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Psychrotrophic *Brocothrix thermosphacta* Bacteriophages Isolated from Beef†

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A total of 15 wild-type *Brocothrix thermosphacta* strains isolated from beef and the type strain, *B. thermosphacta* ATCC 11509, were used as hosts for the isolation of bacteriophages under psychrotrophic conditions (7°C). A total of 21 virulent, psychrotrophic phages were successfully isolated and purified from aqueous extracts of spoiled rib steaks. Phage plaque size and plating efficiency significantly increased as incubation temperature was reduced from 25 to 1°C. Electron microscopy of two homologous *B. thermosphacta* phages showed the virions to consist of hexagonal heads and tails, with terminal appendages clearly visible on one of the phages. On the basis of culture and biochemical data, the wild-type *B. thermosphacta* strains had characteristics identical to those of strain ATCC 11509. However, specific differences in the pattern of susceptibilities to the phages revealed the presence of 14 distinct phage lysotypes. Phage typing may provide a rapid and sensitive means of differentiating *B. thermosphacta* strains.

Brocothrix thermosphacta (25) has been isolated with increasing frequency from a variety of fresh (3, 6, 8, 14, 19, 23) and processed meats (11, 12, 14, 22, 26) and appears to be restricted to the meat environment (18). During growth, metabolic end products can contribute to the objectionable flavors and odors commonly associated with spoiled meats (8, 11, 12, 14, 22, 26). The role of *B. thermosphacta* in meat spoilage has recently been extensively reviewed (12, 14, 22).

Relative to fresh beef, *B. thermosphacta* has been reported to constitute up to 25% of the initial bacterial flora (23, 24). In a previous study (15) about 20% of the psychrotrophic bacteria isolated from spoiled beef had characteristics consistent with those described for *B. thermosphacta* (25), and the remaining 80% were pseudomonads. More recent investigations (16) resulted in the isolation of a number of psychrotrophic bacteriophages lytic for 37 of the beef spoilage *Pseudomonas* strains. However, the existence of *B. thermosphacta* phages has not yet been documented. Virulent *B. thermosphacta* phages may be important to the bacterial ecology of meat spoilage, and a phage typing set would be useful for strain differentiation. In view of this, the objectives of the present study were twofold: to further characterize the wild-type *B. thermosphacta* strains previously isolated (15) from aerobically spoiled beef and to isolate and purify psychrotrophic *B. thermosphacta* phages and determine their taxonomic value in phage typing. The results show that the culture and biochemical characteristics of 15 wild-type *B. thermosphacta* strains were identical to those of strain ATCC 11509, but strain differences could be established on the basis of differential susceptibility to psychrotrophic phages.

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MATERIALS AND METHODS

Bacterial strains. A total of 15 wild-type *B. thermosphacta* strains previously isolated from spoiled retail rib steaks (15) were purified and maintained on tryptic soy agar (Difco Laboratories, Detroit, Mich.) slants. The type strain, *B. thermosphacta* ATCC 11509, originally isolated by McLean and Sulzbacher (19), was obtained from the American Type Culture Collection, Rockville, Md. *Microbacterium lacticum* ATCC 8180, *Corynebacterium flavescens* ATCC 10340 (formerly *Microbacterium flavum* [7]), *Lactobacillus plantarum* ATCC 8014, *Lactobacillus mali* ATCC 27053, *Listeria denitrificans* ATCC 14870, and *Listeria grayii* ATCC 19120 were also obtained from the American Type Culture Collection. The following strains identified as *B. thermosphacta* were generously provided: BL110, BL243, BL253, BL262, and BL264 (A. F. Egan, Commonwealth Scientific and Industrial Research Organization, Division of Food Research, Queensland, Australia); C420 (R. H. Dainty, Agricultural Research Council, Bristol, England); and 467 (C. Vanderzant, Texas Agricultural Experiment Station, College Station, Tex.).

