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Insertion of Bacteriophage SPβ into the *citF* Gene of *Bacillus subtilis* and Specialized Transduction of the *ilvBC-leu* genes

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We isolated a strain of *Bacillus subtilis* in which the SPβc2 prophage is inserted into the *citF* (succinate dehydrogenase) gene. Defective specialized transducing particles for the *ilvBC-leu* genes were isolated from phage-induced lysates of this lysogen. We isolated a group of phages that differ in the amount of genetic material they carry from this region. Also, we incorporated mutant *ilv* and *leu* alleles into the genomes of several transducing phages. Our phage collection enables us to identify the cistron of new *ilv* and *leu* mutations by complementation analysis. In this process we discovered a fourth *leu* cistron, *leuD*. Characterization of the phages confirmed the published gene order: *ilvB-ilvC-leuA-leuC-leuB; leuD* lies to the right of *leuB*.

Lambda specialized transducing phages have proven to be powerful tools for the analysis of the *Escherichia coli* genome (see reference 8). These phages permit the construction of bacterial strains that are heterogenotic for virtually any gene of interest. The cistron relationship of mutations and their dominant or recessive character can then be determined by complementation analysis in the partial diploids. Ultimately, the characterization of specialized transducing phages and their DNA provides detailed information concerning the structure and regulation of bacterial genes.

The development of specialized transducing phages for the gram-positive bacterium *Bacillus subtilis* 168 has lagged behind the *E. coli*-lambda model system. Recently, an in vitro method for the construction of specialized transducing phages from the *B. subtilis* bacteriophages ϕ105 and ρ11 has been described (4, 5). In this laboratory we are using in vivo techniques to construct specialized transducing phages from a temperate bacteriophage of *B. subtilis* called SPβ (12). SPβ-mediated specialized transduction of markers near the normal attachment site, *att*SPβ, has been reported (1, 9, 13). To expand the range of genes transducible by SPβ, we employ strains of *B. subtilis* that lack a functional prophage attachment site, *att*SPβ. SPβ infection of these strains produces lysogens, albeit at a low frequency, that carry the prophage at different sites around the bacterial chromosome. Phage lysates of these lysogens contain transducing phages for nearby genes. We are developing methods that employ these phages to study the *B. subtilis* genome (12a).

For our model system we chose to study a cluster of genes involved in the biosynthesis of isoleucine, valine, and leucine: *ilvB* and *C* and the *leu* genes. Several years ago a genetic map of the *ilvBC-leu* region was published (11). In that report several *leu* mutants were assayed for their leucine biosynthetic enzymes. The mutants were deficient in either α-isopropylmalate (α-IPM) synthetase (*leuA*), IPM isomerase (*leuB*), or β-IPM dehydrogenase (*leuC*). The *leu* mutations are located between *pheA* and the genes encoding the first and second enzymes common to the isoleucine and valine pathways, *ilvB* (α-acetohydroxyacid synthetase) and *ilvC* (α-acetohydroxyacid isomeroreductase). The gene order was reported to be *ilvB-ilvC-leuA-leuC-leuB* (11).

At the time those studies were conducted, there was no way to construct strains that were heterogenotic for *ilv* or *leu*; thus complementation analyses were impossible. Assignment of new *ilv* or *leu* mutations to the proper gene required enzyme assays, and the determination of gene order relied on mapping mutations by transformation. Moreover, there was no way to determine whether IPM isomerase is encoded by two cistrons in *B. subtilis* as it is in *Salmonella typhimurium* and *E. coli* (6, 10).

In this paper we report the isolation and characterization of an SPβ lysogen in which the prophage is inserted into the gene for succinate dehydrogenase (*citF*). We describe six classes of transducing phages that we have isolated from phage lysates of this lysogen. The phages differ in the amount of genetic material they carry from the *ilvBC-leu* region. We also describe the

