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Isolation of Plasmid Deoxyribonucleic Acid from Two Strains of *Bacteroides*

D. G. GUINEY[1] AND C. E. DAVIS*

Department of Pathology, University of California at San Diego, San Diego, California 92103

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Two clinical isolates of *Bacteroides* contained covalently closed circular deoxyribonucleic acid (DNA) as shown by sedimentation in an alkaline sucrose gradient, CsCl ethidium bromide equilibrium centrifugation, and electron microscopy. *Bacteriodes fragilis* N1175 contained a homogeneous species of plasmid DNA with a molecular weight of 25×10^6. *Bacteroides ochraceus* 2228 contained two distinct, covalently closed circular DNA elements. The larger cosedimented with the covalently closed circular DNA form of the R plasmid, R100, corresponding to a molecular weight of 70×10^6; the smaller sedimented as a 58*S* molecule with a calculated molecular weight of 25×10^6. The roles of these plasmids are unknown. Neither strain transferred antibiotic resistance to plasmid-negative *Bacteroides* or *Escherichia coli*, and neither produced bacteriocins active against other *Bacteroides* or sensitive indicator strains of *E. coli*.

Plasmids are stable, extrachromosomal, genetic elements which control many important bacterial properties (26) including conjugation, resistance to antimicrobials and heavy metals, bacteriocin production, and certain determinants of pathogenicity (23, 27). Because of the importance of several of these characteristics to human disease and microbial ecology, plasmids have been sought and found in a wide variety of bacteria including *Pseudomonas* (20), *Bacillus* (4, 24), *Staphylococcus* (26), *Streptococcus* (6, 7, 9, 15), and most genera of *Enterobacteriaceae* (7, 18, 26). These extrachromosomal elements usually exist in the bacterial cell as covalently closed circular deoxyribonucleic acid (DNA) molecules, but the conditions of extraction may result in considerable conversion to the open circular form (7, 18, 26).

Because *Bacteroides* spp. are among the most abundant members of the normal flora of human beings and would be logical reservoirs for R factors and bacteriocins, we were surprised that attempts to transfer antibiotic resistance from *Bacteroides* to sensitive *Enterobacteriaceae* or other *Bacteroides* were unsuccessful (1). When our attempts to transfer antibiotic resistance also failed, we extracted and studied the DNA from 10 strains of *Bacteroides* isolated from human infections. In this report we describe the isolation and characterization of covalently closed circular plasmid DNA from two of eight strains of tetracycline-resistant *Bacteroides*.

[1] Present address: Department of Medicine, University of Southern California, Los Angeles County Hospital, Los Angeles, Calif. 90033.

MATERIALS AND METHODS

Bacteria. All strains of *Bacteroides* were isolated from human infections by the microbiology laboratory at University Hospital, University of California at San Diego, using an anaerobic system that has been described previously (11). The isolates were identified by standard biochemical methods and gas-liquid chromatography (19). Minimal inhibitory concentrations of penicillin, tetracycline, chloramphenicol, erythromycin, clindamycin, and rifampin were determined on agar plates of 5% rabbit blood in blood agar base (Difco Laboratories) containing serial twofold dilutions of antibiotic from 20 to 0.3 μg/ml. Plates were incubated in GasPak jars (BBL). Strains of *Bacteroides* were screened for penicillinase production by a standard technique (28).

The restriction-negative *Escherichia coli* C and the plasmids R-100 and ColE1 were generously provided by Don Helinski, Department of Biology, University of California at San Diego. *E. coli* 53E and *Enterobacter cloacae* 122H are R factor-negative recipients orginally isolated from an antibiotic-free population in Borneo (10). *Enterobacter aerogenes* K3151, a known producer of colicin (3), was used as a positive control in experiments testing for bacteriocin production. *E. coli* C and *E. coli* K-12 were used as colicin-sensitive indicators.

Radioactive labeling. The cells were diluted from an early stationary-phase culture into fresh Trypticase soy broth (Difco). After incubation for 6 h at 37 C, [*methyl*-^{3}H]thymidine (0.01 mCi/ml; specific activity, 55.6 Ci/mmol; New England Nuclear Corp.)

was added, and the cells were grown at 37 C to early stationary phase.

