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Transduction in *Bacillus stearothermophilus*

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Temperate and virulent bacteriophages isolated from soil were shown to carry out generalized transduction of *Bacillus stearothermophilus* NUB36. A transducing frequency of 1×10^{-5} to 7×10^{-4} was obtained for temperate phages TP-42 and TP-56. The transducing frequency for virulent phage TP-68 was two to three orders of magnitude lower. Cotransfer analysis with the three phages showed that *hom-1* is linked to *thr-1* and that *gly-1* is linked to *his-1*.

An efficient protoplast fusion system was developed for *Bacillus stearothermophilus* NUB36 and used to construct isogenic strains and for chromosomal mapping (3). Protoplast fusion is a powerful tool for ordering markers that are not closely linked, but other systems are needed to identify linkage groups and for fine structure mapping. Transduction was the only option because this organism cannot be transformed with chromosomal DNA (3).

There are numerous reports on the isolation and characterization of virulent (1, 4, 6, 8, 10, 12, 14, 16, 22) and temperate (15, 21) bacteriophages that infect various strains of *B. stearothermophilus*. Six of these phages were available for this study, and only one, virulent phage TP-84 (14), formed plaques on NUB36. In preliminary studies, TP-84 was found to mediate the transduction of three auxotrophic markers of NUB36 at frequencies of 2×10^{-8} to 8×10^{-8}. These frequencies are much lower than those obtained with some phages active on mesophilic bacilli (2, 18, 20, 23, 24) and are not sufficient for use in genetic studies.

In this paper I report on the isolation of temperate and virulent phages that carry out generalized transduction of *B. stearothermophilus* NUB36.

Strains of NUB36 were grown in minimal glucose (MG) medium and LB medium (3). Solid LB and MG media and LB top agar contained 1.5 and 0.6% agar, respectively. Bacto-Agar (Difco Laboratories, Detroit, Mich.) was used in solid MG medium. MG medium was supplemented as appropriate with the required amino acids (20 μg/ml for L forms and 40 μg/ml for DL forms) and purines (10 μg/ml). Riboflavin and thiamine were used at a concentration of 5 μg/ml. Streptomycin was used at a concentration of 20 μg/ml, and chloramphenicol and rifampin were used at a concentration of 5 μg/ml. Growth was monitored by using a Klett-Summerson colorimeter with a no. 42 blue filter.

Cells from an LB plate (11 to 14 h at 60°C) were used to inoculate a 300-ml, triple-baffled shake flask containing 20 ml of LB medium. The culture was grown for 2 h at 60°C on a gyratory shaker (170 rpm). Cells (1×10^9 to 5×10^9 CFU/ml) from a 2-h culture were used for the assay and propagation of bacteriophages and for transduction.

Cell suspensions and dilutions were maintained at 60°C.

The bacterial strains used in this study are listed in Table 1, and the phenotype of each genetic marker is shown in Table 2.

Bacteriophages were assayed by a modification of the procedure described by Chen et al. (3). Calcium chloride (2 M) was added to a 2-h culture of the indicator strain to a concentration of 0.02 M, and 0.5 ml of this culture was mixed with 0.1 ml of phage in 1.5 ml of melted LB top agar. The mixture was maintained at 60°C. The final concentration of calcium was approximately 5 mM. The mixture was poured onto the surface of an LB plate that had been incubated for at least 2 h at 60°C followed by 30 min at room temperature. After the top agar had solidified, the surface of each plate was dried for 10 min at 60°C in a mechanical convection incubator with the cover ajar. The plates were incubated for 16 to 18 h at 50°C.

Phages were propagated on Spo^- cells in LB top agar. Cells and 1,500 to 2,000 PFU of phage were mixed in top agar and poured onto the surface of an LB plate. After incubation for 12 to 14 h at 50°C, top agar containing the phage lysate was suspended in LB medium (0.5 ml per plate), and the suspension was stirred for 1 to 2 h at 5°C. The cell debris and agar were removed by centrifugation at 12,000 × *g* for 20 min at 5°C, and the supernatant fluids were sterilized by filtration through 0.45-μm-pore membrane filters and stored at 5°C.