TABLE 1. Bacterial strains used in this study

Strain	Genotype[a]	Source (reference)
CU240	*ilvB2 leuB6 trpC2* (SPβ)	(11)
CU257	*leuB16 trpC2* (SPβ)	(11)
CU258	*ilvB2 leuA5 trpC2* (SPβ)	(11)
CU267	*ilvB2 leuB16 trpC2* (SPβ)	Laboratory collection
CU315	*leuD117 trpC2* (SPβ)	Laboratory collection
CU740	*leuA5 trpC2* (SPβ)	(11)
CU748	*ilvBΔ1 pheA2 trpC2* (SPβ)	Laboratory collection
CU1050	*ade leu metB5 sup-3 thr att*SPβ	(12)
CU1065	*trpC2 att*SPβ	(13)
CU1316	*ilvBΔ1 metB5* (SPβ *c2*)	(9)
CU1846	*ilvC9 leuA169 metB5* (SPβ *c2*)	Laboratory collection
CU1852	*argA2 citD2 metB5 pheA2*	Laboratory collection
CU1881	*argA2 citD2 metB5 pheA2 citF*::SPβ *c2*	SPβ *c2* infection of CU1852
CU1886	*argA2 pheA2 att*SPβ *citF*::SPβ *c2*	CU1065 $\xrightarrow{\text{PBS1}}$ CU1881; Met$^+$ selection
CU2322	*ilvBΔ1 leuD117 trpC2* (SPβ *c2*)	Laboratory collection

[a] *att*SPβ means the strain has a normal, unoccupied SPβ attachment site. Such strains are sensitive to SPβ unless they carry an SPβ prophage elsewhere.

construction of a set of phages, each of which carries a mutant allele for one of the *ilvBC-leu* genes. These phages have permitted us to identify a fourth *leu* cistron, *leuD*. We demonstrate the usefulness of these phages in identifying new *ilv* and *leu* mutations and in determining gene order and cistron number by complementation analysis.

MATERIALS AND METHODS

Bacteria and phage. All bacterial strains used in this study were derived from *B. subtilis* 168 (Table 1; see Tables 2 and 3). Methods for routine culturing of bacteria have been described (11). When strains CU1852, CU1881, and CU1886 were cultured, 0.2% glucose was added to complex medium. Double lysogens, heterogenotic for *ilv* or *leu*, were cultured on minimal medium to maintain selection for the wild-type alleles. PBS1 phage lysates for strain constructions and mapping experiments were prepared and transductions were carried out as described (11). Cit$^+$ transductants were selected for ability to grow with lactate as the sole carbon source (9). DNA-mediated transformations were carried out as described (11).

Bacteria were tested for SPβ lysogeny by immunity to SPβ *c1*, a clear-plaque mutant of SPβ (13), and by betacin production (2). Betacin is a bacteriocin-like substance produced by SPβ lysogens and is active on nonlysogens. Most of the SPβ phages described here contain the *c2* mutation, which results in the production of a thermolabile SPβ repressor (9). SPβ *c2* lysogens were heat-induced by a 10-min incubation in a 50°C temperature block during early logarithmic growth. SPβ *c*$^+$ lysogens were induced with mitomycin C as described (9). The phage lysates were assayed for PFU by the soft-agar overlay technique, using CU1050 as an indicator (9). SPβ transducing particles were assayed by transduction of appropriate *ilv* or *leu* auxotrophs (9).

Isolation of abnormal SPβ lysogens. CU1852 *argA2 citD2 metB5 pheA2* was constructed by PBS1 transduction. The *citD2* mutation is a deletion that extends from within the SPβ prophage through the *kauA* and *citK* genes (R. Z. Korman and P. S. Fink, unpublished data). The lack of *citK* (which encodes α-ketoglutarate dehydrogenase) makes cells that carry *citD2* asporogenous and unable to use lactate as sole carbon source. The normal SPβ attachment site is nonfunctional in *citD2* strains, that is, lysogenization occurs at a low frequency and at sites other than *att*SPβ. A broth culture of CU1852 was infected with SPβ *c2* at a multiplicity of infection of 1. The cells were diluted and spread onto tryptose blood agar base (Difco) plates. The colonies that formed after 24 h of incubation at 37°C were purified by streaking on tryptose blood agar base plates containing SPβ antiserum. The antiserum prevented false-positive betacin tests by pseudolysogens. Thirty-four SPβ lysogens were isolated among 1,000 colonies tested. One lysogen, CU1881, carried SPβ *c2* inserted into the *citF* gene (see Results).

Isolation of SPβ specialized transducing phages and complementation testing. A putative low-frequency transducing lysate for the *ilvBC-leu* genes was prepared by induction of CU1881. The lysate was used to transduce various SPβ lysogens that were auxotrophic for *ilv* or *leu*. SPβ lysogens were used as recipients for two reasons. First, the presence of SPβ repressor protein prevents bacterial lysis caused by superinfecting phage; second, the production of high-frequency-of-transduction (HFT) lysates requires a resident prophage to help package the defective transducing particles.

