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Differentiation of *Bacteroides ovatus* and *Bacteroides thetaiotaomicron* by Means of Bacteriophage

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Two members of the *Bacteroides fragilis* group, *B. ovatus* and *B. thetaiotaomicron*, are difficult to distinguish by biochemical methods. They are currently identified on the basis of their variable ability to ferment salicin. We studied a method of identification for these two species by using cell lysis by bacteriophages. A total of 38 bacteriophages were used to distinguish the two species. Identification by bacteriophages was compared with species identification by prereduced anaerobically sterilized biochemical testing with salicin as the differentiating test. A total of 215 clinical isolates biochemically identified as *B. ovatus* or *B. thetaiotaomicron* were tested. A total of 100% of the strains identified as *B. ovatus* by bacteriophages produced strong acid in salicin (pH ≤ 5.4). However, 40% of the strains identified as *B. thetaiotaomicron* by bacteriophages also produced strong acid in salicin, and an additional 39% produced weak acid (pH 5.5 to 5.7). This study demonstrates that salicin fermentation is an inadequate test for the differentiation of *B. ovatus* and *B. thetaiotaomicron*.

Species of the *Bacteroides fragilis* group are among the anaerobic bacteria most frequently isolated from specimens in clinical bacteriology laboratories (5). The group includes *B. fragilis*, *B. distasonis*, *B. vulgatus*, *B. ovatus*, and *B. thetaiotaomicron*. More recently, *B. uniformis* and *B. eggerthii* have been added to this group. *B. fragilis* is the most frequently isolated in the group. *B. ovatus* and *B. thetaiotaomicron* are second in frequency of isolation from clinical specimens and are the *Bacteroides* spp. (as a group) most resistant to beta-lactam drugs (13).

The members of the *B. fragilis* group are biochemically separated from other *Bacteroides* spp. by their ability to grow in the presence of 20% bile. They are differentiated from each other by the ability to produce indole from tryptophan and to ferment certain carbohydrates (7). *B. ovatus* and *B. thetaiotaomicron*, however, are not easily differentiated by biochemical testing; they show variable activity to the two fermentation tests, salicin and mannitol, currently used to separate them. The objective of this study was to develop and test a method for the accurate identification of *B. ovatus* and *B. thetaiotaomicron* by use of cell lysis by bacteriophages.

MATERIALS AND METHODS

Bacterial strains and media. The *B. fragilis* group strains used in this study were from the Tufts Anaerobic Bacteriology and Research Laboratory collection in the Infectious Disease Section at the New England Medical Center, Boston, Mass. They included clinical isolates from nine medical centers across the United States, four strains from the Anaerobe Laboratory at the Virginia Polytechnic Institute and State University (VPI), Blacksburg, and five strains from the American Type Culture Collection, Rockville, Md. Each strain was isolated from a different patient. These strains were identified by the methods described in the VPI *Anaerobe Laboratory Manual* (6) with prereduced anaerobically sterilized (PRAS) peptone-yeast extract fermentation media (Carr-Scarborough Microbiologicals, Decatur, Ga.). Members of the *B. fragilis* group that were indole positive and trehalose positive were designated as the *B. ovatus-thetaiotaomicron* group and set aside for differentiation by bacteriophages. A total of 366 *B. fragilis* group strains were tested for susceptibility to bacteriophages; 215 of these were *B. ovatus* or *B. thetaiotaomicron* strains. All of the *Bacteroides* spp. were grown on brucella sheep blood agar supplemented with hemin and vitamin K1 (14); all strains were stored on brain heart infusion (BHI) agar slants (Scott Laboratories, Inc., Fiskeville, R.I.).

Isolation of bacteriophages. Samples of sewage water were collected at the Town of Amherst Sewage Plant, Amherst, Mass. Cells and debris were removed by centrifugation (10,000 × *g* for 20 min). The supernatant was then centrifuged (19,000 × *g* for 1 h) to form a bacteriophage pellet. The pellet was washed with 10 ml of buffer (1.0 liter of distilled water, 5.0 g of sodium chloride, 1.0 ml of magnesium sulfate, 20.0 ml of Tris, 10.0 ml of gelatin) and filtered through a 0.2-μm-pore-size membrane filter (Acrodisc; Gelman Sciences, Inc., Ann Arbor, Mich.). The phage pellet-buffer mixture was assayed for bacteriophages by the Adams double-agar-layer method (1). Petri plates (9 cm) containing 1.5% BHI agar supplemented with yeast extract, hemin, and vitamin K1 were overlaid with 1.5 ml of soft BHI agar (0.7% agar at 46°C) containing 0.1 ml of the phage pellet-buffer mixture and 0.2 ml of the host *Bacteroides* sp. grown in PRAS BHI broth to a turbidity of 1.5×10^8 CFU/ml. The plates were incubated anaerobically for 18 h at 37°C and then examined for areas of lysis or plaques. Each bacteriophage was isolated and purified by at least three successive transfers of single plaques. Bacteriophages were diluted in BHI broth, and plaques were counted by the Adams double-agar-layer method (1). Dilutions were made and double-agar plating was done aerobically.