Temperate and virulent phages were isolated from soil by the following procedure. Samples of soil (3 g) were thoroughly suspended in 3 to 6 ml of LB medium and incubated at 80°C for 10 min. Suspended material was collected by low-speed centrifugation (1,800 × *g* to 1,900 × *g* for 5 min at room temperature). The supernatant fluids were used to inoculate (0.1 ml) a tube containing 5 ml of LB medium, and the culture was grown on a gyratory shaker at 60°C. When the culture was turbid (70 to 90 Klett units), mitomycin C was added to a final concentration of 0.5 μg/ml. Incubation was continued for 5 h or until cell lysis was observed (usually 2 to 3 h). Cells and debris were removed by centrifugation at 12,000 × *g* for 20 min at 5°C, and the supernatant fluids were sterilized by filtration as described above. The sterile lysates were examined for the presence of phages with NUB3621 as the indicator strain.

Cells from the center of a turbid plaque were transferred with a fine wire to an LB plate. After two single-colony isolations, the inferred lysogenic nature of each isolate was verified by the treatment of an exponential-phase culture with mitomycin C as described above. Temperate phages were isolated from culture lysates by two single-plaque isolations.

Virulent phages were isolated from the center of a clear plaque, suspended in 1 ml of LB medium, and purified by two single-plaque isolations. Clear-plaque mutants of temperate phages were detected among the turbid plaques when large numbers of temperate phages were plated with NUB36106. Clear-plaque mutants were isolated by two single-plaque isolations.

TABLE 1. *B. stearothermophilus* strains and phages

Strain or phage	Characteristics[a]	Origin or reference
Strain		
NUB36	Wild type	3
NUB361	*thr-1*	3
NUB363	*his-1*	3
NUB364	*hom-1 thi-1*	MNNG of NUB3625
NUB369	*rib-1*	MNNG of NUB36
NUB3611	*asp-1*	MNNG of NUB36
NUB3613	*ilv-1*	MNNG of NUB36
NUB3618	*rif-1*	3
NUB3619	*rif-1 cml-1*	3
NUB3620	Hsr^- Hsm^-	3
NUB3621	Hsr^- Hsm^- Rif^r	3
NUB3625	*hom-1*	3
NUB3645	*rif-1 cml-1 str-1*	Spontaneous mutant of NUB3619
NUB3686	*pur-2 rif-1*	3
NUB3687	*gly-1 rif-1*	3
NUB36102	*rif-1 pur-3*	MNNG of NUB3618
NUB36106	Rif^r Hsr^- Hsm^- Spo^-	Spontaneous mutant of NUB3621
NUB36109	*rif-1 arg-5*	MNNG of NUB3618
NUB36110	*rif-1 gua-4*	MNNG of NUB3618
NUB36141	*thr-1* Spo^-	Spontaneous mutant of NUB361
NUB36143	*pur-2 rif-1* Spo^-	Spontaneous mutant of NUB3686
NUB36144	*gly-1 rif-1* Spo^-	Spontaneous mutant of NUB3687
NUB36155	*rif-1 pur-1* Spo^-	Spontaneous mutant of NUB36102
NUB36157	*his-1* Spo^-	Spontaneous mutant of NUB363
NUB36158	*hom-1* Spo^-	Spontaneous mutant of NUB3625
NUB36167	Spo^-	Spontaneous mutant of NUB36
NUB36169	Hsr^- Hsm^- Spo^-	Spontaneous mutant of NUB3620
NUB36178	*rif-1 cml-1 str-1* Spo^-	Spontaneous mutant of NUB3645
NUB583[b]	Hsr^- Hsm^- Spo^-	New designation for $4SR^-22M^-22$
Phages		
TP-42	Temperate phage	Soil
TP-42C	Clear-plaque mutant	Spontaneous mutant of TP-42
TP-56	Temperate phage	Soil
TP-68	Virulent phage	Soil
TP-84	Virulent phage	14

[a] Abbreviations: Rif^r, rifampin resistance; Hsr, host-specific restriction; Hsm, host-specific modification; Spo^-, cells do not form spores when grown on LB medium at 60°C.

[b] During this study we observed that strain $4SR^-22M^-22$ (NUB583) did not form spores on LB medium. Since this culture has been preserved in LB medium containing 10% glycerol at −85°C since 1979, we assume that the original culture described by Lees and Welker (9) was asporogenic.