The transductants were purified and induced to produce phage. HFT lysates for *ilv* or *leu* were identified by the following simple spot test. Appropriate recipient strains were grown in broth to mid-logarithmic phase. The cultures were washed, and approximately 5×10^7 cells were spread on selective minimal agar plates. Then 0.05-ml samples of the lysates to be

tested were spotted onto the plates. The plates were incubated at 37°C for 24 to 48 h. Lysates that produced heavy growth in the region of inoculation were labeled HFT for the appropriate markers. The ratios of plaque-forming particles to transducing particles were then determined quantitatively to confirm the spot test results.

RESULTS

Screening for a prophage insertion near the *ilvBC-leu* gene cluster. We isolated 34 SPβ lysogens of CU1852 *argA2 citD2 metB5 pheA2*. The *ilvBC-leu* gene cluster is located at position 250° on the *B. subtilis* chromosome map (3). An insertion of SPβ into a secondary attachment site in this region would place the prophage between the *pheA* and *argA* genes, which are located at positions 245° and 260°, respectively. We reasoned that if 1 of the 34 lysogens carried the prophage between the *argA2* and *pheA2* markers, then Arg^+ Phe^+ transductants of such a strain would be cured of SPβ. A PBS1 phage lysate made in strain CU1065 *trpC2 att*SPβ was used to transduce the SPβ lysogens of CU1852 to arginine and phenylalanine prototrophy. Six Arg^+ Phe^+ transductants from each of the 34 crosses were tested for the presence of SPβ. The Arg^+ Phe^+ transductants of one strain, CU1881, were all cured for SPβ. The other 33 strains retained the prophage in the cross. These results indicated that CU1881 carried an SPβ prophage between *argA* and *pheA*. Thus, CU1881 was a possible source of transducing phage of the *ilvBC-leu* genes.

Characterization of CU1881: *argA metB5 citD2 pheA2 citF*::SPβ. We were not aware that the SPβ insertion had occurred in the *citF* gene, giving strain CU1881 two defects in its citric acid cycle. We were therefore surprised to find that we could not transduce CU1881 to Cit^+ by PBS1 transduction. We tried to transduce CU1881 to $MetB^+$, since we knew that *citD2* is cotransduced to $CitD^+$ 60% of the time with *metB5*. All of the Met^+ transductants of CU1881 were still Cit^-. However, about 60% of them produced infrequent revertants to Cit^+. The revertants were detected as papillae in cultures plated on Cit^+-selective media. Cells from the papillae were purified and found to be sensitive to SPβ. One of the $MetB^+$ transductants capable of reverting to Cit^+ was labeled CU1886. It still carried SPβ in the region between *pheA* and *argA*, but it no longer carried the *citD2* deletion. We concluded that the SPβ prophage had inserted into, and thereby inactivated, a *cit* gene located between *argA* and *pheA*. The prophage could occasionally excise accurately, reconstituting the cit^+ gene.

Three *cit* genes are located in this region: *citH* (malate dehydrogenase), *citC* (isocitrate dehydrogenase), and *citF* (succinate dehydrogenase) (3). Strain CU1886 was assayed for the *citF* gene product in the laboratory of L. Rutberg at the Karolinska Institute, Stockholm, Sweden. The results showed that the enzyme activity of succinate dehydrogenase (1a) from CU1886 is 0.008 $U \cdot s^{-1} \cdot mg\ of\ protein^{-1}$, compared to an activity of 0.22 in the wild-type control strain (L. Rutberg, personal communication). Therefore, we believe that the SPβ prophage lies within the *citF* gene.

Isolation of SPβ d*leuD*-1. A potential low-frequency-of-transduction lysate for the *ilvBC-leu* genes was obtained by the induction of CU1881. This lysate was used as the donor in an SPβ transduction of CU1846 *ilvC9 leuA169 metB5* (SPβ *c2*). Selection was for Ilv^+ Leu^+ transductants. Fifteen transductants were isolated, and phage lysates were prepared from all of them. At this time we knew of only three *leu* genes. We tested the lysates for transduction of *ilvB2*, *ilvC4*, *leuA5*, *leuC124*, and *leuB6*. Nine of the 15 lysates were HFT for all of these genes; the other 6 were not HFT for any of them. Subsequently we discovered a fourth *leu* gene, *leuD*, by complementation analysis. We will describe the identification of *leuD* after our discussion of the transducing phages. After our discovery of *leuD*, we tested the nine lysates for their ability to transduce *leuD117*; they were all HFT for *leuD*.