Bacterial characterization. The characteristics of the 15 wild-type *B. thermosphacta* strains were compared with those of ATCC 11509 by the following tests: (i)

motility in moist mounts, (ii) anaerobic growth, (iii) growth on Gardner streptomycin-sulfate-thallous acetate-Acti-dione (cycloheximide, The Upjohn Co., Kalamazoo, Mich.) agar (13), (iv) oxidase (4), (v) catalase (4), (vi) Voges-Proskauer (4), (vii) methyl red (4), (viii) citrate utilization (4), (ix) nitrate reduction (4), (x) oxidative fermentative glucose test (2), and (xi) hydrolysis of starch, Tween 80, gelatin, and skim milk agar (17). Growth at 1°C (21 days), 7°C (10 days), 25°C (48 h), and 37°C (48 h) was determined after streaking on tryptic soy agar plates. Acid production from carbohydrates was determined with differentiation disks carbohydrates (Difco) on a phenol red agar base (Difco). Except when indicated otherwise, test results were determined after incubation at 25°C.

Bacteriophage isolation. At least three attempts were made to isolate bacteriophages for the 15 wild-type *B. thermosphacta* strains and ATCC 11509. The techniques used for the isolation of phages from spoiled retail rib steaks were identical to those previously described (16). Briefly, 10 g of beef longissimus dorsi muscle was homogenized and centrifuged to remove cellular debris, and the supernatant was combined with an equal volume of double-strength tryptic soy broth (Difco) containing 0.001 M $CaCl_2$. This medium was enriched for the bacterial host by the addition of 0.1 ml of an 18-h culture (tryptic soy broth plus 0.001 M $CaCl_2$, 25°C), and incubation was carried out at 7°C for 10 days. After incubation, the medium was centrifuged, and the supernatants were stored over chloroform at 4°C (1). Supernatants suspected of containing phages were spotted on agar overlays (1) containing 0.1 ml of an 18-h culture of the homologous host strain and incubated at 25°C for 24 h. Phages were purified from the supernatant by serial, single plaque isolations (1), and high-titer lysates yielding about 10^9 to 10^{12} PFU/ml were prepared from confluently lysed plates (1). These suspensions of purified phages were stored over chloroform at 4°C.

Phage typing assay. To avoid false-positive reactions, the routine test dilution (RTD) was established for each homologous phage-host pair (see Table 2) as the lowest dilution giving confluent lysis. The typing assay involved spotting the RTD of all phages (4-mm-inside-diameter loop) on each bacterial strain overlaid on grid plates (100 by 15 mm). The pattern of susceptibility of bacteria to phage lysis was determined after incubation at 7°C for 10 days and confirmed in three identical trials. The susceptibility of *Microbacterium*, *Listeria*, and *Lactobacillus* species to lysis by *B. thermosphacta* phages was determined after incubation at 25 and 30°C for 48 h.

Effects of incubation temperature on plaque formation. A high-titer lysate (ca. 10^9 PFU/ml) of phage A19 was diluted by serial 10-fold dilutions (10^{-1} to 10^{-9}) in tryptic soy broth plus 0.001 M $CaCl_2$ and plated by the agar overlay technique with 0.1 ml of an 18-h culture of *B. thermosphacta* ATCC 11509. A total of 10 plates for each dilution were inoculated and incubated at 1, 7, 25, and 30°C until no further increase in plaque size was evident. After incubation, the diameters of 10 randomly selected plaques were determined from plates at each incubation temperature with an ocular micrometer. The efficiency of plating was determined after plaque counts relative to the highest phage titer calculated (efficiency of plating, 1.0).

Electron microscopy. The *B. thermosphacta* ATCC 11509 phages MT and A19 were mounted on carbon-coated copper grids and negatively stained with phosphotungstic acid for transmission electron microscopic observation. Phage dimensions were reported as the means and standard errors of the means for measurements made on five virions.