Cell lysis and sucrose gradient and CsCl-ethidium bromide centrifugation. Lysozyme spheroplasts were prepared, lysed with sodium dodecyl sulfate, and treated with NaCl to precipitate the chromosome as described by Guerry et al. (17). For sucrose gradient centrifugation, the lysate was diluted with 0.05 M tris(hydroxymethyl)aminomethane, pH 8.0, 0.05 M NaCl, and 0.005 M ethylenediaminetetraacetic acid, layered onto a 5 to 20% linear sucrose gradient in 0.05 M tris(hydroxymethyl)aminomethane, pH 8.0, 0.5 M NaCl, and 0.005 M ethylenediaminetetraacetic acid, and centrifuged in the Spinco SW50.1 rotor at 50,000 rpm for 55 min at 15 C. CsCl-ethidium bromide centrifugation, collection, scintillation counting, pooling, and dialysis of samples were performed as described by Helinski and co-workers (2, 5). Greater than 95% recovery of input counts was obtained from all velocity sedimentation runs.

Alkaline sucrose sedimentation. Samples pooled from the neutral 5 to 20% sucrose gradient were layered directly onto 20 to 31% alkaline sucrose gradients containing 0.3 N NaOH, 1.0 M NaCl, and 0.005 M ethylenediaminetetraacetic acid. Centrifugation in the Spinco SW50.1 rotor was for 55 min at 50,000 rpm and 15 C.

Electron microscopy. DNA samples, pooled from a preparative 5 to 20% neutral sucrose gradient, were prepared for electron microscopy by a modification of the Kleinschmidt procedure, as described by Davis et al. (12).

Conjugation experiments. These experiments were designed to detect transfer of antibiotic resistance from either tetracycline-resistant or multiply resistant *Bacteroides fragilis* to *E. coli* C, *E. coli* 53E, *Enterobactor cloacae* 122H, *Bacteroides melaninogenicus*, or to tetracycline-sensitive strains of *B. fragilis*. The general techniques for detection of transferable drug resistance (10, 31) were modified as appropriate for an anaerobic system. Stationary-phase cultures were prepared in brain heart infusion broth incubated in a GasPak jar, and 0.5 ml of the resistant strain was mixed with 3.5 ml of the sensitive strain. After overnight incubation, 0.5 ml of the mixture was inoculated over the surface of blood agar plates with and without antibiotics. When *B. fragilis* was the prospective donor and *E. coli* or *Enterobacter cloacae* the recipient, the inoculum was taken from the undiluted mixture and incubated aerobically. When resistant strains of *Bacteroides* were mated with sensitive *B. melaninogenicus*, 0.05 ml of the undiluted, the 10^{-3}, and the 10^{-6} dilutions of the mixture were plated onto antibiotic-containing 5% blood agar. In all experiments involving *B. melaninogenicus*, the media was supplemented with a terminal concentration of 0.5 μg of menadione per ml (Abbot Laboratories). *B. melaninogenicus* could be identified by pigment production. One multiply resistant strain, *B. ochraceus* 2228, was sensitive to 2 μg of colistin per ml. This organism was mated with several sensitive strains of *B. fragilis* and suppressed from subcultures of the undiluted mixture by incorporating into the agar 10 μg of colistin per ml, as well as the antibiotics under study.

Mating mixtures were plated onto 5% blood agar containing 10 μg of either tetracycline, chloramphenicol, clindamycin, rifampin, or erythromycin per ml. In experiments with *B. melaninogenicus*, mixtures were also plated onto agar containing 2 μg of penicillin per ml.

Bacteriocin experiments. Production of bacteriocins by two resistant strains of *Bacteroides* sp. were studied by the method of Fredericq (16). Prospective bacteriocinogenic strains were stabbed on plates of brain heart infusion agar and incubated for 48 h in a GasPak jar at 37 C. *Enterobacter aerogenes* K3151, a known colicin producer, was included on each plate. Colonies were sterilized over chloroform vapor, and a plasmid-negative strain of *B. fragilis*, *B. melaninogenicus*, or a colicin-sensitive *E. coli* K-12 was seeded over the surface of the plate from an overnight culture. The confluent cultures of the indicator strains were examined after 24 h of incubation for zones of inhibition surrounding the sterilized colonies.