One of the strains that produced HFT lysates for *ilvB*, *ilvC*, *leuA*, *leuC*, *leuB*, and *leuD* was labeled CU1889. Phage lysates of CU1889 contain both PFU of SPβ and defective specialized transducing units in a ratio of approximately 500 to 1. The specialized transducing phage in CU1889 was named SPβ d*leuD*-1.

Characterization of SPβ d*leuD*-1 transductants. Twenty Ilv^+ Leu^+ transductants produced in an SPβ d*leuD*-1 transduction of strain CU240 *ilvB2 leuB6 trpC2* (SPβ) were picked at random, and phage lysates were prepared from each of them. Fourteen of the transductants produced HFT lysates for *ilvBC-leu*. The six remaining transductants did not. The 14 HFT lysates contained both SPβ and SPβ d*leuD*-1 phage particles.

We wished to determine whether SPβ d*leuD*-1 was integrated into the region of bacterial DNA homology ("B" configuration) or the region of phage DNA homology ("P" configuration) in the 14 double lysogens. One way to distinguish these two arrangements is based on gene linkage analysis. If SPβ d*leuD*-1 were in the B configuration, then the $ilvB^+$ gene carried by the phage would be linked to $pheA^+$ by PBS1. Similarly, if SPβ d*leuD*-1 were in the P configuration, then $ilvB^+$ would be linked to $metB^+$.

PBS1 phage lysates produced in each of the 14

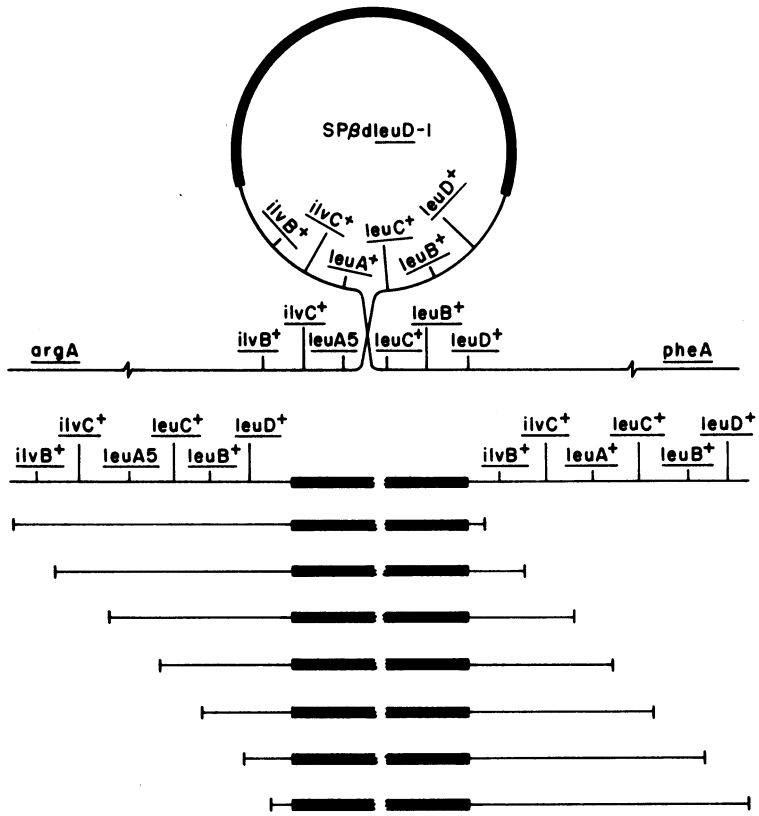

FIG. 1. Model of SPβ d*leuD*-1 inserting into the B configuration. The seven parallel lines represent different defective transducing phage genomes that may be produced upon induction. Three of the seven carry the *leuA5* mutation.

double lysogens were used to transduce CU1316 *ilvBΔ1 metB5* (SPβ *c2*) and CU748 *ilvBΔ1 pheA2* (SPβ). Ilv$^+$ Met$^+$ transductants of CU1316 and Ilv$^+$ Phe$^+$ transductants of CU748 were selected. In 7 of the 14 double lysogens, *ilvB*$^+$ was linked to *pheA* and not to *metB*. In five of the double lysogens, *ilvB*$^+$ was linked to *metB* and not to *pheA*. Linkage of *ilvB*$^+$ to both *metB* and *pheA* was detected in the two remaining lysogens.

