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Genome Organization of Spβ *c*2 Bacteriophage Carrying the *thyP3* Gene

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Thymine auxotrophs of *Bacillus subtilis* strains lysogenic for temperate bacteriophage SPβ *c*2 were transformed to prototrophy by DNA from related phage φ3T. During transformation, the φ3T-encoded thymidylate synthetase gene, *thyP3*, became integrated into the extreme right end of the SPβ *c*2 prophage near the bacterial *citK* gene. Upon heat induction, the transformed *B. subtilis* cells released SPβ *c*2T phages that could lysogenize thymine auxotrophs and convert them to prototrophy. Comparison of restriction endonuclease fragments of DNAs from SPβ *c*2 and SPβ *c*2T phages revealed that the latter contained a large region of deletion and substitution near the center of the chromosome. This region included the phage attachment site on the SPβ *c*2 genome.

The genome of *Bacillus subtilis* 168 contains two structural genes, *thyA* and *thyB*, for thymidylate synthetases (8). Thymine auxotrophy in this bacterium results when mutations occur in both of these genes. If such thymine auxotrophs are lysogenized by the temperate bacteriophage φ3T, they are converted to prototrophy (14). This observation is explained by the fact that the phage DNA also contains a thymidylate synthetase gene (the *thyP3* gene), and the latter is expressed even in the prophage state.

More surprisingly, *B. subtilis thyA thyB* mutants can be transformed to thymine prototrophy by intact or fragmented DNA isolated from φ3T. Studies by Stroynowski (13) demonstrate that the genome of *B. subtilis* contains two regions of homology which may undergo recombination with transforming DNA containing the *thyP3* gene. First, the bacterial *thyA* gene has considerable sequence homology with the viral gene. When φ3T DNA that has been fragmented by restriction endonucleases is used for transformation, the *thyP3* gene occasionally becomes inserted at the position of the *thyA* gene. Second, if thymine auxotrophs are transformed with intact φ3T DNA, the *thyP3* gene is usually incorporated into the prophage of another closely related bacteriophage, SPβ, which is present in most strains derived from *B. subtilis* 168.

Despite similarities to φ3T, SPβ does not possess an active thymidylate synthetase gene. However, when the *thyP3* gene of the former becomes inserted after transformation, the substituted SPβ prophage is usually still capable of producing viable phage particles upon induction (13). The latter, which we have designated as SPβ T phages, are capable of lysogenizing other thymine auxotrophs of *B. subtilis* and subsequently converting these bacteria to prototrophy. Recently, restriction fragment maps of SPβ *c*2, a heat-inducible mutant of SPβ, have been prepared (4), and these have allowed us to determine more precisely where the *thyP3* gene becomes inserted into the SPβ genome during transformation. In the present paper, we report the results of mapping studies and also describe some of the unusual properties of hybrid phages containing the *thyP3* gene.

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MATERIALS AND METHODS

Bacterial and phage strains. The bacteria used were derived from *B. subtilis* 168 (Table 1). Bacteriophage SPβ *c*2 has been described previously (10). SPβ *c*2::Tn*917* was obtained from Philip Youngman. φ3T was provided by Donald Dean. Bacteriophage φ1m, a mutant of φ1, was isolated in this laboratory (9).

Growth conditions. The liquid medium used for routine growth of *B. subtilis* was M broth (17) without added $CaCl_2$. Plaque assays and subculturing were done on the same medium with agar added to 1.5%. For growth of thymine auxotrophs, both media were supplemented with 25 μg of thymidine per ml. Selection of transformants was carried out on synthetic medium (1) supplemented with 20 μg of tryptophan per ml and 0.1% Casamino Acids (Difco Laboratories). This same medium with the addition of 25 μg each of thymine and thymidine per ml plus 10 μg of trimethoprim (Calbiochem-Behring) per ml were used in the isolation of thymine auxotrophs. Synthetic medium without Casamino Acids and with 0.4% sodium glutamate substituted for glucose was used in the detection of bacteria lacking alpha-ketoglutarate dehydrogenase (mutants in the *citK* gene). Media used in transformation are described below.

All incubations were at 37°C unless otherwise noted. Small volumes of liquid cultures were aerated in tubes (16 by 125 mm) on a rotary shaker. Large-volume cultures were grown in 2,800-ml Fernbach flasks on a rotary shaker.

Spores were prepared by growing bacteria for 3 days on M agar supplemented with 25 μg of thymidine per ml. Spores were scraped from plates, suspended in 0.5% NaCl, and treated with lysozyme (100 μg/ml) for 1 h at 37°C. The preparation was then washed three times with distilled water, and spores were stored frozen until used. Before plating, spores were diluted in distilled water and heated at 65°C for 30 min.