RESULTS

Bacterial characterization. The culture and biochemical characteristics of the 15 wild-type strains isolated from beef and *B. thermosphacta* ATCC 11509 were identical. These strains were nonmotile, facultatively anaerobic, gram positive pleomorphic rods capable of growth at 1, 7, and 25°C but not at 37°C. They were also positive in the catalase, Voges-Proskauer, and methyl red tests and fermentative in the oxidative-fermentative glucose test, and they grew on Gardner streptomycin-sulfate-thallous acetate-Acti-dione agar selective medium (13). In addition, they were oxidase negative and could not utilize citrate, reduce nitrates, or hydrolyze starch, Tween 80, gelatin, or casein. All strains were able to produce acid through the fermentation of inositol, mannitol, dextrose, mannose, levulose, salicin, sucrose, maltose, and trehalose. On the basis of these findings, the wild-type strains were considered to be *B. thermosphacta* (14, 25).

Phage typing. The data in Table 1 show that a total of 21 psychrotrophic bacteriophages lytic for 13 of the *B. thermosphacta* host strains were successfully isolated from beef. *B. thermosphacta* B1 and B16 were the only wild-type strains for which no phages could be isolated, but these strains were found to be susceptible to heterologous phage lysis (Table 1). The following phages had identical host ranges: A2 and A3, A10 and A12, A8 and MT, A11 and A16, and A19 and A21 (Table 1). The percentage of wild-type bacteria susceptible to lysis by a single phage ranged from 13 (2/15) for phages A5, A7, A11, A15, and A16 to 87 (13/15) for phages A6 and A14. It should be noted that phages also lysed their homologous hosts under anaerobic conditions at 7°C.

RTD titers established for use in phage typing ranged from 4.6×10^3 to 6.1×10^{10} PFU/ml (Table 1). On the basis of differential susceptibility to phages, 18 *B. thermosphacta* strains were found to comprise 14 distinct phage lysotypes (Table 1). The only *B. thermosphacta* strains which could be grouped according to identical patterns of phage sensitivity were B1 and B5, B8 and B10, and B4, B7, and B16. *B. thermosphacta* 467, BL110, BL243, BL253, and BL264 were not susceptible to lysis by any phage at the RTD or at 100 times the RTD.

None of the high-titer lysates of any of the *B. thermosphacta* phages were capable of lysing

TABLE 1. Phage typing patterns of *B. thermosphacta* strains

Phage	Homologous host strain	Phage RTD titer (PFU/ml)	Lysis of *B. thermosphacta* test strain[a]:												
			B1 and B5	B8 and B10	B4, B7, and B16	B2	B3	B9	B11	B12	B13	B14	B15	ATCC 11509	C420	BL262
A2	B2	3.5×10^6	−	+	−	+	−	−	+	+	−	−	−	−	−	−
A3	B2	1.1×10^8	−	+	−	+	−	−	+	+	−	−	−	−	−	−
A10	B8	4.6×10^3	−	+	−	+	−	−	+	+	−	−	+	−	−	−
A12	B10	3.6×10^{10}	−	+	−	+	−	−	+	+	−	−	+	−	−	−
A8	B2	5.5×10^6	−	+	+	+	+	+	+	+	+	−	+	+	−	+
MT	ATCC 11509	3.6×10^5	−	+	+	+	+	+	+	+	+	−	+	+	−	+
A11	B9	7.3×10^6	−	−	−	−	−	+	−	−	+	−	−	−	−	−
A16	B13	6.1×10^{10}	−	−	−	−	−	+	−	−	+	−	−	−	−	−
A19	ATCC 11509	2.1×10^6	−	−	−	−	+	+	+	−	+	+	+	+	−	+
A21	ATCC 11509	2.9×10^8	−	−	−	−	+	+	+	−	+	+	+	+	−	+
A4	B3	8.1×10^4	−	−	−	−	+	−	+	+	−	−	+	−	−	−
A5	B3	1.8×10^8	−	−	−	−	+	−	−	−	−	−	+	−	−	−
A6	B4	1.3×10^6	−	+	+	+	+	+	+	+	+	+	+	−	+	+
A7	B5	3.8×10^7	+	−	−	−	−	−	−	−	−	−	−	−	−	−
A9	B7	2.3×10^{10}	−	−	+	−	+	+	+	+	+	+	+	−	−	+
A13	B11	6.5×10^7	−	−	−	+	−	−	+	+	−	−	−	−	−	−
A14	B11	2.0×10^9	−	+	+	+	+	+	+	+	+	+	+	−	−	+
A15	B12	7.5×10^8	−	−	−	−	−	−	+	+	−	−	−	−	−	−
A17	B14	3.4×10^8	−	−	−	−	−	−	+	+	−	+	+	−	−	−
A18	B15	1.1×10^5	−	−	−	−	+	−	−	+	−	−	+	−	−	−
A20	ATCC 11509	9.6×10^7	−	−	−	−	+	+	+	−	+	+	+	+	+	+