Isolation of SPβ d*leuD*-1 phage particles that carry negative alleles for *ilvBC-leu*. To conduct complementation tests, we incorporated *ilv* and *leu* mutations into the genome of SPβ d*leuD*-1. The method we developed to accomplish this was based on the following assumptions. When a double lysogen is induced to produce phage, excision by homologous recombination produces an array of recombinant phage genomes. The recombinant classes present depend on the arrangement of genetic markers in the lysogen. To isolate a recombinant of SPβ d*leuD*-1 that carries a particular *ilv* or *leu* mutation, the transducing phage genome should be integrated into the *ilvBC-leu* region (B configuration) of a strain that contains that particular mutation. Upon induction, some fraction of the defective phage particles will incorporate the mutant allele into their genomes (Fig. 1).

We isolated a recombinant of SPβ d*leuD*-1 that carries the *leuA5* mutation by the following manipulations. CU740 *leuA5 trpC2* (SPβ) was transduced to Leu$^+$ with SPβ d*leuD*-1. About 100 Leu$^+$ transductants were pooled and induced to produce phage. We expected that about two-thirds of the transductants were double lysogens and that, of those, approximately one-half carried the SPβ d*leuD*-1 genome in the B configuration. The phage lysate produced from the pool of transductants was used to transduce CU258 *ilvB2 leuA5 trpC2* (SPβ). Ilv$^+$ transductants were selected and scored for their Leu phenotype. Nine of 50 Ilv$^+$ transductants were still Leu$^-$. Four of the nine were induced to see whether they were double lysogens. Two strains gave HFT lysates for each of the *ilvBC-*

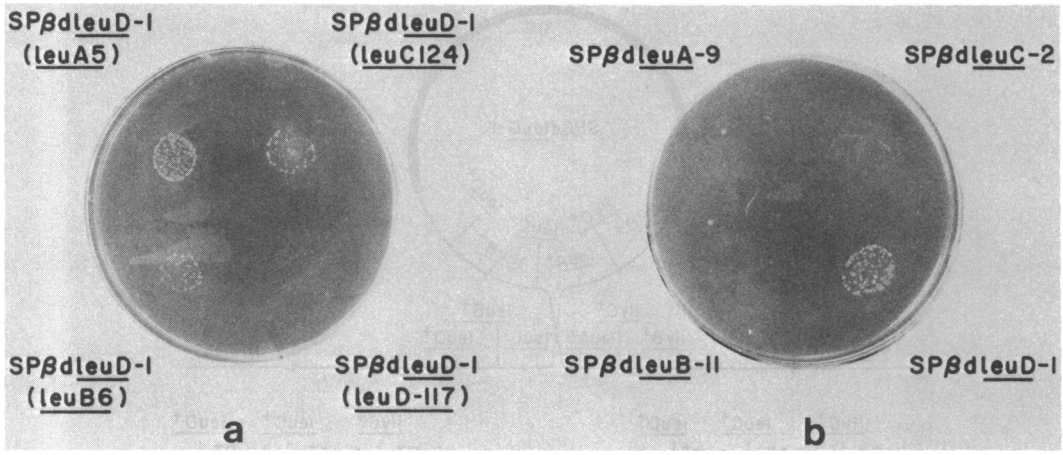

FIG. 2. Complementation test. A culture of the recipient strain CU2463 *ilvB2 leuD117 metB5* (SPβ *c*2) was spread onto minimal agar plates containing methionine, isoleucine, and valine, but lacking leucine. About 0.05 ml of sterile SPβ lysates was placed onto the recipient lawn. The plates were scored for complementation after 36 h of incubation at 37°C. (a) Null phage lysates; (b) short phage lysates.

leu genes except *leuA*. One of these strains was labeled CU2084. It carries two copies of *leuA5*. The defective phage carried by CU2084 was named SPβ d*leuD*-1 [leuA5]. By similar manipulations we have isolated recombinants of SPβ d*leuD*-1, each of which carries a mutant allele for one of the *ilvBC-leu* genes (Table 2). We used these "null" phages in complementation tests to determine in which cistron *ilv* or *leu* mutations lie (Fig. 2a).