Selection of thymine auxotrophs. Mutants of *B. subtilis* that required thymine were isolated by the procedure of Neuhard et al. (8) with trimethoprim-containing medium (12).

Isolation and purification of phage. SPβ *c*2 or φ3T was isolated by mitomycin C induction of strain CU1147 or NR1004 as described by Warner et al. (15). The cultures

TABLE 1. Bacterial strains used

Strain	Genotype	Origin/Reference
CU1050	*sup-3 metB5 thr leu ade* SPβ^s	20
CU1065	*trpC2* SPβ^s	20
CU1147	*sup-3 metB5 thr leu ade* (SPβ *c2*)	SPβ *c2* infection of CU1050
CU2332	*trpC2 thyB5 thyA5 ilvA2* (SPβ *c2 pilvA10*)	SPβ *c2 pilvA10* infection of strain CU1430 (3)
NR1000	*trpC2 thyA25 thyB25* SPβ^s	Trimethoprim selection of CU1065 (8)
NR1001	*trpC2 thyA25 thyB25* (SPβ *c2*)	SPβ *c2* infection of NR1000
NR1002	*trpC2 thyA25 thyB25* (SPβ *c2T-1*)	Transformation of NR1001 with φ3T DNA
NR1003	*trpC2 thyA25 thyB25* (SPβ *c2T-2*)	Transformation of NR1001 with φ3T DNA
NR1004	*trpC2 thyA25 thyB25* φ3T	φ3T infection of NR1000
NR1010	*trpC2 thyA25 thyB25* (SPβ *c2Ta*)	SPβ *c2Ta* infection of NR1000
NR1011	*trpC2 thyA25 thyB25* (SPβ *c2Tz*)	SPβ *c2Tz* infection of NR1000

Bacteriophage SPβ c2Ta and SPβ c2Tz were isolated from individual plaques obtained from lysates of, respectively, strains NR1002 and NR1003, which had been plated on M agar with *B. subtilis* CU1050 as the indicator strain. These phages were cultivated lytically on strain CU1050 but were purified from lysates by the procedure described above.

Isolation of DNAs. DNA from *B. subtilis* was isolated and purified by the method of Marmur (7). Bacteriophage DNA was obtained by phenol extraction as described in Warner et al. (15). The aqueous phase from the phenol extraction was layered with two volumes of 95% ethanol, and the DNA was precipitated by mixing the two phases with a glass rod. The DNA was redissolved in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (7), and the solution was subsequently adjusted to 0.5× SSC for storage.

Plasmid pGS101 and pGS102 DNAs were isolated from *Escherichia coli* cells as described (6). Plasmid pCJM622 DNA was a gift from C. J. Mackey in the laboratory of S. A. Zahler.

Transformation of thymine auxotrophs of *B. subtilis*. Conditions for transformation were approximately those of Anagnostopoulos and Spizizen (1). The bacteria to be transformed were grown overnight on M agar supplemented with 25 μg of thymidine per ml and transferred to synthetic medium (1) supplemented with 0.5% glucose, 5 mM $MgSO_4$, 0.01% Casamino Acids and 50 μg of tryptophan and 200 μg of thymidine per ml. The inoculum contained at least 10^8 cells per ml, and 1 ml of the cell suspension was placed in a tube (16 by 125 mm). The culture was incubated with shaking for 5 h at 37°C. The cells were then diluted 10fold into synthetic medium supplemented with 0.5% glucose, 5 mM $MgSO_4$, 0.01% Casamino Acids, and, with the dilution of nutrients from the original growth medium, about 5 μg of tryptophan and 20 μg of thymidine per ml. To 0.45 ml of the diluted culture, 0.05 ml of DNA solution was added, and incubation with shaking was continued for 1 h at 37°C. The transformed cultures were then diluted and plated on appropriate selective media.

Construction of chimeric plasmids containing the *thyP3* gene. Bacteriophage φ3T DNA was digested with restriction endonuclease *Eco*RI, and the DNA fragments were ligated into the *Eco*RI site of plasmid pBR322. A thymine auxotroph of *E. coli* HB101 was made competent (6) and transformed with the chimeric plasmids. Clones containing plasmids which had incorporated the *thyP3* gene were selected for thymine independence and ampicillin resistance on minimal medium supplemented with 20 μg of tryptophan per ml, 0.1% Casamino Acids, and 100 μg of ampicillin per ml. The plasmid obtained by this procedure (pGS101) was isolated and digested with restriction nuclease *Eco*RI and found to contain a 7.4 kilobase pair (kb) insertion.

