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Defective Specialized SPβ Transducing Bacteriophages of *Bacillus subtilis* That Carry the *sup-3* or *sup-44* Gene

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We isolated defective specialized transducing phages of SPβ that carry one of the extracistronic suppressors, *sup-3* or *sup-44*. Lysates containing these phages can be used in a simple spot test to determine whether an auxotrophic mutation can be suppressed. The *sup-3* and *sup-44* mutations are distinct, in that their suppression patterns differ for the markers *hisA1*, *metC3*, and *thr-5*; and they are not alleles.

SPβ is a temperate bacteriophage of *Bacillus subtilis* (13). The normal prophage attachment site, *attSPβ*, is located between the *ilvA* and *kauA* genes (map position 190°) on the *B. subtilis* chromosome (7, 10, 15). Like phage λ in *Escherichia coli*, SPβ is capable of mediating high-frequency specialized transduction of bacterial genes that flank the normal attachment site (10, 15). To transduce bacterial genes that are not adjacent to this site, the phage must first integrate at secondary sites on the bacterial chromosome. One approach to obtaining such lysogens utilizes an integration-defective mutant of SPβ, SPβ *c2 int5* (manuscript in preparation). The *c2* allele on this phage codes for a heat-labile repressor which causes phage induction after brief incubation at 50°C (10). The *int5* dysfunction prevents lysogenization at the normal attachment site and allows occasional lysogenization at other sites.

In *B. subtilis* the mutations *sup-3* and *sup-44* apparently suppress nonsense mutations (5, 8, 11). No information is available on the nature of the codons suppressed or the biochemical mechanism of suppression in *B. subtilis* (11).

We constructed strains of *B. subtilis* that were doubly lysogenic for SPβ *c2* and for a defective transducing phage that carries either the *sup-3* or *sup-44* gene. The transducing particles carrying an extracistronic suppressor were used in a simple test to determine whether particular auxotrophic markers were suppressible. Each suppressor was also characterized by its ability to overcome particular auxotrophic mutations.

All bacterial strains were derived from *B. subtilis* strain 168, which carries the *trpC2* marker. The strains used in this study are listed in Table 1. Routine culturing of bacterial strains for PBS1-mediated transductions, DNA-mediated transformations, and for the preparation of SPβ phage lysates have been described elsewhere (10, 12, 15).

B. subtilis strain CU1698 (Table 1) was produced by infecting the nonlysogenic strain CU1065 with the integration-deficient phage mutant SPβ *c2 int5*. Lysogens of SPβ *c2 int5* were detected because they excrete a bacteriocin-like substance (betacin) that kills nonlysogens (6). Furthermore, they are all immune to infection by clear-plaque mutant SPβ *c1*, as determined in a cross-streak test (10). Details of the isolation of CU1698 will be published elsewhere. Strain CU1698 was found to carry SPβ *c2 int5* between its *purB* and *dal* genes, close to *dal*. The prophage could be contransduced with *dal* by the large generalized transducing phage PBS1 (P. A. Toye, unpublished data). This permitted us to construct first strain CU1802 (Table 1) and then strain CU1968 *metB5* (*zbb*::SPβ *c2 int5*). (Nomenclature is explained in Table 1.)

Strain CU1968 was transformed to Met^+ by DNA extracted from strain CU1063 (*sup-3*) and also from strain CU1965 (*sup-44*). Both donor and recipient strains carried the suppressible *metB5* marker; thus all Met^+ transformants resulted from the presence of a suppressor gene. The two strains (CU2056 and CU2057) constructed in this manner carried the SPβ *c2 int5* prophage between the *dal* and *purB* genes. Strain CU2056 also carried the extracistronic suppressor gene *sup-3*; CU2057 carried *sup-44* (Table 1). Both suppressor genes lie near *dal*, which codes for alanine racemase (4), on the side away from the prophage.