Propagation of bacteriophages. Stock cultures of bacteriophages (titers, 10^8 to 10^{10} PFU/ml) were prepared by harvesting the soft-agar plates as described by Adams (1). PRAS BHI broth (15 ml) was added to the surface of a semiconfluently lysed lawn of bacteria and bacteriophages. The soft-agar layer was scraped off and mixed in a centrifuge tube with a vortex mixer. After 2 h of incubation at room temperature with occasional gentle mixing, the bacterio-

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phage-agar mixture was centrifuged (10,000 × *g* for 20 min) to remove agar and bacterial debris. The supernatant was then filtered through a 0.2-μm-pore-size membrane filter (Acrodisc) and stored at 4°C (1, 2, 12).

Identification by bacteriophages with the MIC-2000. Each *Bacteroides* strain to be tested was grown in PRAS BHI broth at 37°C until a turbidity of 1.5×10^8 CFU/ml was obtained. This culture (0.2 ml) was used with 1.5 ml of soft BHI agar to make the soft-agar layer test plate. The bacteriophage stock suspensions were placed in wells of a 96-well microtiter plate. By using the MIC-2000 (Dynatech Laboratories, Inc., Alexandria, Va.), we were able to spot 38 bacteriophage suspensions onto the test plate at the same time. The MIC-2000 has a movable rectangular hand with inoculator prongs that correspond in position to the wells of the microtiter plate. Each prong picks up 0.001 ml of each bacteriophage suspension and delivers it to the soft-agar layer containing the test *Bacteroides* strain. The test plates were incubated anaerobically at 37°C, and the bacterial lawns were examined for zones of lysis after 18 h.

RESULTS

Host range and bacteriophage specificity. A total of 33 bacteriophages were isolated from sewage water; one bacteriophage was lost during transfer or storage. Five bacteriophages specific for *B. thetaiotaomicron* were obtained from D. R. Woods of Rhodes University, Grahamstown, South Africa (4); the host strain had been confirmed as *B. thetaiotaomicron* by the Anaerobe Laboratory at VPI. The 38 bacteriophages were divided into two groups on the basis of their lytic activity against the 22 host strains and the fermentation of salicin by their own host strains. Eighteen bacteriophages were placed in group 1; their nine host strains had salicin pH values from 5.0 to 5.3, indicating that the strains were probably *B. ovatus*. Twenty other bacteriophages were placed in group 2; their 13 host strains had salicin pH values from 5.4 to 6.1, indicating that the strains were probably *B. thetaiotaomicron* (Fig. 1). Each bacteriophage was specific for host strains within its own group.

Four strains, two *B. ovatus* (8605 and 3524) and two *B. thetaiotaomicron* (6212 and 2808) identified by DNA homology studies at VPI, and five strains, four *B. thetaiotaomicron* (12290, 29148 [type strain], 29741, and 29742) and one *B. ovatus* (8483 [type strain]) obtained from the American Type Culture Collection, were tested with bacteriophages. Eight of nine of these strains were susceptible to bacteriophages. Their identification by bacteriophages agreed with their previous VPI or American Type Culture Collection identification. One strain of *B. ovatus* (8483) was not susceptible to any of the bacteriophages.

Certain bacteriophages showed variable lytic ability upon repeated testing with the same *Bacteroides* strain. Visible cell lysis varied from clear to turbid from day to day. This phenomenon kept us from reducing the number of bacteriophages used in the method.

Identification of clinical isolates by bacteriophages. A total of 215 strains, identified by PRAS biochemical testing as either *B. ovatus* or *B. thetaiotaomicron*, were tested for their susceptibility to the 38 bacteriophages. A total of 185 strains (86%) were susceptible to one or more bacteriophages. All strains susceptible to group 1 bacteriophages were presumptively called *B. ovatus*; all strains susceptible to group 2 bacteriophages were presumptively called *B. thetaiotaomicron* (Fig. 2). All of the strains identified by bacteriophages as *B. ovatus* (31 strains) produced a strong acid reaction in salicin (pH ≤ 5.4). A total of 154 other strains were identified by bacteriophages as *B. thetaiotaomicron*. Of these strains, 63 (40.9%) produced a strong acid reaction in salicin, 60 (38.9%) produced a weak acid reaction in salicin (pH 5.5 to 5.7), and only 31 (20.1%) did not ferment salicin at all (pH ≥ 5.8). Of the 215 *B. ovatus-thetaiotaomicron* group strains, 30 (14%) were not susceptible to any of the 38 bacteriophages; the majority of these strains had salicin pH values from 5.1 to 5.5.