A second plasmid (pGS102) containing the *thyP3* gene on a smaller DNA fragment was also constructed. To do this, the 7.4-kb insert from pGS101 was digested with *Eco*RI under conditions which produce *Eco*RI* fragments (I. T. Stroynowski, Ph.D thesis, Stanford University, Stanford, Calif., 1980). These fragments were ligated into the *Eco*RI site of pBR322, and bacterial clones containing chimeric plasmids incorporating *thyP3* were selected as described above. Plasmid pGS102 obtained from this procedure contained a 1.3-kb insertion.

Restriction enzyme cleavage and electrophoresis. DNA digestions were performed according to methods outlined by the endonuclease supplier (Bethesda Research Laboratories). After overnight digestion at 37°C, equal volumes of loading buffer (40 mM Tris, pH 7.8, 1 mM disodium EDTA, 5 mM sodium acetate, 0.08% bromphenol blue, 1.0% sodium dodecyl sulfate, 2.0% Ficoll) were added to the reaction mixtures. Twenty-microliter samples were applied to a 0.7 or 1.4% agarose slab gel. Electrophoresis in a running buffer of 40 mM Tris (pH 7.8)–1 mM disodium EDTA–5 mM sodium acetate was carried out for 18 to 24 h at 1.5 V/cm. The gels were stained for 20 min in 0.4 μg of ethidium bromide per ml and examined on a UV transilluminator (Spectroline). The fluorescent bands were photographed by using a red filter and Polaroid type 57 film. Molecular sizes were determined by comparison with known fragments of SPβ *c2* and bacteriophage lambda DNA cleaved with *Sal*I, *Pst*I, and *Sac*I (2).

DNA-DNA Southern hybridizations. Fragments of endonuclease-digested DNA that were separated by electrophoresis in agarose gels were denatured and transferred onto nitrocellulose filters as described by Southern (11). The filters were dried for 2 h under vacuum at 80°C. To reduce nonspecific binding of DNA during hybridization, the filters were soaked in 3× SSC for 1 h and in hybridization buffer containing 50% formamide, 10 mM 1,4 piperazinediethanesulfonic acid (pH 6.4), and 0.006% each of polyvinylpyrrolidone, bovine serum albumin and Ficoll for 2 h at 50°C. Plasmid DNAs to be used as probes were labeled with ^{32}P by nick translation as described by the vendor of the reagents (Bethesda Research Laboratories). The radioactive plasmids were heat-denatured in hybridization buffer supplemented with 0.2% sodium dodecyl sulfate and hybridized to the DNA on the nitrocellulose filters at 50°C for 36 h. The filters were washed in two changes of SSC followed by two changes of a 2 mM EDTA solution. After washing, the filters were dried and autoradiographed with Kodak X-Omat AR film and Dupont Cronex Hi-plus intensifying screens.

RESULTS

Transformation of SPβ *c2* lysogens with φ3T DNA. Table 2 presents data on the frequency with which thymine auxotrophs of *B. subtilis* were transformed to prototrophy by bacterial and phage DNAs. Strain NR1000, a thymine auxotroph which is not lysogenic for SPβ, was readily trans-

TABLE 2. Transformation of strains NR1000 and NR1001 to thymine prototrophy[a]

Strain transformed	Source of transforming DNA	Transformants per ml
NR1000	*B. subtilis* CU1065	6,840
NR1000	φ3T	60
NR1001	*B. subtilis* CU1065	2,100
NR1001	φ3T	9,750

[a] Transformations were carried out as described in the text. φ3T DNA (17 μg) or *B. subtilis* CU1065 DNA (16 μg) was added to a 0.5-ml culture (about 10^9 CFU/ml) of competent bacteria.

formed by DNA from prototrophic *B. subtilis* CU1065 but was only rarely transformed by φ3T DNA. However, when NR1000 was lysogenized with SPβ *c*2, a temperature-inducible mutant of SPβ, the resulting strain NR1001 was transformed by viral and bacterial DNAs at comparable frequencies.

Several lines of evidence suggest that transformation of strain NR1001 involved a recombination event in which the *thyP3* gene of φ3T became incorporated into the prophage of SPβ *c*2. (i) Upon heat induction, the bacteria transformed with φ3T DNA released viable phage particles (which we call SPβ *c*2T phages). When the latter were used to create new lysogens of strain NR1000, they converted this thymine auxotroph to prototrophy. SPβ *c*2T virions were also released spontaneously from transformed lysogens. (ii) When SPβ *c*2 lysogens transformed by φ3T DNA were grown at 49°C, they lost the prophage and were simultaneously converted to thymine auxotrophy. (iii) DNA isolated from SPβ *c*2T phages transformed NR1001 cells to thymine prototrophy at high frequency.

