated with SDS and high salt, and the supernatants were subjected to CsCl-EB centrifugation as described in Materials and Methods. Note the change of scale at fraction 25 in panel A. Increasing density is from right to left. (A) V214; (B) V217; (C) V289.*

represent contaminating linear chromosomal DNA and/or open circular plasmid DNA (8). Such upper bands varied by as much as 25% from experiment to experiment, presumably reflecting day-to-day variations in the efficiency of the SDS-high salt-mediated chromosomal precipitation. Figure 1C shows a typical profile of a strain (*Bacteroides distasonis* V289) in which no heavy satellite band was detected. This strain, as well as all others (see below) that contained no detectable CCC plasmid DNA, was examined at least three times.

Both of the DNA homology groups of the species *Bacteroides thetaiotaomicron* (Table 1) were also found to contain plasmid DNA, as judged by analysis on CsCl-EB density gradients (Fig. 2). Strains V212 (Fig. 2A) and V210 (Fig. 2B) both show dense satellite bands at approximately fraction 20. V218 (Fig. 2C), shown as a negative control, contained no detectable plasmid DNA.

All of the remaining strains presented in Table 1 were negative for the presence of plasmid DNA.

**Plasmids of V214 and V217 (*B. fragilis*).** The fractions representing the dense satellite bands seen on CsCl-EB gradient analysis of strains V214 and V217 were pooled and purified by dialysis. This material was then co-sedimented through neutral sucrose gradients with purified $^{14}$C-labeled R6K plasmid DNA (sedi-

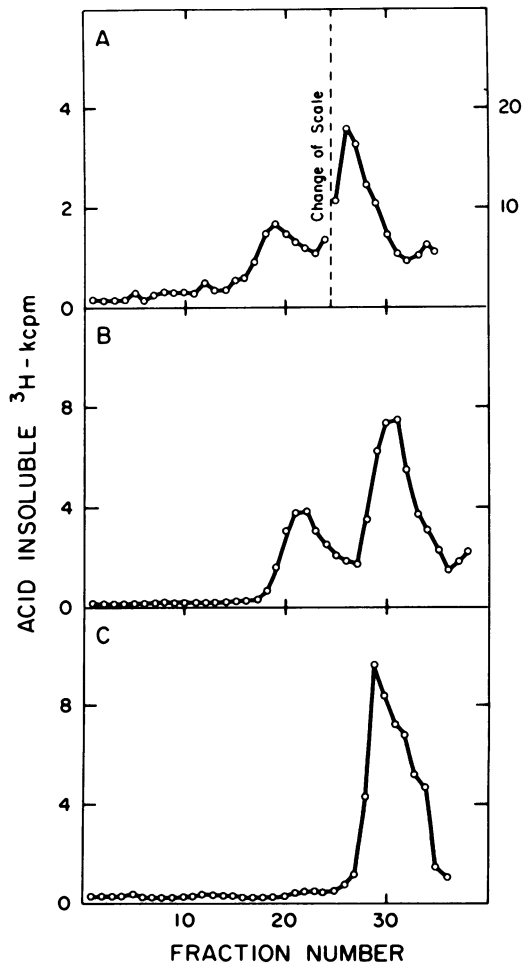


FIG. 2. *$^3$H profiles of CsCl-EB gradients of V212, V210, and V218. [$^3$H]thymidine-labeled lysates were precipitated with SDS and high salt, and the supernatants were subjected to CsCl-EB centrifugation as described in Materials and Methods. Note the change of scale at fraction 24 in (A). Increasing density is from right to left. (A) V212; (B) V210; (C) V218.*

mentation coefficient of CCC form equal to 51S). A radioactivity profile of such a gradient may be seen in Fig. 3A). The major component is seen at about fraction 19, and this corresponds to a sedimentation coefficient of 48S using the 51S $^{14}$C-labeled R6K DNA as an internal reference. A small component is seen at fraction 14, having a sedimentation coefficient of 32S. The 48S and 32S components are assumed to represent, respectively, the closed and open circular forms of this plasmid DNA, which we designated pVA100 in accordance with the rules for plasmid nomenclature suggested by Novick et al. (33). Similar gradients from older preparations of purified pVA100 (VA signifying Virginia) show an increase in the size of the 32S peak consistent with the spontaneous conversion of the CCC form to the open circular configuration.