An additional 151 strains, including *B. fragilis* (92 strains),

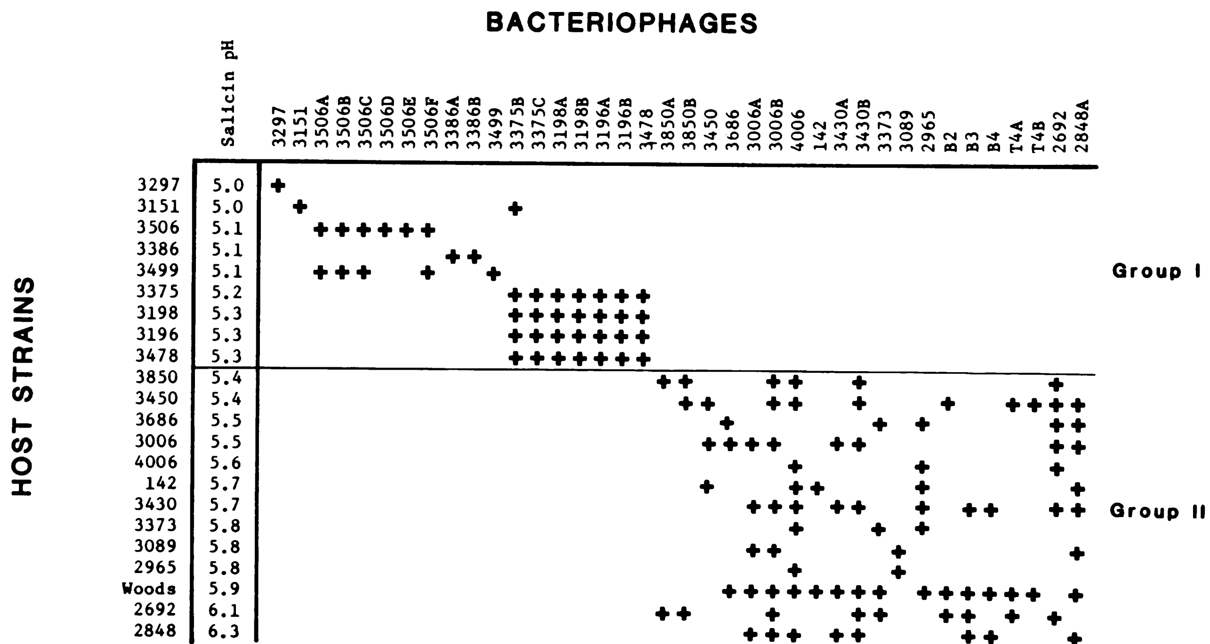

FIG. 1. Host range determinations. Group 1, *B. ovatus* bacteriophages; group 2, *B. thetaiotaomicron* bacteriophages.