Transformation of SPβ *c*2 lysogens did not involve incorporation of the entire φ3T genome to produce double lysogens. The transformed bacteria continued to demonstrate immunity to superinfection by SPβ *c*2 but were not immune to φ3T. Moreover, *B. subtilis* thymine auxotrophs lysogenic for SPβ *c*2::Tn*917*, a prophage carrying a transposon conferring resistance to macrolide antibiotics (19), were also transformed by φ3T DNA. The phage particles released from these transformants simultaneously transduced strain NR1000 to thymine prototrophy and to antibiotic resistance.

Position of the *thyP3* insertion in the SPβ *c*2 prophage. Figure 1 presents a restriction fragment map of SPβ *c*2 aligned in the prophage configuration. This map provided a guide in approximating the position at which the *thyP3* gene of φ3T became incorporated into the genome of SPβ *c*2.

We attempted to determine whether there was homology between the *thyP3* gene and the SPβ *c*2 genome. To do this, SPβ *c*2 DNA was digested with restriction endonuclease *Sal*I, the restriction fragments were separated by electrophoresis, and the latter were transferred to nitrocellulose paper for a Southern DNA-DNA hybridization experiment. Plasmids pGS101 and pGS102, which contain, respectively, 7.4- and 1.3-kb inserts of φ3T DNA inclusive of the *thyP3* gene, were radioactively labeled by nick translation and hybridized to the DNA on the filter paper. Plasmid pGS102 showed no homology to SPβ *c*2 DNA, indicating that SPβ *c*2 contained no DNA sequences closely matching those of the *thyP3* gene. On the other hand, pGS101, which contains *thyP3* in a larger DNA fragment, hybridized to *Sal*I fragment E. This suggests that there was homology between regions of the φ3T genome flanking the *thyP3* gene and the right end of the SPβ *c*2 prophage (Fig. 1).

We next digested DNA from *B. subtilis* strains NR1002 and NR1003, both of which are SPβ *c*2T lysogens, with *Sal*I. The resulting restriction fragments were separated by electrophoresis and transferred to nitrocellulose paper. The transfers were hybridized to radioactively labeled DNA from SPβ *c*2 (Fig. 2). The results established that *Sal*I fragments F&A (which is formed by the joining of the two ends of the phage chromosome), B, C, and D were present in the SPβ *c*2T lysogens. However, *Sal*I fragment E was not found, although a novel fragment of about 16.3 kb which did not occur in digests of SPβ *c*2 lysogens was present. In a separate experiment, transfers of *Sal*I digests of DNA from strains CU1147 (an SPβ *c*2 lysogen), NR1002, and NR1003 were also hybridized to radioactively labeled DNA from plasmid pCJM622. The latter contains *Sal*I fragment G of SPβ *c*2 DNA, a sequence known to include the phage attachment site of SPβ. The plasmid hybridized to an apparently identical 6.7-kb fragment in the digests of the three lysogens (data not shown). This fragment is known from other studies to include the left prophage junction *Sal*I-G'' (P. S. Fink and S. A. Zahler, manuscript in preparation). Together these results indicate that the left end of the