[a] Lysis was determined after 10 days of incubation at 7°C.

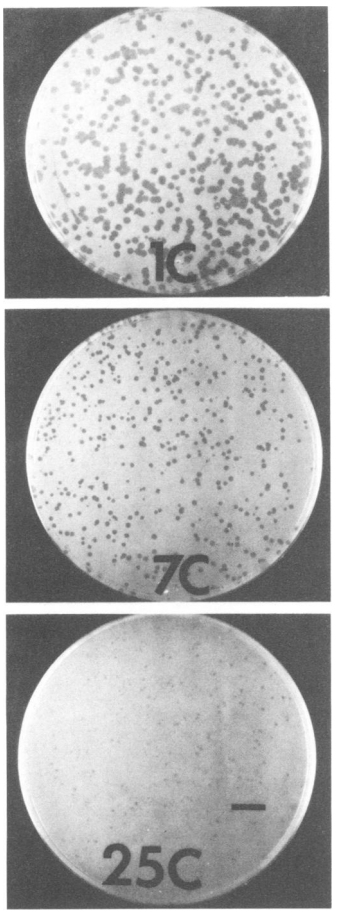

FIG. 1. Effect of temperature on *B. thermosphacta* phage A19 plaque size. Various temperatures are indicated. Bar = 10 mm.

M. lacticum, C. flavescens, L. plantarum, L. mali, L. denitrificans, or *L. grayii* at 25 or 30°C.

Effect of temperature on phage plaques. Throughout the course of this investigation, phage plaques produced at incubation temperatures of 25°C were considerably smaller than those produced during incubation at 7°C. This finding is substantiated in Fig. 1; the plaques produced by phage A19 on *B. thermosphacta* ATCC 11509 progressively decreased concomitantly with an increase in incubation temperature. Consequently, plaque diameters were significantly reduced ($P < 0.001$) as incubation temperatures were increased from 1°C (1.94 ± 0.10 mm) to 7°C (1.18 ± 0.08 mm) and 25°C (0.68 ± 0.05 mm). Bacterial growth was poor, and no plaques were observed after incubation at 30°C.

The efficiency of plating was high but not significantly different ($P < 0.05$) at incubation temperatures of 1°C (0.82 ± 0.06) and 7°C (0.96 ± 0.03). However, the efficiency of plating was significantly reduced ($P < 0.01$) when incubation was carried out at 25°C (0.24 + 0.06).

Phage morphology. The structures of *B. thermosphacta* ATCC 11509 phages MT and A19 are compared in the electron photomicrographs in Fig. 2a and 2b, respectively. Both phages appear to consist of hexagonal heads with tails. Terminal tail appendages are clearly visible on phage MT (Fig. 2a). The head diameters of phages MT (98.8 ± 3.3 nM) and A19 (93.6 ± 1.5 nM) were not significantly different ($P > 0.1$). However, phage MT had a significantly ($P < 0.05$) longer tail (183.6 ± 11.5 nM) than did phage A19 (116.4 ± 1.5 nM).