Alkaline sucrose gradient analysis of preparations of pVA100 displayed count profiles consistent with the CCC nature of the plasmid. Figure 3B shows a typical gradient, in which a fast-moving component is seen at about fraction 10. This represents the denatured collapsed CCC molecule, which is known to sediment much faster than single-stranded DNA derived from alkali denaturation of corresponding open circular plasmid DNA (8).

The satellite band obtained from CsCl-EB gradients of strain V217 was also analyzed by velocity sedimentation in sucrose gradients. Figure 4A shows a typical count profile of a neutral sucrose gradient, and a well-defined peak occurring at fraction 10 is seen. This corresponds to a sedimentation coefficient of about 20S. Alkaline sucrose gradient centrifugation (Fig. 4B) reveals that this component sediments rapidly, thus indicating that this DNA is in the CCC form. This plasmid has been designated pVA101.

**Plasmids of V212 and V210 (*B. thetaiotaomicron*).** Plasmid DNA from V212 and V210 isolated by CsCl-EB centrifugation (Fig. 2) and purified by dialysis was also examined by velocity sedimentation analysis. Figure 5A shows a typical profile obtained from 5 to 20% neutral sucrose gradient analysis of CCC DNA from V212. Closed and open circular forms are seen at about fractions 20 and 14, respectively, corresponding to sedimentation coefficients of 55S and 36S. In addition, a shoulder is seen at about fraction 26. This rapidly moving component, although always minor in nature, is seen consistently in CCC preparations from V212 and may represent high-molecular-weight forms of this plasmid (e.g., catenated dimer forms). This possibility is currently being explored using electron microscopy. The 55S plasmid of strain V212 has been designated pVA102.