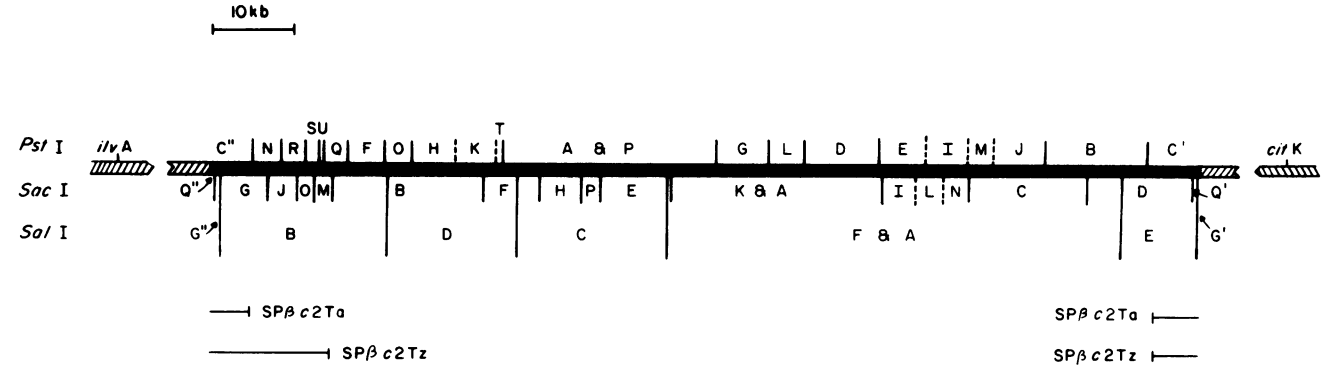

FIG. 1. Restriction endonuclease fragment map of the SPβ *c*2 genome arranged in the prophage configuration. Sites of cleavage by enzyme *Pst*I are shown by the vertical lines above the solid bar; sites for *Sac*I and *Sal*I cleavage are shown by short and longer vertical lines below the bar, respectively. Dashed lines indicate the boundaries of fragments whose order is not unequivocally known. The fragments are lettered in order of decreasing size. C', C'', Q', Q'', G', and G'' are parts of the C, Q, and G fragments from the virion DNA separated when in the prophage form. The fragments designated A&P, K&A, and F&A result from joining of the ends of the vegetative chromosome before integration of the prophage.

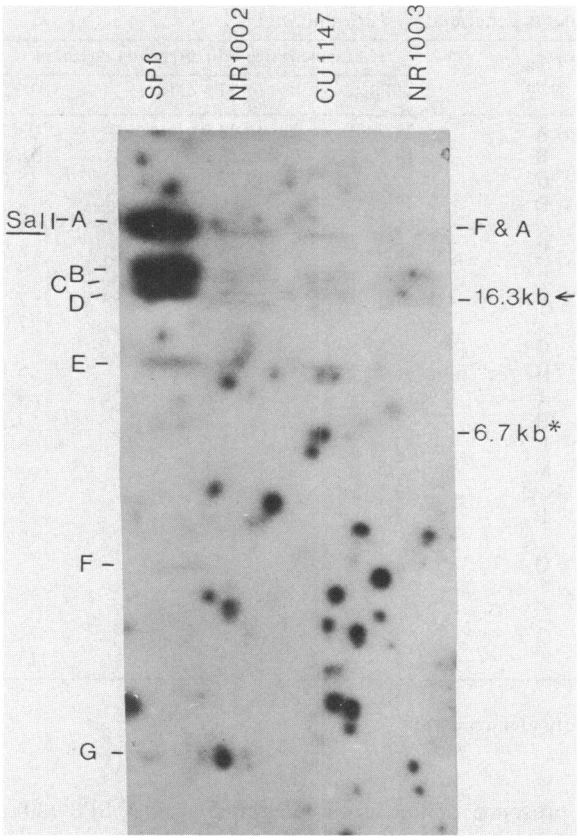

FIG. 2. Autoradiogram of ^{32}P-labeled SPβ c2 DNA hybridized to *Sal*I digests of phage and bacterial DNAs. The first lane shows the position of seven *Sal*I fragments generated from SPβ c2 DNA. Lanes designated NR1002 and NR1003 each contained 1.5 μg of digested DNA from SPβ c2T lysogens. The lane marked CU1147 was a control which included 1.5 μg of DNA from an SPβ c2 lysogen. The 16.3-kb fragment (arrow) also hybridized to the *thyP3*-containing plasmid pGS102. The position of a 6.7-kb fragment which hybridized to plasmid pCJM622, which contains the phage attachment site region, is indicated with an asterisk.

prophage and all regions up to at least the left boundary of *Sal*I fragment E were unchanged by the insertion of the *thyP3* gene into the SPβ c2 prophage. The incorporation of ϕ3T-derived DNA sequences after transformation affected only the *Sal*I E fragment and possibly *Sal*I-G'. Finally, to directly determine the presence of the *thyP3* gene in *Sal*I digests of SPβ c2T lysogens NR1002 and NR1003, Southern DNA transfers were hybridized with radioactively labeled pGS102 DNA. The latter showed homology to a 16.3-kb *Sal*I fragment which appeared to be identical to the novel *Sal*I fragment of the same size that hybridized to SPβ c2 DNA.

Additional evidence that the ϕ3T DNA containing the *thyP3* gene underwent recombination with the *Sal*I-E region of the SPβ c2 chromosome comes from studies with *B. subtilis* CU2332. This strain is a thymine auxotroph which is lysogenic for SPβ c2 p*ilvA*10, a specialized transducing phage which contains a deletion of most of *Sal*I fragment E (3; P. S. Fink and S. A. Zahler, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, H32, p. 111). CU2332 was transformed to thymine prototrophy when *B. subtilis* CU1065 DNA was used as a source of transforming DNA. However, CU2332 was transformed with ϕ3T DNA no better than was the nonlysogenic strain NR1000. Thus, the right end of SPβ c2 prophage apparently must be present for successful transformation with intact ϕ3T DNA.