Isolation of "short" transducing phages. In addition to SPβ d*leuD*-1, which carries all of the *ilvBC-leu* genes, we isolated specialized transducing phages that carry only some of these genes. To do this we transduced CU267 *ilvB2 leuB16 trpC2* (SPβ) with a lysate from the *citF*::SPβ strain CU1881. Ilv^+ transductants were selected and scored for their Leu phenotypes. Thirty-two of 100 Ilv^+ transductants were still Leu^-. SPβ lysates were prepared from 20 of them. Eleven of the 20 did not give HFT lysates for *ilvB*. Presumably they were haploid transductants, the result of replacement transductions in which ilv^+ alleles from the transducing phage replaced the recipients' *ilvB2* alleles. The other nine transductants gave rise to HFT lysates for $ilvB^+$. Two of the nine complemented mutations in *ilvB* but not in *ilvC*. Therefore, the genomes of the transducing phages in those lysates included a complete *ilvB* gene, and extended into but did not include all of *ilvC*; nor were any of the *leu* genes present. Similarly, four transducing phages were isolated that ended in *leuA*, one ended in *leuC*, and two ended in *leuB* (Fig. 3 and Table 3). After our discovery of *leuD* (see below), we isolated a transducing phage that ended in *leuD*, using CU2322 *ilvBΔ1 leuD117 trpC2* (SPβ *c*2) as a recipient. The use of the short phages in a complementation test is shown in Fig. 2b.

Identification of a fourth *leu* cistron, *leuD*. At the start of this work we knew of only three *leu* genes for the three leucine biosynthetic enzymes. While trying to classify a *leu* mutation in our collection we discovered that it was complemented by SPβ d*leuD*-1[*leuA5*], SPβ d*leuD*-1[*leuC124*], and SPβ d*leuD*-1[*leuB6*]. Thus this mutation, *leu-117*, was in a fourth *leu* complementation group which we named *leuD*. Subsequently, we identified two more mutations in the *leuD* complementation group.

TABLE 2. Bacterial strains lysogenic for null phages

Strain	Genotype[a]
CU2083	*ilvB2 leuA5 trpC2* (SPβ) (SPβ d*leuD*-1[*ilvB2*])
CU2084	*ilvB2 leuA5 trpC2* (SPβ) (SPβ d*leuD*-1[*leuA5*])
CU2085	*ilvC4 leuC124 trpC2* (SPβ) (SPβ d*leuD*-1[*ilvC4*])
CU2086	*ilvC4 leuC124 trpC2* (SPβ) (SPβ d*leuD*-1[*leuC124*])
CU2087	*ilvB2 leuB6 trpC2* (SPβ) (SPβ d*leuD*-1[*leuB6*])
CU2089	*ilvBΔ1 leuD117 trpC2* (SPβ) (SPβ d*leuD*-1[*leuD117*])

[a] The null phages are derivatives of SPβ *c*2 d*leuD*-1 that were produced in bacteria lysogenic for SPβ c^+. Since recombination occurs between whole phage genomes and defective phage genomes during phage replication (9), the null phages may carry either the *c*2 or c^+ allele.

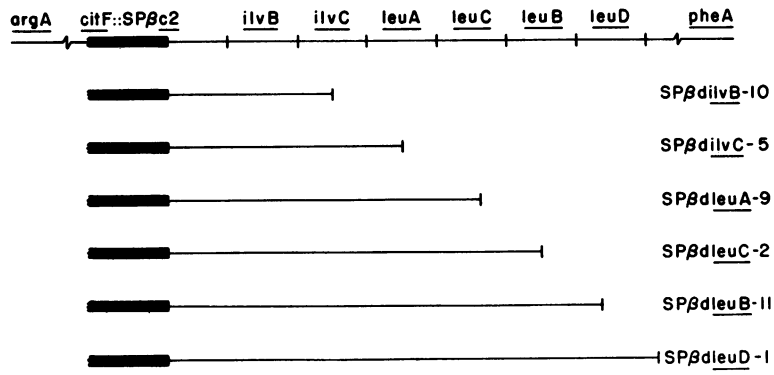

FIG. 3. Short defective specialized transducing phages. SPβ DNA is indicated by heavy lines. Horizontal lines indicate the extent of bacterial DNA included in different transducing phages.