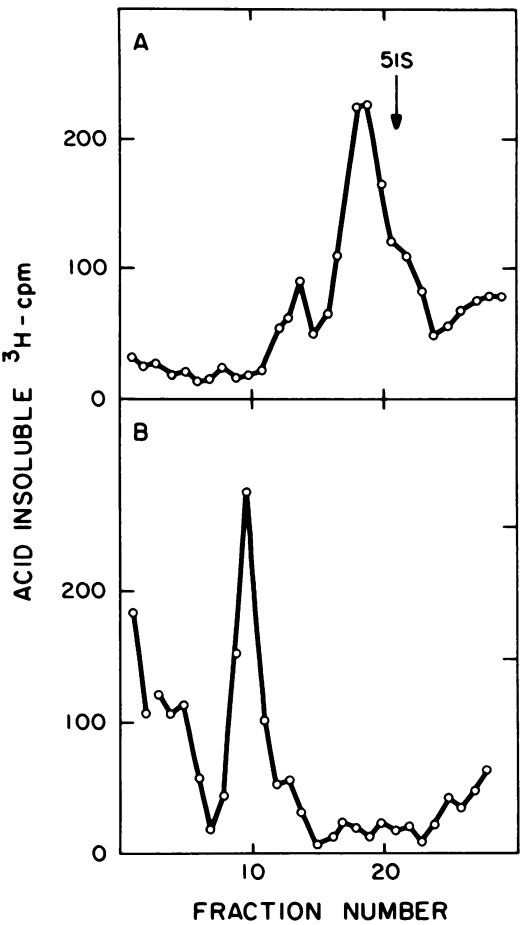


FIG. 3. *Velocity sedimentation studies of plasmid DNA (pVA 100) from B. fragilis V214. Purified plasmid DNA from V214 was centrifuged through neutral and alkaline sucrose gradients. (A) Neutral sucrose, 5 to 20%, Beckman SW50.1 rotor, 45,000 rpm, 15°C for 120 min. (B) Alkaline sucrose, 5 to 20%, Beckman SW50.1 rotor, 35,000 rpm, 23°C for 50 min. Two-tenths milliliter of purified $^{3}$H-labeled pVA100 DNA was layered directly onto the gradient in each case. For the neutral gradient, 0.1 ml of $^{14}$C-labeled R6K CCC marker DNA also was layered on the gradient. Its position in the gradient (51S) is noted by the arrow. Sedimentation in both cases is from left to right. Recovery of input $^{3}$H and $^{14}$C radioactivity was always greater than 85%.*

Alkaline sucrose gradients of pVA102 revealed a fast-moving component (fraction 14; Fig. 5B), consistent with CCC DNA, and an additional fast-moving minor component (fraction 17) was seen in these gradients also. This presence of a second possible fast-moving (CCC) species is consistent with the existence of a

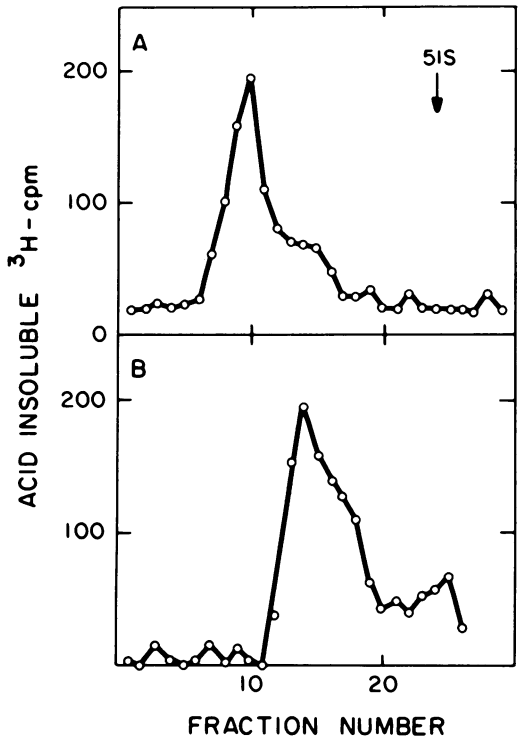


FIG. 4. *Velocity sedimentation studies of plasmid DNA (pVA 101) from B. fragilis V217. Purified plasmid DNA from V217 was centrifuged through neutral and alkaline sucrose gradients. (A) Neutral sucrose, 5 to 20%, Beckman SW50.1 rotor, 45,000 rpm, 15°C, for 120 min. (B) Alkaline sucrose, 5 to 20%, Beckman SW50.1 rotor, 45,000 rpm, 23°C, for 100 min. Two-tenths milliliter of purified $^3$H-labeled pVA 101 DNA was layered directly onto the gradient in each case. For the neutral gradient, 0.1 ml of $^{14}$C-labeled R6K CCC marker DNA also was layered on the gradient. Its position in the gradient (51S) is noted by the arrow. Sedimentation in both cases is from left to right. Recovery of input $^3$H and $^{14}$C radioactivity was always greater than 85%.*

minor component seen in the neutral sucrose gradients.

Figure 6 presents our velocity sedimentation studies with plasmid material obtained from CsCl-EB gradient analysis of *B. thetaiotaomicron* V210. The neutral sucrose gradient profile (Fig. 6A) shows a single peak (fraction 9) with an estimated sedimentation coefficient of 20*S*. The alkaline gradient profile (Fig. 6B) verifies this peak as being CCC DNA; it shows a rapidly sedimenting form at about fraction 15. We thus conclude that there is a single plasmid species in V210 and have designated this extrachromosomal element pVA103.