The phages used in transduction experiments are listed in Table 1.

Mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and the isolation of auxotrophic mutants were carried out as described by Chen et al. (3). Antibiotic-resistant mutants were isolated by plating concentrated exponential-phase cells on LB plates containing the appropriate antibiotic. Spo^- mutants were isolated by plating exponential-phase cultures to obtain isolated colonies on LB plates. After incubation for 12 to 15 h at 60°C, the plates were examined with the aid of a stereomicroscope. Colonies with a texture or color different from that of the parental colony were picked with a fine wire and purified by two single-colony isolations. The absence of spores in cultures grown on LB plates for 24 h at 60°C was verified with the aid of a phase-contrast microscope.

For transduction, cells from a 2-h culture were used to inoculate a 300-ml, triple-baffled nephelometer flask containing 20 ml of LB medium to a cell density of 2×10^7 to 3×10^7 CFU/ml. The culture was grown on a gyratory shaker (170 rpm) at 60°C to a cell density of 5×10^8 to 8×10^8 CFU/ml. Cells from 5 ml of culture were collected by centrifugation at 1,800 × *g* to 1,900 × *g* for 5 min at room temperature and

TABLE 2. Genetic markers in *B. stearothermophilus*

Marker	Phenotype[a]
arg-5	Arginine requirement
asp-1	Aspartate requirement
cml-1	Resistance to chloramphenicol (5 μg/ml)
gly-1	Glycine or serine requirement
gua-4	Guanine requirement
his-1	Histidine requirement
hom-1	Homoserine or threonine and methionine requirement
ilv-1	Isoleucine and valine requirement
pur-2	Adenine requirement
pur-1, *pur-3*	Hypoxanthine or guanine requirement
rib-1	Riboflavin requirement
rif-1	Resistance to rifampin (10 μg/ml)
str-1	Resistance to streptomycin (100 μg/ml)
thi-1	Thiamine requirement
thr-1	Threonine requirement

[a] The highest concentration of antibiotic in LB plates that did not inhibit growth after 13 to 16 h at 60°C is in parentheses.

TABLE 3. Transduction of *B. stearothermophilus* NUB36 with phages TP-42, TP-42C, TP-56, and TP-68[a]

Relevant genotype or phenotype of recipient[b]	Transduction frequency with phage[c]:			
	TP-42	TP-42C	TP-56	TP-68
arg-5	2.3×10^{-5}	1.0×10^{-5}	6.5×10^{-4}	3.4×10^{-7}
asp-1	4.1×10^{-4}			
Cml^s	3.9×10^{-5}			
gly-1	4.1×10^{-5}	3.7×10^{-5}	2.6×10^{-5}	
gua-4	1.1×10^{-5}			
his-1	4.3×10^{-5}			
hom-1	3.9×10^{-4}	1.7×10^{-4}	2.7×10^{-4}	1.0×10^{-7}
ilv-1	3.7×10^{-4}			
pur-2	8.7×10^{-5}	4.9×10^{-5}	5.7×10^{-5}	
pur-3	5.2×10^{-5}			
rib-1	3.0×10^{-4}	4.7×10^{-4}	5.2×10^{-4}	5.3×10^{-7}
Rif^s	6.4×10^{-5}	5.8×10^{-5}	2.3×10^{-5}	
Str^s	6.7×10^{-5}			
thi-1	4.0×10^{-5}			
thr-1	4.9×10^{-4}	5.1×10^{-4}	2.3×10^{-4}	2.5×10^{-7}

[a] For transduction of auxotrophic markers, the donor strain was NUB36167 and the recipient strains were those listed in Table 1. For transduction of antibiotic resistance markers, NUB36178 was the donor strain and NUB3620 was the recipient strain. Phages TP-42 and TP-56 are temperate phages, phage TP-42 is a clear-plaque mutant of phage TP-42, and phage TP-68 is a virulent phage.

[b] Abbreviations: Cml, chloramphenicol; Rif, rifampin; Str, streptomycin.