Finally, mutants of SPβ c2T lysogen *B. subtilis* NR1003 which reverted to auxotrophy were isolated. Although these variants still released SPβ-type phage, they appeared to contain a deletion of the right end of the prophage which extended past the bacterial *citK* gene. This suggests that loss of this region of the SPβ c2T prophage correlated with loss of the thymidylate synthetase gene.

Position of the *thyP3* gene in the genome of SPβ c2T phages. SPβ c2 lysogens which had been transformed with ϕ3T DNA released phage particles carrying the *thyP3* gene. Two such phages, SPβ c2Ta and SPβ c2Tz, were obtained by heat induction of strains NR1002 and NR1003, respectively. Table 3 compares the molecular sizes of restriction fragments generated by *Pst*I and *Sac*I digestion of the DNAs from SPβ c2Ta, SPβ c2Tz, and SPβ c2. The chromosomes of both SPβ c2T phages were missing restriction fragments found in their antecedent, SPβ c2, and several novel fragments were observed. SPβ c2Ta and SPβ c2Tz genomes had, respectively, about 1 and 8% less DNA than SPβ c2. The missing or altered fragments were located in a continuous region near the middle of the SPβ c2 chromosome, but as shown in Fig. 1, corresponded to two regions at the opposite ends of the prophage. Both SPβ c2Ta and SPβ c2Tz had an identical modified region in the portion of the SPβ c2 genome corresponding to the right extremity of the prophage. Indeed, since the mobility of the *Pst*I fragment B was unchanged, it is likely that the ϕ3T DNA containing the thymidylate synthetase gene replaced part of the *Pst*I-C' region of the prophage. Thus, the region of homology between ϕ3T and SPβ c2 is probably very close to the right prophage junction.

SPβ c2Ta and SPβ c2Tz also had altered or missing restriction fragments in a portion of the genome which corresponds to the left end of the SPβ c2 prophage, although the two phages differed substantially in the amounts of DNA in this region that were affected. Since our analyses of SPβ c2T prophages in *B. subtilis* strains NR1002 and NR1003 demonstrated that the left ends were intact, it is likely that the additional modifications observed in SPβ c2Ta and SPβ c2Tz occurred during or after induction.

Stability of ϕ3T and SPβc2T lysogens. When spores of strain NR1004, a ϕ3T lysogen of thymine auxotroph NR1000, were plated on trimethoprim-containing medium, about 1 spore in 600 gave rise to a colony (Table 4). An analysis of 12 such clones revealed that all required thymine or thymidine for growth. Moreover, they no longer released ϕ3T phage, but were now sensitive to this virus.

Spores of strains NR1002 and NR1003, both of which were constructed by transformation of SPβ c2 lysogens with ϕ3T DNA, gave rise to trimethoprim-resistant colonies at a frequency of about 1 spore in 300. Table 5 summarizes the results when bacteria from 21 of these colonies were characterized with respect to thymine auxotrophy and the presence of SPβ c2. Although all required thymine or thymidine for growth, 14 of the isolates were still resistant to infection by SPβ c2, and four of these also did not support the growth of virulent phage ϕ1m. This latter interference is known to be controlled by the *mpi* gene of SPβ prophage (9). Two strains still released SPβ-type phages, although analyses of the DNA of these viruses showed them to be deletion mutants lacking the region corresponding to the right end of SPβ c2 prophage. Only seven of the trimethoprim-resistant strains showed no evidence by our criteria of still possessing SPβ genes.

TABLE 3. Molecular sizes of restriction fragments generated by *Pst*I and *Sac*I

Fragment[a]	Fragment size (kb) after *Pst*I digestion			Fragment[a]	Fragment size (kb) after *Sac*I digestion		
	SPβ *c*2	SPβ *c*2Ta	SPβ *c*2Tz		SPβ *c*2	SPβ *c*2Ta	SPβ *c*2Tz
A	22.4	22.4	22.4	A	23.7	23.7	23.7
B	15.0	15.0	15.0	B	18.6	18.6	18.6
			14.0	C	15.0	15.0	15.0
C	13.5			D	12.0		
		11.4				10.2	10.2
D	10.0	10.0	10.0	E	8.9	8.9	8.9
E	7.5	7.5	7.5			8.1	
F,G	6.6 (2×)[b]	6.6 (2×)	6.6 (2×)	F	7.5	7.5	7.5
H	6.0	6.0	6.0				7.0
I	5.7	5.7	5.7	G	6.8		
J	5.5	5.5	5.5	H	5.5	5.5	5.5
K	5.1	5.1	5.1	I	4.3	4.3	4.3
L	4.4	4.4	4.4	J,K	3.7 (2×)	3.7 (2×)	3.7
M	3.9	3.9	3.9	L	3.5	3.5	3.5
N,O	3.7 (2×)	3.7 (2×)	3.7	M	2.8	2.8	
P	3.4	3.4	3.4	N,O	2.5 (2×)	2.5 (2×)	2.5
Q	3.2	3.2		P	2.3	2.3	2.3
R	2.3	2.3					2.0
S	2.2	2.2		Q	1.1		
T	0.8	0.8	0.8				
U	0.03	0.03	?				
Total	132	129	121		124	123	115

[a] Letters correspond to restriction fragments shown in Fig. 1.
[b] (2×) indicates twice the expected intensity, suggesting two bands of nearly identical size.