**Estimation of plasmid copy number.** To estimate the relative amount of extrachromosomal DNA present in each of the four plasmid-containing strains, we prepared [$^3$H]thymidine-labeled crude lysates of each strain, and subjected samples from these preparation to CsCl-EB equilibrium centrifugation. Such analysis quantitatively reveals plasmid and chromosomal DNA in their existing proportions in the lysate. Earlier gradients (Fig. 1 and 2) used in plasmid screening experiments were prepared from lysates in which most of the chromosomal DNA had been selectively removed by precipi-

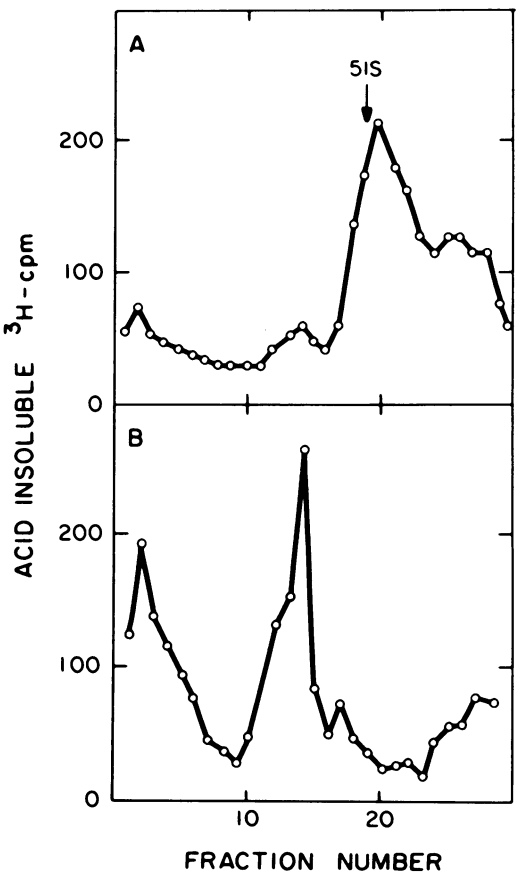


FIG. 5. *Velocity sedimentation studies of plasmid DNA (pVA102) from B. thetaiotaomicron V212. Purified plasmid DNA from V212 was centrifuged through neutral and alkaline sucrose gradients. (A) Neutral sucrose, 5 to 20%, Beckman SW50.1 rotor, 45,000 rpm, 15°C for 120 min. (B) Alkaline sucrose, 5 to 20%, Beckman SW50.1 rotor, 35,000 rpm, 23°C for 50 min. Two-tenths milliliter of purified $^3$H-labeled pVA 102 DNA was layered directly onto the gradient in each case. For the neutral gradient, 0.1 ml of $^{14}$C-labeled R6K CCC marker DNA also was layered onto the gradient. Its position in the gradient (51S) is noted by the arrow. Sedimentation in both cases is from left to right. Recovery of input $^3$H and $^{14}$C radioactivity was always greater than 85%.*