RESULTS

Characteristics of Bacteroides strains tested. The 10 strains of *Bacteroides* selected for study included seven tetracycline-resistant *B. fragilis* (resistant to 20 μg/ml), two tetracycline-sensitive *B. fragilis* (sensitive to 1.0 μg/ml), and 1 multiply resistant strain of *B. ochraceus* (syn. *B. oralis* subsp. *elongatus*) designated 2228. All strains were typically resistant to aminoglycoside antibiotics, polymyxins, and penicillin except *B. ochraceus* 2228, which was sensitive to 2 μg of colistin per ml. All produced penicillinase. One strain of *B. melaninogenicus* was used as a sensitive recipient of drug resistance studies and was sensitive to 0.3 μg of penicillin, tetracycline, chloramphenicol, clindamycin, rifampin, and erythromycin per ml. *B. ochraceus* 2228 was resistant to 20 μg of tetracycline, chloramphenicol, clindamycin, erythromycin, and rifampin per ml. All strains of *B. fragilis* were sensitive to 1.0 μg or less of these five drugs per ml except for tetracycline, as indicated above.

Of these 10 strains, two were shown to contain plasmids: a typical strain of *B. fragilis* subsp. *fragilis* (tetracycline resistant) designated N1175 and the multiply resistant *B. ochraceus* 2228. This strain of *B. ochraceus* was microaerophilic, producing visible colonies in a candle jar after 48 h of incubation. It did not produce spores, was consistently gram negative, and displayed the typical carbohydrate fermentation pattern of this organism (19), except that it fermented mannitol. In peptone-yeast-glucose broth it produced predominantly acetic, lactic, and succinic acids, as well as an unidentified short-chain alcohol with a retention time rela-

tive to acetic acid of 0.56.

The tetracycline-sensitive, plasmid-negative bacteria used in conjugation and bacteriocin experiments were designated *B. fragilis* A, B, and C.

Plasmids of B. fragilis strain N1175. The neutral sucrose gradient analysis of the cleared lysate from *B. fragilis* strain N1175 is shown in Fig. 1. Three discrete peaks of DNA, designated 1, 2, and 3, are seen. Using R100 and ColE1 superhelical DNA as internal markers, the sedimentation coefficients of the three peaks were determined to be as follows: 1, 54*S*; 2, 41*S*; and 3, 22*S*. The *S* values of R100 and ColE1 are 77*S* and 23*S*, respectively, corresponding to molecular weights of 70×10^6 and 5×10^6 (2, 29). To assay for covalently closed circular DNA, the three peaks were pooled separately and sedimented through alkaline sucrose gradients (Fig. 2). Under these conditions, covalently closed circular DNA collapses into a rapidly sedimenting structure, whereas open circular and linear DNA denature into slower sedimenting single strands. Most of the DNA of peak 1 forms a sharp, rapidly sedimenting band characteristic of closed circular DNA (Fig. 2A), whereas the DNA in peaks 2 and 3 sediments much slower, approximately as expected for denatured, single strands (Fig. 2B and C). Analysis of peaks 1, 2, and 3 by CsCl-ethidium bromide equilibrium density gradient centrifugation confirmed that only peak 1 contained covalently closed circular DNA. The radioactivity in peak 1 represented between 1.0 to 1.2% of the total counts in the crude lysate.

The ratio of the sedimentation coefficients of peaks 1 and 2 suggested that 2 is the open circular form of 1. To confirm this conclusion each DNA peak was examined by electron microscopy. The vast majority of the DNA from peak 1 appeared as individual superhelical molecules (Fig. 3A), as expected for closed circular DNA. Peak 2 consisted mostly of open circular DNA molecules (Fig. 3B) of a single size that corresponded to the open circular DNA form of the superhelical DNA in peak 1. In addition, a large number of heterogeneous linear DNA molecules were seen in peak 2, probably representing chromosomal DNA fragments. Peak 3 contained primarily linear DNA fragments but also a number of small open circular DNA molecules, No small superhelical DNA was seen, nor could any small covalently closed circular DNA molecules be isolated from CsCl-ethidium bromide equilibrium density gradi-