[c] Expressed as the quotient of the number of transductants per plate divided by the number of input PFU per plate.

suspended in 5 ml of fresh LB medium. Transduction was accomplished by mixing equal volumes of cells and phages (10^7 to 10^8 PFU/ml) at a multiplicity of infection of 0.05 to 0.5. The transduction mixture was maintained at 60°C. Samples (0.1 ml) were plated in quadruplicate on appropriately supplemented MG plates that were equilibrated at 60°C. Each sample was evenly applied to the outer edge of the plate and spread over the entire surface of the plate, starting at the outer edge and moving towards the center. The surface of each plate was dried for 10 min at 60°C in a mechanical convection incubator with the cover ajar. For the transduction of auxotrophic markers, the plates were incubated for 20 to 24 h at 65°C. Transductant colonies were transferred to the same medium with sterile toothpicks to form master plates. After incubation at 60°C, the master plates were velveteen replicated onto appropriate selective media to score the unselected phenotypes. For transduction of antibiotic resistance markers, the plates were incubated for 8 h at 65°C. Each plate was velveteen replicated onto LB medium containing the appropriate antibiotic, and incubation at 65°C was continued for 12 to 16 h. Plates were equilibrated and dried at 60°C as described above.

All of the temperate phages and approximately 30% of the virulent phages isolated from soil were shown to carry out generalized transduction. Two temperate phages and one virulent phage were selected for these studies.

Transduction frequencies for temperate phages TP-42 and TP-56 ranged from 1.1×10^{-5} to 6.5×10^{-4}/PFU for 12 auxotrophic markers (6 for phage TP-56) and 3 antibiotic resistance markers (1 for phage TP-56) (Table 3). The transduction frequencies for phage TP-42C, a clear-plaque mutant of phage TP-42, were similar to those obtained with phage TP-42 for the seven markers tested. The frequencies of transduction for the temperate phages were similar to or higher than the frequencies obtained for transducing phages of mesophilic bacilli (2, 17, 18, 20, 23). It was not necessary to inactivate PFU with UV irradiation to recover transductants or to achieve the relatively high transduction frequencies observed with these phages. Virulent phage TP-68 also carried out generalized transduction; however, the frequency for four auxotrophic markers was two to three orders of magnitude lower than that observed with the temperate phages. The highest frequency of transduction with the temperate phages was observed with Spo$^+$ strains at a multiplicity of infection of 0.05 to 0.5. At multiplicities of infection of 1.0, 2.5, and 14, the frequencies of transduction were reduced 2.5-, 6-, and 33-fold, respectively. These results indicate that excess phages in the transduction mixture inhibit the recovery of transductants.

TABLE 4. Cotransduction of markers in the region from *hom-1* to *pur-2* in the *B. stearothermophilus* chromosome[a]

Donor genotype	Recipient genotype	Cotransduction (%)[b] with phage:		
		TP-42	TP-56	TP-68
hom-1	*thr-1*	475/1,321 (36)	171/348 (49)	95/178 (53)
thr-1	*hom-1*	422/1,299 (33)	144/307 (47)	159/294 (54)
gly-1	*his-1*	0/487 (0)	14/375 (4)	37/245 (15)
his-1	*gly-1*	0/440 (0)	29/417 (7)	43/250 (17)
hom-1	*gly-1*	0/397 (0)	0/395 (0)	0/187 (0)
thr-1	*gly-1*	0/429 (0)	0/275 (0)	0/217 (0)
gly-1	*pur-3*	0/397 (0)	0/390 (0)	0/145 (0)
his-1	*pur-3*	0/276 (0)	0/270 (0)	0/150 (0)
pur-3, *pur-1*	*pur-2*	0/450 (0)	0/265 (0)	0/176 (0)

[a] Donor strains were Spo$^-$, and recipient strains were Spo$^+$.

[b] Fractions represent number of transductants carrying the unselected marker/total number of transductants tested. Values in parentheses represent the percent cotransduction, obtained by multiplying the fraction by 100.