DISCUSSION

There are only a limited number of reports (9, 10, 27, 28) documenting psychrotrophic bacteriophage-host systems in refrigerated foods and even less information concerning phages in meats (27, 28). In this regard, Whitman and Marshall (27, 28) isolated psychrotrophic phages from ground beef and pork sausage, and, more recently, Greer (16) isolated and purified a number of psychrotrophic *Pseudomonas*-specific phages from spoiled beef steaks. The results of the present study have shown, for the first time, that psychrotrophic *B. thermosphacta* phages also exist in aerobically spoiled beef. A total of 21 virulent, psychrotrophic bacteriophages were isolated for 14 *B. thermosphacta* strains, including *B. thermosphacta* ATCC 11509.

The true psychrotrophy of the phages was demonstrated by their ability to lyse bacterial strains at temperatures as low as 1°C. Moreover, plaque size and plating efficiency increased as incubation temperatures reduced from 25 to 1°C. These findings are in accordance with those of other investigators, who reported an increase in plaque diameter for psychrotrophic *Pseudomonas* phages as incubation temperatures were decreased (10, 20, 21, 28).

Electron microscopic observations of phages MT and A19 isolated for *B. thermosphacta* ATCC 11509 showed the virions to consist of a hexagonal head and a tail; in the case of phage MT, the tail supported terminal appendages. On the basis of this morphology, however, it was not possible to differentiate between Bradley's (5) morphological group A or B.

In the present study, the culture and biochemical characteristics of the 15 wild-type *B. thermosphacta* strains were identical to those of the type strain, *B. thermosphacta* ATCC 11509. Strain differences, however, could be demonstrated on the basis of the patterns of susceptibility to phages. In this manner, the 15 wild-type strains and 3 previously identified strains of *B.*