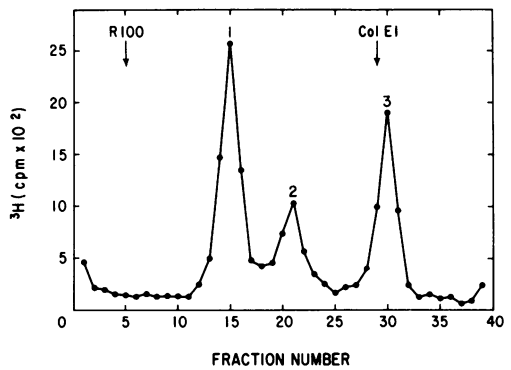

FIG. 1. *Neutral sucrose gradient analysis of a cleared lysate from B. fragilis strain N1175. The lysate was prepared and sedimented through a 5 to 20% neutral sucrose gradient as described in Materials and Methods. ^{14}C-labeled covalently closed DNA forms of R100 and ColE1 were added as internal markers.*

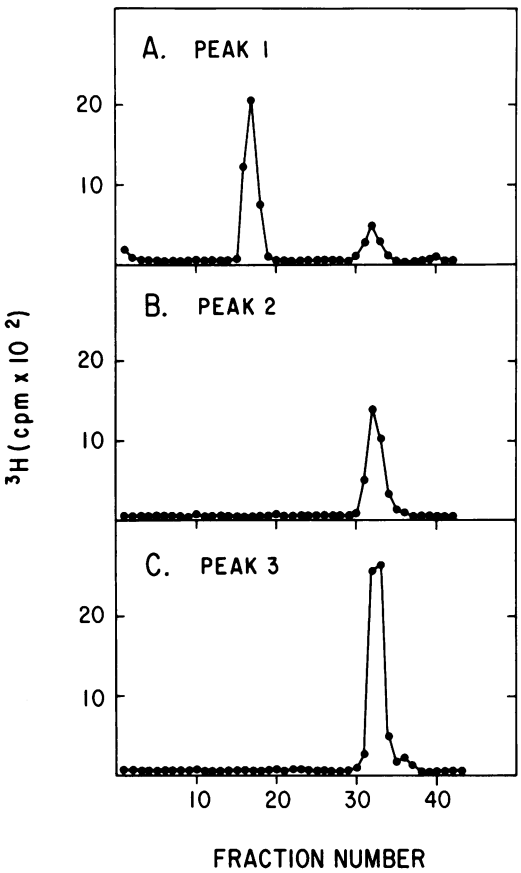

FIG. 2. *Alkaline sucrose gradient analysis of the three DNA peaks shown in Fig. 1. Samples from each peak in Fig. 1 were layered directly onto separate 20 to 31% alkaline sucrose gradients and centrifuged as described in Materials and Methods. (A) Peak 1; (B) peak 2; (C) peak 3.*