Heat induction of strains CU2056 and CU2057 yielded some transducing particles that carried both the *dal* gene and a *sup* gene. The lysates containing the suppressor-carrying defective phage were used to construct double lysogens carrying SPβ *c2* and one of the transducing

TABLE 1. *Bacterial and phage strains used in this study*[a]

Strain	Genotype	Source/origin
CU974	*trpC2 dal-1* (SPβ)	This laboratory
CU1063	*sup-3 metB5 thr leu ade attSPβ*[b]	(15)
CU1064	*metB5 attSPβ*	(15)
CU1065	*trpC2 attSPβ*	(15)
CU1142	*metB5 dal-1 attSPβ*	CU1064 $\xrightarrow{\text{PBS1}}$ CU974; Trp$^+$ selection
CU1182	*trpC2 aroI906 purB33 dal-1* (SPβ)	kit2 of R. Dedonder (3)
CU1232	*metB5 dal-1* (SPβ *c2*)	SPβ *c2* → CU1142
CU1442	*metB5 aroI906 purB33 dal-1 attSPβ*	CU1064 $\xrightarrow{\text{PBS1}}$ CU1182; Trp$^+$ selection
CU1698	*trpC2* (*zbb*::SPβ *c2 int5*)[c]	SPβ *c2 int5* → CU1065
CU1802	*metB5 aroI906 purB33* (*zbb*::SPβ *c2 int5*)	CU1698 $\xrightarrow{\text{PBS1}}$ CU1442; Dal$^+$ selection
CU1805	*metB5 leuD117 attSPβ*	This laboratory
CU1964	[*hisA1*][d] *thr-5 sup-44* (SPβ)	QB1180 of R. Dedonder (3)
CU1965	[*metB5*] *sup-44 attSPβ*	CU1964 $\xrightarrow{\text{PBS1}}$ CU1064; Met$^+$ selection
CU1968	*metB5* (*zbb*::SPβ *c2 int5*)	CU1802 $\xrightarrow{\text{PBS1}}$ CU1142; Dal$^+$ selection
CU2054	*metB5 hisA1 attSPβ*	This laboratory
CU2056	[*metB5*] *sup-3* (*zbb*::SPβ *c2 int5*)	CU1063 $\xrightarrow{\text{DNA}}$ CU1968; Met$^+$ selection
CU2057	[*metB5*] *sup-44* (*zbb*::SPβ *c2 int5*)	CU1965 $\xrightarrow{\text{DNA}}$ CU1968; Met$^+$ selection
CU2058[e]	[*metB5*] *dal-1* (SPβ *c2*) (SPβ *c2 int5* d*sup3*-1)	CU2056 $\xrightarrow{\text{SPβ}}$ CU1232; Dal$^+$ Met$^+$ selection
CU2059[e]	[*metB5*] *dal-1* (SPβ *c2*) (SPβ *c2 int5* d*sup44*-1)	CU2057 $\xrightarrow{\text{SPβ}}$ CU1232; Dal$^+$ Met$^+$ selection
CU2681	*purA16 metB5 ilvA1 ddl-1475* (SPβ)	R. Buxton (1)

[a] All SPβ phages described in the text carry the *c2* mutation (thermosensitive repressor).

[b] *attSPβ* designates sensitivity to clear-plaque mutant SPβ *c1* and lack of SPβ prophage in the normal attachment site.

[c] *zbb* means insertion into an unknown gene (*z*) at a position 0.11 chromosome lengths clockwise from the top of the *B subtilis* genetic map (2, 7). It corresponds to 46° on the Henner-Hoch map (7).

[d] Markers in brackets are suppressed.

[e] These strains are Dal$^+$ because their defective prophages carry the *dal*$^+$ gene.

particles. The recipient strain was CU1232 *metB5 dal-1* (SPβ *c2*), and selection was for Dal$^+$ Met$^+$.

The double lysogens were labeled CU2058 and CU2059, and they produced high-frequency transducing lysates for *sup-3* and *sup-44*, respectively. Both transducing particles carried the *dal*$^+$ gene. Heat induction of the double lysogens released more than 10^5 transducing particles per ml carrying the *dal* gene and either *sup-3* or *sup-44*. The transducing particles, however, differed in the amount of bacterial DNA they contained. Only SPβ *c2 int5* d*sup*44-1 carries the *ddl*$^+$ gene (D-alanine ligase) (1). Strain CU2681 (Table 1) was transduced to Met$^+$ by the high-frequency transducing lysates containing *sup-3* and *sup-44*-defective phage. Only the *sup-44*-containing defective phage also transduced the recipient to Ddl$^+$. Since the *ddl-1475* marker is temperature sensitive, it is very unlikely that *sup-44* is suppressing it.

As evidence that suppression was occurring, a second, unlinked suppressible marker, *leuD117*, was examined with the lysate of CU2058 (which carries *sup-3*); CU1805 *metB5 leuD117* was recipient. All of 24 Met$^+$ transductants tested were also Leu$^+$. Because the *leuD117* mutation is unlinked to either *metB5* or *sup-3*, we concluded that the selection was for *sup-3*, as expected. The unlinked markers *metB5* and *hisA1* in strain CU2054 were also examined, using a high-frequency transducing lysate prepared from CU2059. All of the 24 Met$^+$ transductants tested were also His$^+$, providing evidence that suppression by *sup-44* had occurred.