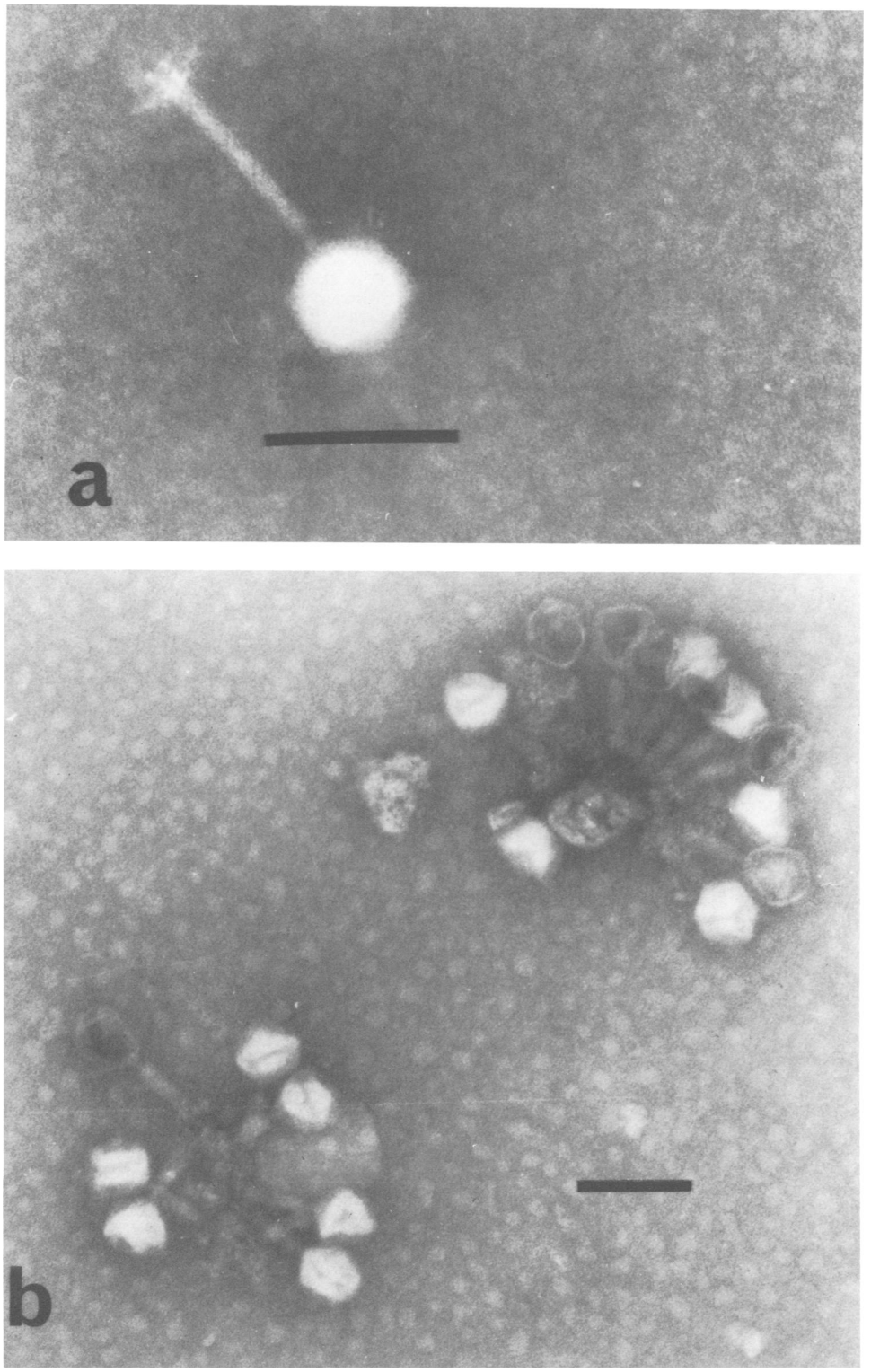

FIG. 2. Electron photomicrographs of *B. thermosphacta* phages MT (a) and A19 (b). Phages were negatively stained with phosphotungstic acid. Bar = 150 nM.

thermosphacta were found to constitute 14 distinct phage lysotypes. Thus, although classical culture and biochemical taxonomic data may not distinguish *B. thermosphacta* strains, phage typing would provide a rapid and sensitive means of detecting subtle differences among closely related strains. On the basis of phage typing, the population of *B. thermosphacta* associated with spoiled beef appears to be comprised of a large number of distinct strains.

It was of interest that *B. thermosphacta* ATCC 11509 isolated from pork sausage (19), C420 isolated from fresh beef (8), and BL262 isolated from vacuum-packaged beef (A. F. Egan, personal communication) were all susceptible to lysis by phage A20 (isolated from aerobically spoiled beef in the present study). This suggested that some *B. thermosphacta* strains isolated by independent investigators from different meats under distinct packaging conditions show some degree of relatedness.

In establishing the specificity of a phage typing scheme it becomes necessary to assess the phage sensitivities of related genera and species. However, *Brocothrix* is a new genus recently proposed for *B. thermosphacta* (25). Originally, this organism was placed in the genus *Microbacterium* (19), but when dissimilarities between this organism and members of the genus *Microbacterium* became evident (7, 14, 25) and when this organism was found to be more closely related to lactic acid bacteria and *Listeria* species (14, 25, 29), it was tentatively placed in the family *Lactobacillaceae* (25). The specificity of the *B. thermosphacta* phages was demonstrated in the present study by their lack of cross-reactivity with the *Microbacterium, Lactobacillus*, and *Listeria* species tested.

Although some investigators have contended that *B. thermosphacta* is restricted to the meat environment (18), Gardner (14) noted that this organism has been isolated from a variety of sources, including vegetables, dairy products, and fish, and stressed that the natural habitat remains to be established. Relative to this, a phage typing set such as that developed in the current study may provide a sensitive means of determining the source of *B. thermosphacta* contamination, of tracing the fate of specific strains during food processing, and of examining strain distribution during the spoilage of meats and other refrigerated foods.

It would also be of value to investigate the consequences of psychrotrophic bacteriophage-host interactions within the meat environment. Conceivably, bacteriophages could alter the ecological development of specific bacterial strains during refrigerated storage. This type of interaction may have a pronounced effect on the time course and characteristics of spoilage.

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