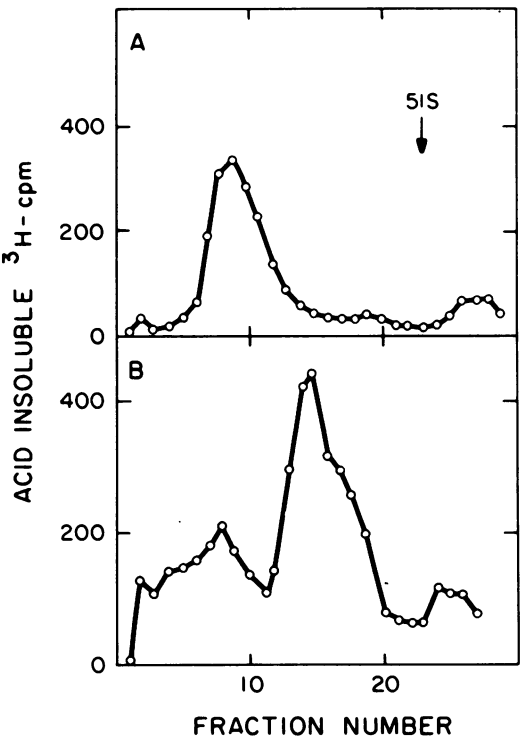


FIG. 6. *Velocity sedimentation studies of plasmid DNA (pVA103) from B. thetaiotaomicron V210. Purified plasmid DNA from V210 was centrifuged through neutral and alkaline sucrose gradients. (A) Neutral sucrose, 5 to 20%, Beckman SW50.1 rotor, 45,000 rpm, 15°C for 120 min. (B) Alkaline sucrose, 5 to 20% Beckman SW50.1 rotor, 45,000 rpm, 23°C for 100 min. Two-tenths milliliter of purified $^3$H-labeled pVA 103 DNA was layered directly onto the gradient in each case. For the neutral gradient, 0.1 ml of $^{14}$C-labeled R6K CCC marker DNA also was layered on the gradient. Its position in the gradient is noted by the arrow. Sedimentation in both cases is from left to right. Recovery of input $^3$H and $^{14}$C radioactivity was always greater than 85%.*

tation. Representative gradient radioactivity profiles of crude lysates of two strains are shown in Fig. 7. Strain V210 (Fig. 7A) (*B. fragilis*) shows a relatively dense satellite band at about fraction 18, corresponding to the position of CCC DNA. A major peak is seen at about fraction 30, representing the chromosomal (and any open circular plasmid) DNA. A similar profile is seen in Fig. 7 for *B. thetaiotaomicron* V210.

Using such gradient profiles for these and the other two plasmid-containing strains (V217 and V212), we have calculated the proportion of extrachromosomal DNA relative to that of chromosomal DNA. These data are seen in Table 2 (column 5). In each case, the numbers were calculated by summation of the counts under the peak representing the CCC DNA and expressing this value as a percentage of the counts in the chromosomal peak.

Molecular weights of each plasmid have been calculated from their sedimentation coefficients using the equation of Bazaral and Helinski (3). The respective *s* values and corresponding molecular weights are seen in columns 3 and 4 of Table 2. These molecular weights were then used in connection with the amount of plasmid DNA (percentage of chromosomal DNA; Table 2, column 4) to estimate the number of plasmid copies per chromosomal equivalent. Free-solution kinetic studies of DNA association have enabled us to estimate the molecular weight of