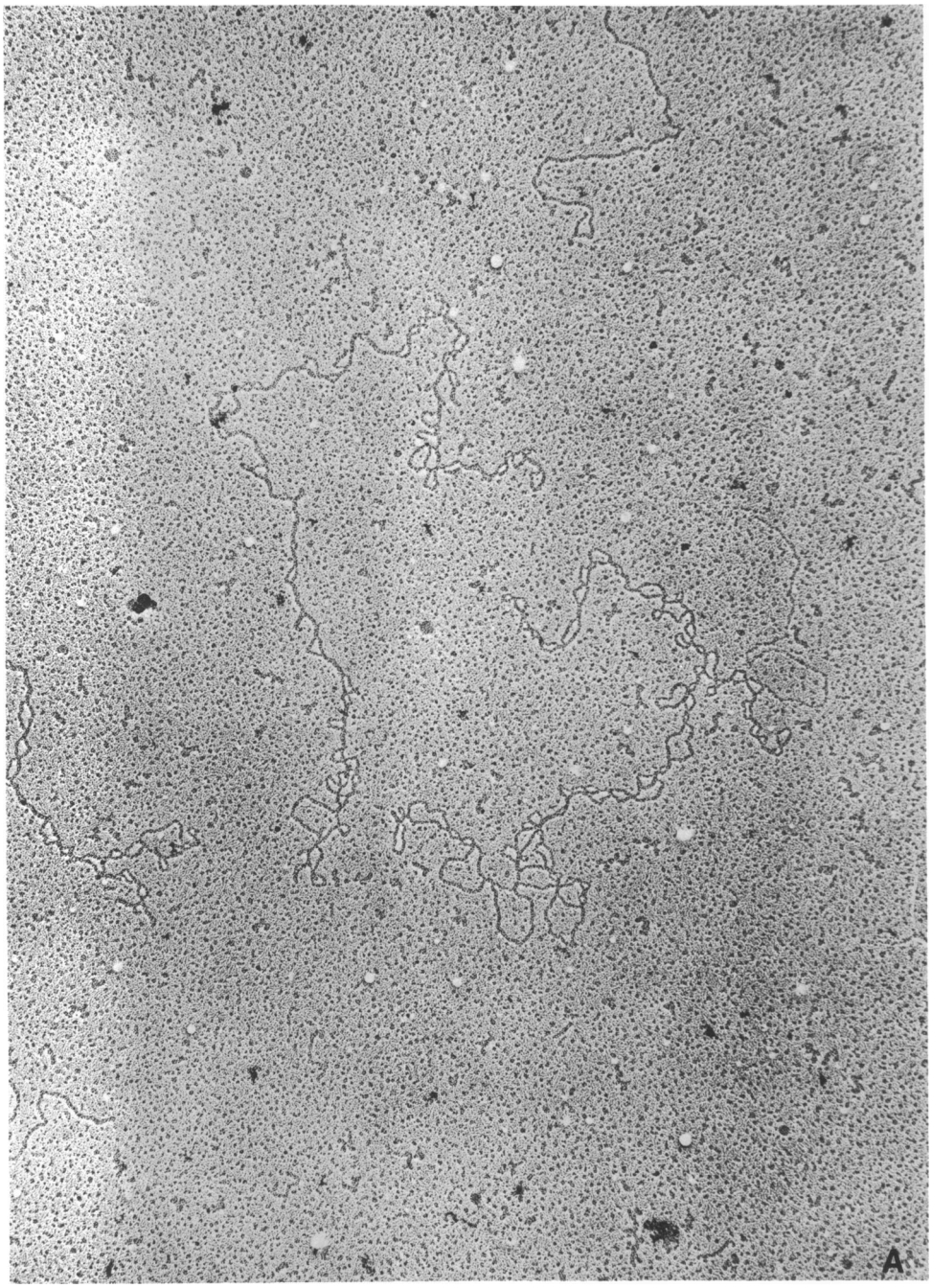

FIG. 3. *Electron micrographs of the B. fragilis plasmid DNA. (A) and (B) represent the DNA of peaks 1 and 2, respectively, described in Fig. 1. Both micrographs were taken at the same magnification.*

ents carried out on cleared lysates. Thus, the significance of these small DNA circles in peak 3 is not clear.

Plasmids of B. ochraceus strain 2228. This strain of *Bacteroides* was studied because of its multiple antibiotic resistance. Figure 4 shows the CsCl-ethidium bromide density gradient of the cleared lysate from strain 2228. The satellite