It is also notable that many of the thymine auxotrophs derived from NR1002 and NR1003 were unable to grow on sodium glutamate as sole source of carbon. This indicates that these variants no longer had an active *citK* gene (18), consistent with a deletion extending from the right end of the prophage through the bacterial *citK* marker.

Strains NR1010 and NR1011 were constructed by lysogenization of thymine auxotroph NR1000 with SPβ *c*2Ta and SPβ *c*2Tz, respectively. As can be seen in Table 4, these strains were very unstable; about one spore in two that was inoculated on trimethoprim-containing medium gave rise to a colony. Twenty of these isolates were analyzed as described above. All were thymine auxotrophs; however, they were sensitive to SPβ *c*2 and ϕ1m. None showed a *citK* phenotype. Thus, these variants appeared to have been cured of the prophages.

The marked instability of the SPβ *c*2T prophages in *B. subtilis* NR1010 and NR1011 suggests that these bacteria might be pseudolysogens (5). We cultivated these strains in the presence of antiserum prepared against SPβ (20) for several passages (about 20 generations) and then plated cultures on M agar supplemented with thymidine. Individual colonies were subsequently checked for thymine prototrophy. In the case of NR1010, none of the 25 colonies tested were thymine auxotrophs, and we concluded that few bacteria had lost the SPβ *c*2Ta prophage during growth in antiserum. On the other hand, 18 of 25 colonies derived from NR1011 under the same conditions were thymine auxotrophs. Two of these were tested and found to be sensitive to SPβ *c*2 and to ϕ1m. Thus, the SPβ *c*2Tz prophage in NR1011 was lost at high frequency when the bacteria were grown in the presence of SPβ antiserum.

TABLE 4. Spores derived from ϕ3T and SPβ *c*2T lysogens which yield trimethoprim-resistant colonies

Strain[a]	Prophage	% Trimethoprim-resistant clones[b]
NR1004	(ϕ3T)	0.15
NR1002	(SPβ *c*2T-1)	0.33
NR1003	(SPβ *c*2T-2)	0.31
NR1010	(SPβ *c*2Ta)	50
NR1011	(SPβ *c*2Tz)	68

[a] All strains were *thyA25 thyB25*.
[b] The percentage of resistant clones was the number of CFU per milliliter measured when a spore preparation titer was determined on trimethoprim-containing medium divided by CFU per milliliter when the titer was determined on the same medium lacking the drug times 100.

TABLE 5. Properties of 21 trimethoprim-resistant clones derived from SPβ *c*2T lysogens

No. of clones in category	Phage sensitivity[a]		Release of SPβ[b]	Growth on glutamate as carbon source	Requirement for thymine
	SPβ *c*2	ϕ1m			
2	s	s	−	Yes	Yes
5	s	s	−	No	Yes
1	r	r	+	No	Yes
3	r	r	−	Yes	Yes
9	r	s	−	No	Yes
1	r	s	+	No	Yes

[a] A drop of a diluted SPβ *c*2 lysate (about 10^5 PFU/ml) or ϕ1m lysate (about 10^4 PFU/ml) was placed on a lawn of bactera to be tested. s, A zone of killing or growth inhibition visible after 24 h of incubation; r, the strain was resistant to the phage.
[b] The culture to be tested was grown in broth and induced by a 5-min temperature jump to 50°C. The culture was incubated an additional 90 min and centrifuged to remove unlysed cells. The supernatant was diluted and plated with strain CU1050 as indicator. +, Plaques formed on lawns of CU1050; −, no evidence that viable phage were released.

DISCUSSION

Thymine auxotrophs of *B. subtilis* 168 can be transformed to prototrophy by DNA from bacteriophage ϕ3T. This transformation usually results from the incorporation of a phage structural gene for thymidylate synthetase, the *thyP3* gene, into the prophage of SPβ. This latter phage is closely related to ϕ3T and is found in virtually all derivatives of *B. subtilis* 168 (13, 15).