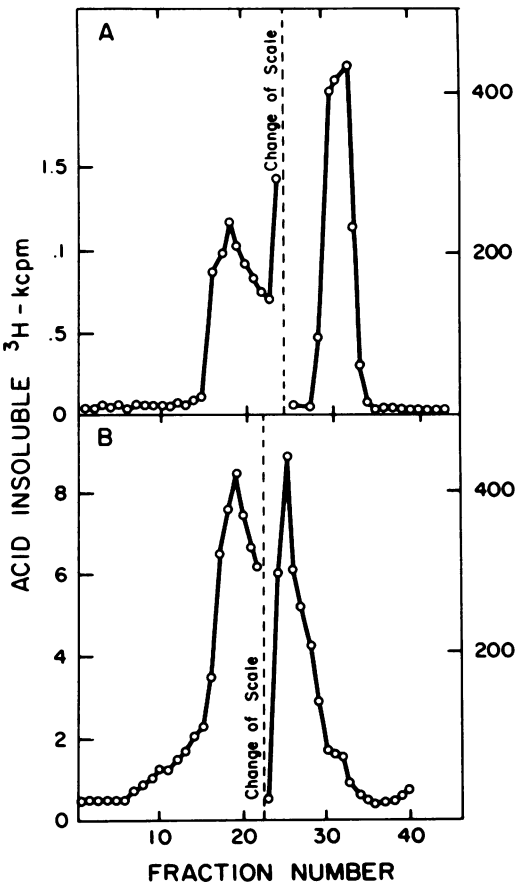


FIG. 7. *CsCl-EB gradient radioactivity profiles of crude cell lysates of V214 and V210. [$^3$H]thymidine-labeled, Sarkosyl-lysed, sheared cell lysates were prepared as outlined in Materials and Methods. Centrifugation was accomplished in a Beckman 50Ti rotor, for 48 h, at 42,000 rpm, 20°C. Density increases from right to left. Note the change of scale in each case. (A) B. fragilis (V214); (B) B. thetaiotaomicron (V210).*

TABLE 2. *Plasmid copy number estimates*

| Strain | Plasmid | Sedimentation coefficient of CCC form | Plasmid molecular wt[a] | Plasmid DNA as % of chromosomal DNA[b] | Estimated plasmid copies/ $2.5 \times 10^9$ genomic equivalent |
|---|---|---|---|---|---|
| *B. fragilis* | | | | | |
| V214 | pVA100 | 48*S* | $23 \times 10^6$ | 1 | ~1 |
| V217 | pVA101 | 20*S* | $3 \times 10^6$ | 1.8 | ~15 |
| *B. thetaiotaomicron* | | | | | |
| V212 | pVA102 | 55*S* | $31 \times 10^6$ | 1.5 | ~1 |
| V210 | pVA103 | 20*S* | $3 \times 10^6$ | 2 | ~15 |

[a] Calculated from the sedimentation coefficient of the CCC form using the equation of Bazaral and Helinski (3).

[b] Based on an average of two experiments.

the *Bacteroides* genome. Preliminary studies (unpublished) with one strain (V218) demonstrated a molecular weight of $2.5 \times 10^9$, a value equal to that of *E. coli* B (9, 13). More rigorous examination of a number of strains representing the DNA homology groups is currently underway, and these data will appear in a future publication. Using this value, the plasmid molecular weight and amount of plasmid DNA per chromosomal equivalent, we have estimated the number of plasmid copies per genome. These results are seen in column 6 of Table 2. Both of the two large plasmids (pVA100 and 102) appear to be present in very low numbers, approximating one plasmid molecule per chromosomal equivalent. Both small-molecular-weight plasmids (pVA101 and 103), on the other hand, appear to be present in multicopy pools (~15 copies/genome equivalent). This correlation between small molecular size and multicopy pool correlation is known to exist in a variety of other bacterial plasmid systems (8). Finally, it should be noted that the CsCl-EB technique would not enable us to recover plasmid DNA present in (or converted to) the open circular form (8). Thus, our values of plasmid DNA amounts should be viewed as minimum estimates.

## DISCUSSION

The unique ecological niche of *Bacteroides*, its potentially important role in human intestinal physiology, and its importance as an opportunistic pathogen have made this genus the subject of much current investigation. We are approaching the study of the genetics of this group by studying their plasmid DNA, since a variety of plasmid-conferred phenotypes could have bearing on these ecological and medical implications.

Stiffler et al. (35) first reported the presence of plasmid DNA in three clinical isolates of *B. fragilis*. A fourth strain they examined (also a *B. fragilis* species) contained no plasmid DNA. Two of these strains carried multicopy pools of plasmids of molecular weight about $4 \times 10^6$, whereas the third contained multiple copies of a $2.7 \times 10^6$-molecular-weight plasmid. A second plasmid of molecular weight $16 \times 10^6$ was present in only a few copies per chromosomal equivalent. Next, Damle and Syed (10) reported plasmids in two oral *Bacteroides* strains. A clinical isolate of *Bacteroides ochraceus* was found to contain two plasmids, one of molecular weight approximating $17 \times 10^6$ and the other of molecular weight of about $5 \times 10^6$. Additionally, a clinical isolate of *Bacteroides melaninogenicus* was found to contain a plasmid of molecular weight approximately $17 \times 10^6$. Most recently, Guiney and Davis (18) have reported a $25 \times 10^6$-molecular-weight plasmid in one of seven clinically isolated strains of *B. fragilis* and two plasmids (molecular weights equal to $25 \times 10^6$ and $70 \times 10^6$) in one strain of *B. ochraceus*.