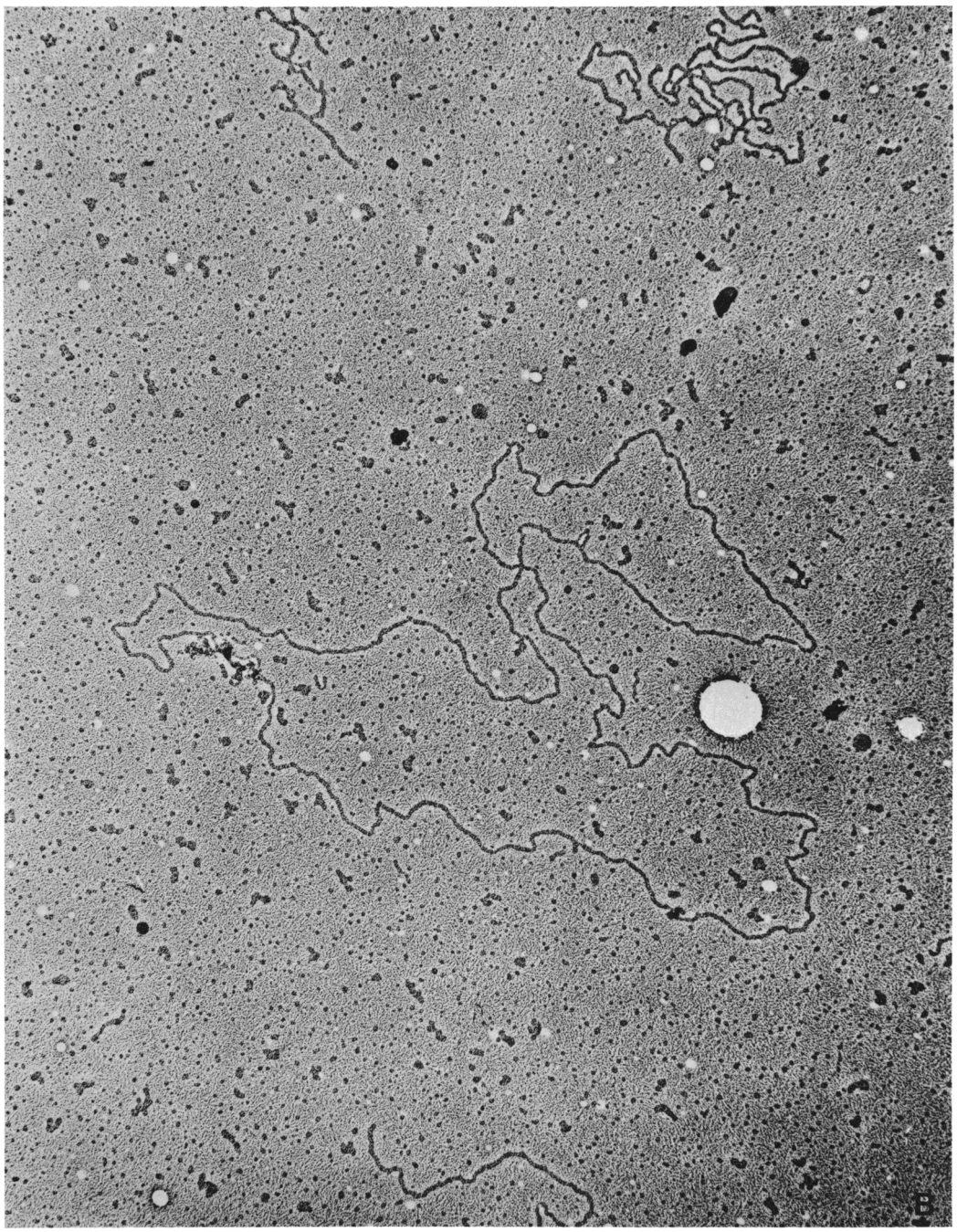

FIG. 3B

peak of closed circular DNA was pooled, dialyzed, and then centrifuged through a neutral sucrose gradient (Fig. 5). This gradient revealed two sharp plasmid peaks at 77*S* and 58*S*, with a broad peak of contaminating chromosomal DNA at 25 to 45*S*. Each plasmid peak contained about 1% of the total radioactivity present in the crude lysate. The superhelical nature of the plasmid peaks was confirmed by sedimenting the pooled samples from the gradients in Fig. 5 through separate alkaline sucrose gradients. The results (Fig. 6) demonstrate that

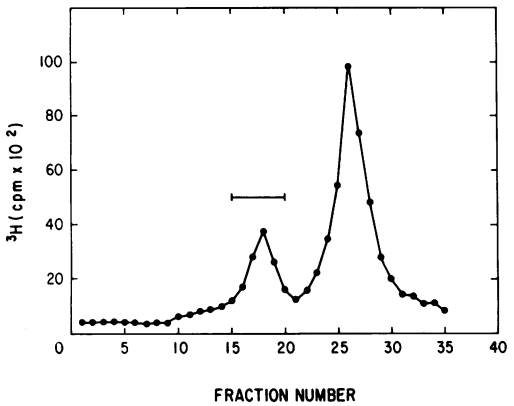

FIG. 4. *CsCl-ethidium bromide equilibrium density gradient centrifugation of the cleared lysate from B. ochraceus strain 2228. The bar indicates the plasmid DNA fractions which were pooled for the neutral sucrose gradient analysis.*