Our data suggest that the *thyP3* gene became incorporated near the right SPβ *c*2 prophage junction and close to the bacterial gene *citK*. The extent of homology between *thyP3* and SPβ *c*2 DNA is not clear. SPβ *c*2 DNA hybridized to a 7.4-kb fragment of ϕ3T DNA which contained the *thyP3* gene. However, a 1.3-kb fragment containing *thyP3* did not hybridize to SPβ *c*2 DNA. Thus, the regions of homology presumbly needed to facilitate recombination between the *thyP3* region of ϕ3T and SPβ *c*2 are probably in DNA sequences flanking the *thyP3* gene.

SPβ *c*2 lysogens which were transformed with ϕ3T DNA (hereafter called primary SPβ *c*2T lysogens) could be induced to release viable phage. The latter contained the *thyP3* gene, and upon lysogenization converted *B. subtilis* thymine auxotrophs to prototrophy. The DNAs of six of these SPβ *c*2T phages derived from induction of primary lysogens were analyzed with retriction endonucleases and found to fall into two classes. One group, represented by SPβ *c*2Ta, was missing restriction fragments corresponding to about 8% of the genome. The other class, represented by SPβ *c*2Tz, was lacking fragments from a 22-kb region (16% of the genome) near the middle of the phage chromosome. However, the total loss of DNA in these phages was less than could be accounted for by the missing fragments. It is likely, therefore, that the central regions of these phages were partly replaced by a large fragment of ϕ3T DNA (perhaps as much as 10 kb) or by bacterial DNA gained from regions proximal to the prophage.

Curiously, the affected regions in both SPβ *c*2T phages overlapped the opposing ends of the prophage state of SPβ *c*2 (Fig. 1). Since our data suggest that transformation of the original SPβ *c*2 lysogen affected only the right end of the prophages, it is probable that changes which altered the left end occurred during or after excision. Induction of SPβ *c*2 prophage is thought to involve a circularization event, because the vegetative and prophage genomes are circularly permutated. Perhaps insertion of the *thyP3* gene affects induction, resulting in deletions or replacement of some of the left end of the prophage during circularization. Indeed, we found that when SPβ *c*2 lysogens were induced, many of the progeny phage were missing a large portion of the genome corresponding to the left end of the prophage (data not shown). This suggests an instability in that region of the SPβ *c*2 chromosome.

Lysogens of ϕ3T or SPβ *c*2T lost some or all of the prophage genome at a fairly high frequency. In the case of ϕ3T, our data suggest that bacteria are actually cured of the prophage and can be readily lysogenized with the same phage upon reinfection. The mechanism by which the prophage is excised from the bacterial genome without inducing a lytic sequence is unknown.

When spores of a primary lysogen of SPβ *c*2T were plated on a medium containing trimethoprim, about 1 spore in 300 gave rise to a thymine-requiring auxotroph. Analyses of these isolates indicated that most still contained part of the SPβ *c*2 prophage but had deleted the DNA segment containing the *thyP3* gene. Moreover, in many instances the loss of the thymidylate synthetase gene correlated with loss of neighboring bacterial markers, suggesting a large deletion spanning the right end of the prophage through at least the *B. subtilis citK* gene. Only occasionally did we find evidence of the entire prophage being lost from a primary lysogen, and even these apparent exceptions may have contained portions of SPβ that we could not detect by the expression of known prophage genes.

As noted above, SPβ *c*2Ta and SPβ *c*2Tz phages can infect thymine auxotrophs of *B. subtilis* to produce secondary lysogens which are prototrophic. When spores of the secondary lysogens were plated on trimethoprim, about half gave rise to resistant colonies, which appeared to have been cured of the prophage. In these instances, we could find no evidence that any part of the SPβ *c*2 prophage was still present. Moreover, lysogens of SPβ *c*2Tz also lost the phage at high frequency when grown in a medium containing SPβ antiserum. Together these results suggest that at least some secondary lysogens may be pseudolysogens in which the prophage is not actually integrated into the bacterial DNA (5). Such a finding would not be surprising since SPβ *c*2Ta and SPβ *c*2Tz are apparently missing the normal prophage attachment site.

Overall, our results are consistent with a model in which the *thyP3* gene and some flanking sequences of ϕ3T DNA underwent recombination with the right end of the SPβ *c*2 prophage genome. This part of the SPβ *c*2 chromosome and a segment of DNA on the left end of the prophage (which together comprise a 22-kb region of the vegetative phage genome) are unstable in the sense that they can be deleted or replaced at high frequency. Since phages missing substantial portions of this area of the chromosome were viable, it seems clear that few if any genetic functions required for lytic growth of SPβ *c*2 are encoded in this region. We are now endeavoring to determine what, if any, functional genetic information is contained in this region and the role it might serve in the life cycle of SPβ.

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