Johnson (24) has established that the intestinal anaerobe *B. fragilis* comprises a heterogeneous group of organisms when examined using nucleic acid association studies. At least nine DNA homology groups have been established, and corresponding species (or group) names have been applied so that each homology group may be conveniently designated (5, 24).

Since much physiological and molecular taxonomic data were available, we believed that these strains afforded a significant basis for our studies on the occurrence and function of plasmids in this genus. The work presented in this report provides information on the extrachromosomal DNA content of representatives of each of the precisely defined (DNA homology group and physiological biotype) representatives of this *Bacteroides* group. Consistent with the findings of Stiffler et al. (35), we have iden-

tified *B. fragilis* as a frequently occurring plasmid reservoir. The representative from DNA homology group 1 of species *B. fragilis* (V214) has a plasmid of molecular weight $23 \times 10^6$ that is present in no more than a few copies per chromosomal equivalent. This is in contrast to the somewhat related DNA homology group 2 representative of species *B. fragilis* (V217), which contains a multicopy pool of a $3 \times 10^6$-molecular-weight plasmid. The occurrence of a variety of plasmids in species *B. fragilis* is particularly significant because such strains are the most frequently isolated anaerobic species from infections of soft tissues in man (29). It is reasonable to propose that such a prevalence of extrachromosomal DNA in this subspecies might play a role in equipping such strains with respect to virulence and/or drug resistance.

*B. thetaiotaomicron* has been shown in one study to be the sixth most frequently isolated species from human feces (and thus considered to occupy the same position of hierarchy in the intestinal tract). Populations of this subspecies have been estimated at about $2 \times 10^{10}$ organisms/g of fecal material (30). Both of the unique *B. thetaiotaomicron* species (Table 1) were found to contain plasmids. Strain V212 contained a plasmid of molecular weight $31 \times 10^6$, whereas strain V210 contained a plasmid of molecular weight equal to $3 \times 10^6$. As in the case of the two *B. fragilis* species (V214 and V217), the large plasmid of V212 was found to be present in very few copies per chromosomal equivalent, whereas the relatively small plasmid of V210 was present to the extent of about 15 copies per chromosomal equivalent.

The correlation of molecular size and plasmid copy number seen in the *Bacteroides* strains reported here is reminiscent of a variety of other plasmid systems seen in both gram-negative and gram-positive bacteria. Namely, plasmids of molecular size less that $20 \times 10^6$ tend to be present in multiple copies per chromosomal equivalent, whereas larger plasmids tend to be present in only a few copies per chromosomal equivalent. The significance and biological basis of this stringent (few copies) versus relaxed (many copies) plasmid replication remains nebulous at this time.

The absence of plasmid DNA in the other five groups of *Bacteroides* examined in this study should not be used to draw conclusions presently. Additional representatives will have to be examined for plasmid DNA before any generalizations can be made. Given the current body of evidence (10, 18, 35; this paper), it is anticipated that plasmids will be found in a number of these other species.

Attempts have been made to identify possible plasmid-linked phenotypes in *Bacteroides*, including conjugal transfer of drug resistance and bacteriocin production (1, 18). No success has been met in this area as yet, however. As additional information is compiled regarding plasmid occurrence and plasmid type in *Bacteroides* strains representing the DNA homology groups, it is believed that we may begin a more meaningful search for plasmid function in these well-characterized isolates of known molecular relationship. At present, however, the plasmids reported in this paper must be considered as cryptic (33), having no known phenotypic function.

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