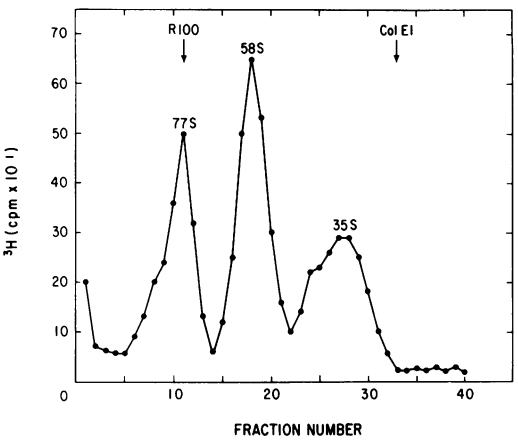

FIG. 5. *Neutral sucrose gradient analysis of the plasmid DNA peak from Fig. 4. The pooled and dialyzed plasmid DNA was centrifuged through a neutral 5 to 20% sucrose gradient as described in Materials and Methods. ^{14}C-labeled superhelical R100 and ColE1 were added as internal markers.*

the 77*S* and 58*S* peaks sediment rapidly as closed circular DNA species in alkaline sucrose, and that the 25 to 45*S* peak is probably contamination from the chromosomal band in the CsCl-ethidium bromide preparative gradient. The second rapidly sedimenting component in Fig. 6A represents contamination of the 77*S* peak with 58*S* DNA.

Conjugation experiments. The following attempts at transfer of antibiotic resistance were unsuccessful: (i) transfer of tetracycline resistance from *B. fragilis* N1175 to *E. coli* C, *E. coli* 53E, and *Enterobacter cloacae* 122H; (ii) transfer of tetracycline resistance and penicillinase production from *B. fragilis* N1175 to *B. melaninogenicus;* (iii) transfer of tetracycline, chloramphenicol, rifampin, erythromycin, and clindamycin resistance from *B. ochraceus* 2228 to *B. melaninogenicus* and *B. fragilis* A, B, and C; (iv) transfer of tetracycline and chloramphenicol resistance from *B. ochraceus* to *E. coli* C or *E. coli* 53E.

Bacteriocin production. *Enterobacter aerogenes* K3151 produced a bacteriocin (colicin V) active against *E. coii* C and *E. coli* K-12 but inactive against the plasmid-negative strains of *B. fragilis* and *B. melaninogenicus*. Sterilized colonies of *B. fragilis* N1175 and *B. ochraceus* 2228 did not inhibit either strain of *Bacteroides*, *E. coli* K-12, or *E. coli* C.