Chen et al. (3) used protoplast fusion for chromosomal mapping of *B. stearothermophilus* NUB36. Six markers in the region from *hom-1* to *pur-2* were mapped, and the order was *hom-1 thr-1 his-1 gly-1* (or *gly-2*) *pur-1 pur-2*. Representative strains were used to verify the relative linkages in this region by transduction with phages TP-42, TP-56, and TP-68. Two-factor transduction crosses mediated by the three phages in the region from *hom-1* to *pur-2* are shown in Table 4. Linkage was observed only between *hom-1* and *thr-1* and between *his-1* and *gly-1*. The cotransfer frequencies of *hom-1* and *thr-1* by phages TP-42, TP-56, and TP-68 were 33 to 36%, 47 to 49%, and 53 to 54%, respectively. The cotransfer frequencies between *gly-1* and *his-1* by phages TP-56 and TP-68 were 4 to 7% and 15 to 17%, respectively. No transfer of these two markers was observed with phage TP-42. Cotransduction values for the markers did not significantly vary with the direction of the cross.

Comparison of the cotransduction values for *hom-1* and *thr-1* and for *gly-1* and *his-1* indicates that the three phages may carry different-sized segments of the chromosome. The different cotransduction values may be related to differences in the size of the phage heads and the molecular weight of the phage DNA. Restriction analyses were used to estimate the molecular weight of the DNA of each phage. Phage DNA was isolated by the procedure described by Lees and Welker (9). DNA (1.5 μg) was digested with *Bgl*II and *Xho*I restriction endonucleases under the conditions recommended by the supplier (International Biotechnologies, Inc., New Haven, Conn.). Digests were analyzed by standard agarose gel electrophoresis with 1% gels. The molecular weights of the DNA fragments were estimated from the mobility of the fragments relative to those of fragments of *Hin*dIII digests of lambda DNA. The molecular weights of the DNAs from phages TP-42, TP-56, and TP-68 were estimated to be 17.9×10^6, 19.7×10^6, and 26.1×10^6, respectively, based on the

TABLE 5. Effect of restriction and modification on transduction[a]

Multiplicity of infection	Transduction frequency with[b]: Modified phage	Transduction frequency with[b]: Nonmodified phage	Efficiency of transduction[c]
1.30	2.9×10^{-4}	4.7×10^{-5}	0.16
0.65	5.5×10^{-4}	9.4×10^{-5}	0.17
0.32	6.4×10^{-4}	1.3×10^{-4}	0.20

[a] Transduction experiments were carried out with phage TP-42 and NUB361 *thr-1* Hsr^+ Hsm^+ as the recipient strain.

[b] See Table 3, footnote *c*. Modified phage, Phage propagated on NUB36167 Hsr^+ Hsm^+ Spo^-; nonmodified phage, phage propagated on NUB36169 Hsr^- Hsm^- Spo^-.

[c] Expressed as the quotient of the transduction frequency with nonmodified phage divided by the transduction frequency with modified phage.

addition of the linear DNA fragments after digestion with either *Bgl*II or *Xho*I or both.

Adding the molecular weights of the fragments produced by digestion of virulent phage TP-84 (14) DNA with *Bgl*II yielded a value of 22.2×10^6 for the molecular weight of the TP-84 genome. This value is close to the molecular weight of TP-84 DNA determined from sedimentation (22.6×10^6) and intrinsic viscosity (22.4×10^6) measurements (13). These results indicate that the molecular weight of phage DNA estimated from restriction analyses is in good agreement with the molecular weight determined by physical methods.

The estimated molecular weights of the three phage genomes were lower than those reported for the genomes of phages active on mesophilic bacilli. Large mesophilic bacillus phages TP-13 (250×10^6 [11]), SP-15 (250×10^6 [19]), PBS-1 (190×10^6 [7]), SP-10 (59×10^6 [5]), and CP-51 (56×10^6 [24]) have been useful for scanning large segments of the chromosome.

*Bsm*I-modified phage TP-42 and nonmodified phage TP-42 were used to transduce NUB361, a restriction-proficient auxotroph, to prototrophy. The results (Table 5) demonstrate that 80 to 84% of the transductants were not recovered when the transducing phage was propagated on a modification-deficient donor strain. Restriction and modification also had an effect on the recovery of fusion recombinants from protoplast fusion experiments with this strain (3).

The availability of phages that transduce chromosomal DNA fragments that cover a wide range of sizes will make it possible to identify linkage groups and to determine the order of closely linked markers. Combined with protoplast fusion, this method of genetic analysis will be used to construct isogenic mutant strains and map chromosomal markers in *B. stearothermophilus*.

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