Lysates which contained large numbers of transducing particles carrying either *sup-3* or *sup-44* were used in a simple spot test to characterize these two suppressor mutations. A crude lysate could be filter-sterilized and used for suppression tests. However, the stability of defective phage in broth was poor (half-life of about 7 days). Moreover, the results from suppression tests conducted on auxotrophs with these lysates tended to be ambiguous due to nutrient carryover from the culture medium.

To minimize interference caused by carryover and to improve lysate stability, the phage was recovered and concentrated by a variation of the method of Yamamoto et al. (14). NaCl (25 g) was dissolved in 1 liter of lysate supernatant. Polyethylene glycol (Carbowax 6000), 60 g per liter, was dissolved, and the treated supernatant was refrigerated for 12 to 18 h at 4°C. Phage was harvested by centrifugation at 10,500 × *g* for 30 min at 4°C. The supernatant fluid was discarded, and all excess liquid was removed. Three milliliters of phage dilution buffer (0.1 M NaCl, 0.01 M $MgCl_2$, 0.1 M Tris-hydrochloride, 0.7% gelatin, pH 7.0) was pipetted over the pellet and allowed to stand for 12 to 18 h at 4°C. The pellet was suspended in the overlay buffer and filter-sterilized. Concentrated phage preparations containing either SPβ *c2 int5* d*sup3*-1 or SPβ *c2 int5* d*sup*44-1 had a half-life of approximately 27 days when stored at 4°C. Storage in glycerol (25% wt/vol) at −20°C stabilized the phage with no appreciable loss of infectivity over a 2-month period. The final phage preparation was diluted in phage dilution buffer for testing an auxotroph for suppressibility.

To test for suppressibility, an auxotrophic strain was grown in antibiotic medium no. 3 (Difco Laboratories) to early stationary phase, washed, and suspended in standard saline citrate buffer (0.1 M NaCl, 0.05 M sodium citrate, pH 7.2) to the original volume. A 0.1-ml sample of the culture was spread on a minimal selective agar plate. When the plate had dried, 0.05-ml droplets of the diluted lysates were added to the plate. The plates were allowed to dry and then incubated at 37°C. The plates were examined for growth at 18 and 42 h. After incubation, heavy growth within the area of the dried droplet was interpreted as a positive test for suppression.

Using this method, we tested a number of auxotrophs for suppressibility. The following markers were suppressed by both *sup-3* and *sup-44*: *metB5*, *purB6*, *gltA2*, and *leuD117*. The markers *metC3* and *thr-5* were suppressed by *sup-3*, but not by *sup-44*. Conversely, only *sup-44* suppressed the *hisA1* marker. The markers *ilvA3*, *ilvD15*, and *hisH2* were not suppressed by either *sup-3* or *sup-44*. Suppression was easily detected whether or not the auxotroph was lysogenic for SPβ, or if the recipient strain carried the *recE4* mutation.

Mellado et al. (9) reported the isolation of a strong suppressor of nonsense mutations in *B. subtilis* (*sup-44*) that differed from *sup-3* by its inability to suppress the phage ϕ29 mutation, *susB47*. They estimated that the efficiency of suppression in the *sup-44* strain was approximately 50%, in contrast to only about 10% for the *sup-3* strain of Georgopoulos (5).

Our results show that *sup-3* and *sup-44* are genetically distinct, in that their suppression patterns differ for the markers *hisA-1*, *metC3*, and *thr-5*. This conclusion supports the findings of Mellado and co-workers (9) that *sup-3* and *sup-44* are not identical. The *sup-3* and *sup-44* mutations are not alleles, since we have constructed strains that carry both extracistronic suppressors (unpublished data).

By using the simple spot test outlined in this report, in conjunction with the proper controls, one can examine any auxotrophic mutation from *B. subtilis* for suppression, provided that the mutation does not lie within the fragment of bacterial DNA carried on the transducing particle.

We are constructing a genetic map for the position of *sup-44* with respect to *sup-3* and other nearby markers. Strains CU2058 and CU2059 have been deposited in the *Bacillus* Genetic Stock Center (Department of Microbiology, Ohio State University, Columbus 43210).

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