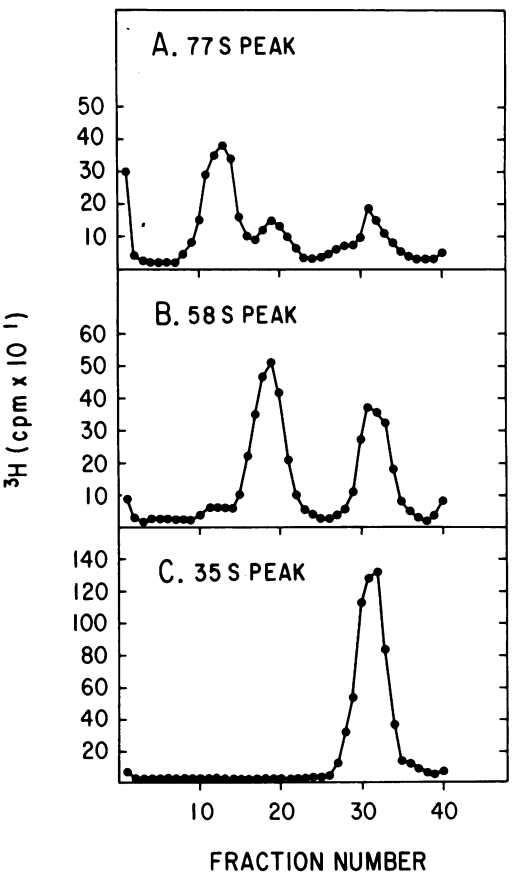

FIG. 6. *Alkaline sucrose gradient analysis of the three DNA peaks shown in Fig. 5. Samples from each peak in Fig. 5 were layered directly onto separate 20 to 31% alkaline sucrose gradients and centrifuged for 50 min at 50,000 rpm in the Spinco SW50.1 rotor at 15 C. (A) 77S peak; (B) 58S peak; (C) 35S peak.*

DISCUSSION

The demonstration of multiple plasmids from two strains of *Bacteroides* is of particular interest in view of the prominence of these bacteria among the normal flora. *B. fragilis* is by far the most common organism in the normal intestine (13, 14) and would be a logical reservoir for plasmids controlling the production of colicins, antibiotic resistance, or other properties vital to the maintenance of normal microbial ecology. There have been few studies of the distribution of *B. ochraceus* (*B. oralis* subsp. *elongatus*), but it appears to have been isolated primarily from subprimate animals (19).

B. fragilis strain N1175 contains a single species of covalently closed circular DNA with a sedimentation coefficient of 55*S* for the superhelical form, corresponding to a calculated molecular weight of 25×10^6 (21). The plasmid DNA bands slightly less dense than the *B. fragilis* chromosome (40% guanine plus cytosine; J. Ryan, C. E. Davis, and A. I. Braude, unpublished data) in neutral CsCl equilibrium centrifugation. *B. ochraceus* 2228 was shown to contain two distinct closed circular DNA elements, the larger cosedimenting with the R100 marker, corresponding to a molecular weight of 70×10^6 (25), and the smaller sedimenting at 58*S*, with a calculated molecular weight of 27×10^6 (21). Several cryptic plasmids recently isolated by Stiffler and colleagues from three strains of *B. fragilis* were much smaller, with molecular weights ranging from 1.6×10^6 to 4×10^6 (30). Although we found no evidence of small closed circular DNA in our strains, it is possible that the open circular forms in peak 3 of *B. fragilis* N1175 represent closed circular DNA which has been converted to open circular DNA by the conditions of study. Similarly, low concentrations of small plasmids could be present in the 25 to 45*S* chromosomal peak of *B. ochraceus* 2228. In a recent abstract Damle and Syed (J. Dent. Res. **54**:104, special issue A, 1975) reported 42 to 45*S* and 24 to 26*S* plasmid DNA in a strain of *B. ochraceus* and a 24 to 26*S* plasmid in *B. melaninogenicus*.

The physiological roles of these plasmids are unknown. Although the two plasmid-containing strains of *Bacteroides* were tetracycline resistant, we could not isolate closed circular DNA from six other resistant isolates. *B. ochraceus* 2228 was also resistant to chloramphenicol, erythromycin, clindamycin, and rifampin, drugs to which most *Bacteroides* are sensitive. Attempts to transfer antibiotic resistance from N1175 and 2228 to *Enterobacteriaceae* and other strains of sensitive *Bacteroides* were unsuccessful. Neither strain produced bacteriocins active against either a universally sensitive indicator strain of *E. coli* or plasmid-negative strains of *Bacteroides*. Although our experiments do not rule out the possibility that these plasmids are associated with resistance to antibiotics or other antibacterial agents, our inability to detect plasmids in six other strains of tetracycline-resistant *B. fragilis* with the method employed suggests that resistance to this antibiotic may not be plasmid-mediated in most strains of *Bacteroides*. These plasmids are large enough to contain sex factors, but it is possible that they mediate nontransmissible drug resistance.

Other possible functions of *Bacteroides* plasmids might include genetic control of properties important to bacteria that colonize mucosal surfaces. We plan to examine these strains for pili which could promote adherence to surfaces, for resistance to nitrites and other potentially toxic perservatives in food, and for resistance to heavy metals and the serum bactericidal system.

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