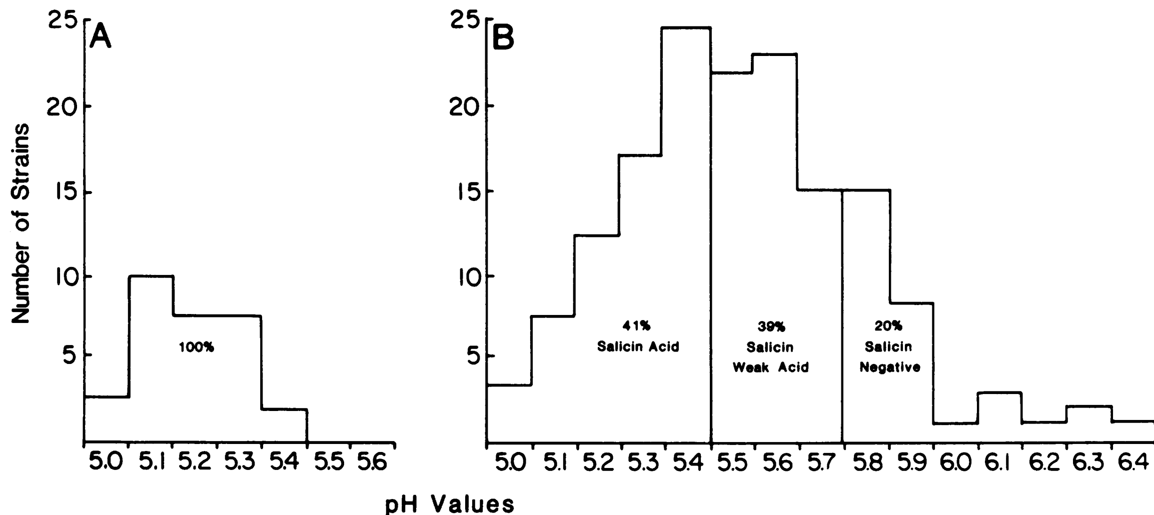

FIG. 2. Salicin pH values of *Bacteroides* spp. identified by group 1 (A) and group 2 (B) bacteriophages.

B. distasonis (19 strains), *B. vulgatus* (19 strains), *B. uniformis* (15 strains), and *B. eggerthii* (6 strains), were tested against the bacteriophages. None of these *Bacteroides* spp. was susceptible to any of the group 1 or 2 bacteriophages.

DISCUSSION

The first isolation of a bacteriophage for a *Bacteroides* species was reported by Sabiston and Cohl in 1969 (10). They isolated a bacteriophage from sewage that was active against *B. distasonis* (10). In 1972, Nacecu et al. (9) isolated two bacteriophages from sewage that lysed 23 of 68 *B. fragilis* strains. Strains of other species were not susceptible to either phage (9). In 1974, Keller and Traub (8) isolated bacteriophages specific for *B. fragilis* from a number of different animal sera, including calf, lamb, chicken, and rabbit. All 25 bacteriophages were specific for *B. fragilis*; no lytic activity was detected with 42 strains of other *Bacteroides* species or 7 *Fusobacterium* species (8). In 1979, Booth et al. (3) reported the isolation of 68 bacteriophages from sewage over a 3-year period. The bacteriophages were specific for nine *Bacteroides* species; each bacteriophage was specific for strains within a given species (3). In this study, we isolated 38 bacteriophages specific for *B. ovatus* or *B. thetaiotaomicron* from sewage, used these bacteriophages to separate the two species, and compared the results of their identification by bacteriophages with that by salicin fermentation testing.

A total of 33 bacteriophages specific for *B. ovatus* or *B. thetaiotaomicron* were isolated from sewage water. The bacteriophages were completely specific for the species from which they were isolated. Of 215 strains belonging to the *B. ovatus-thetaiotaomicron* group, 86% were susceptible to the bacteriophages. All of the *Bacteroides* spp. designated *B. ovatus* by bacteriophages produced a strong acid reaction in salicin; this agrees with the currently used identification keys for the *B. fragilis* group that state that *B. ovatus* produces a strong acid reaction in salicin (6). However, 40% of the *B. thetaiotaomicron* strains identified by bacteriophages also produced a strong acid reaction in salicin, and 39% produced a weak acid reaction, indicating that the fermentation of salicin is not an adequate test for the differentiation of the two species.

The variable lytic ability of many of the bacteriophages may have been due to pseudolysogeny (bacteriophage carrier state), as described by Keller and Traub (8), who thought that the carrier state could be due to the continuous development of bacteriophage-sensitive organisms from a resistant population (8). Identification by bacteriophages provides useful information only when one obtains a positive result, i.e., when the test strain is susceptible to the bacteriophages and an area of lysis can be seen. When the test strain does not appear to be susceptible to any of the bacteriophages, it still can be a member of the susceptible species, but one that is resistant. The development of an identification method that uses bacteriophages for the identification of *Bacteroides* spp. should include the isolation of more stable bacteriophages and the application of factors and conditions that encourage bacteriophage attachment and cell lysis and discourage the bacteriophage carrier state.

The identification of anaerobic bacteria usually includes the study of morphological characteristics such as colony and cellular morphology, staining reactions, cell shape and arrangement, and biochemical reactions that are based on the enzymatic activity of the bacteria. These methods test phenotypic markers and may vary. At the present time, *B. ovatus* and *B. thetaiotaomicron* cannot be distinguished by these methods. The identification of these two species by bacteriophages also presented problems. Too many bacteriophages (38 phages) were needed to type 86% of the test strains, and the lytic activity of many of the bacteriophages was highly variable.

One of the most promising new methods developed is that of Salyers et al. (11), who used randomly cloned fragments of DNA from *B. thetaiotaomicron* as hybridization probes for the differentiation of *B. thetaiotaomicron* from other *Bacteroides* spp. This is a method based on genetic relatedness (DNA hybridization). It is highly specific and not dependent upon gene expression. The application of this method to the identification of clinical isolates should be developed and tested.

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