The short phage SPβ d*leuB*-11 complements mutations in *ilvB*, *ilvC*, *leuA*, *leuC*, and *leuB*, but not in *leuD*. SPβ d*leuD*-1 complements all the mutations complemented by SPβ d*leuB*-11, plus mutations in *leuD*. Neither phage transduces *pheA*. Based on the gene order *citF-ilvB-leuB-pheA* (11) and the complementation pattern of the short phages, we predicted that *leuD* lies between *leuB* and *pheA*. To corroborate this evidence, we determined the gene order of *ilvB*, *leuD*, and *pheA* by transductional analysis. A PBS1 phage lysate produced in a wild-type strain was used to transduce CU232 *trpC2 ilvB2 pheA2*. Ilv$^+$ transductants were selected and scored for Phe$^+$. Of 100 Ilv$^+$ transductants, 65 were also Phe$^+$. Therefore, the distance between *ilvB2* and *pheA2* in PBS1 transduction units (1 − fraction cotransduced) is 0.35. When CU315 *trpC2 leuD117* was used as the donor, Ilv$^+$ Leu$^+$ transductants were selected and scored for Phe$^+$. Phe$^+$ transductants were also selected. We obtained 114 Ilv$^+$ Leu$^+$ transductants compared to 980 Phe$^+$ transductants. This ratio, 0.12, reflects the recombination distance between *ilvB2* and *leuD117*. Only 6 of 114 Ilv$^+$ Leu$^+$ transductants were also Phe$^+$. Since *ilvB2* and *pheA2* are only 0.35 transduction units apart, and *ilvB2* and *leuD117* are 0.12 transduction units apart, the small number of Phe$^+$ transductants suggests that a double recombination event is required to generate Ilv$^+$ Leu$^+$ Phe$^+$ transductants. These data strongly suggest the gene order *ilvB2-leuD117-pheA2*.

We then mapped *leuD117* with respect to *leuB6* and *ilvB2* by transformation. DNA from CU315 *leu-117 trpC2* (SPβ) was used to transform CU240 *ilvB2 leuB6 trpC2* (SPβ). Both Ilv$^+$ and Leu$^+$ transformants were selected. Of 100 Leu$^+$ transformants, 56 were also Ilv$^+$. This result suggests the order *ilvB2–leuB6–leu-117*.

DISCUSSION

We have isolated a strain of *Bacillus subtilis*, CU1881, in which the SPβ prophage is inserted into the *citF* gene. The prophage insertion prevents the formation of the *citF* gene product, succinate dehydrogenase. Gene function can be restored upon prophage excision. This is the first example of an insertion of the SPβ prophage into a gene, and it suggests the use of SPβ to generate insertion mutations in *B. subtilis*.

SPβ lysates of CU1881 contain specialized transducing phages for the *ilvBC-leu* genes. One phage, SPβ d*leuD*-1, carries all of the *ilvBC-leu* genes. In addition, SPβ d*leuD*-1 carries the *gerE* gene (7), which lies between *citF* and *ilvB* (Anne Moir, personal communication).

We have analyzed the transductants produced by SPβ d*leuD*-1 and found that two classes are produced. Haploid recombinants result from the replacement of chromosomal DNA sequences of the recipient by homologous sequences carried by the phage. We call such events replacement

TABLE 3. Bacterial strains lysogenic for short phages

Strain	Genotype
CU1889	*ilvC9 leuA169 metB5* (SPβ *c2*) (SPβ *c2* d*leuD*-1)
CU2073	*ilvB2 leuB16 trpC2* (SPβ) (SPβ *c2* d*ilvB*-10)
CU2075	*ilvB2 leuB16 trpC2* (SPβ) (SPβ *c2* d*ilvC*-5)
CU2078	*ilvB2 leuB16 trpC2* (SPβ) (SPβ *c2* d*leuA*-9)
CU2080	*ilvB2 leuB16 trpC2* (SPβ) (SPβ *c2* d*leuC*-2)
CU2092	*argA2 ilvB2 leuD117 trpC2* (SPβ) (SPβ *c2* d*leuB*-11)

transductions. Partial diploids are generated by the addition of the transducing phage DNA to the recipient's genome. We call such events addition transductions. When SPβ d*leuD*-1 was used as the donor, about two-thirds of the transductants resulted from addition transductions.

The site for integration of the transducing phage genome in an addition transduction is determined by DNA homology. The defective specialized transducing phage genome can insert into the region of bacterial homology in a nonlysogenic recipient strain (9). SPβ d*leuD*-1 may integrate into either of two regions in a lysogenic recipient: the *ilvBC-leu* region (B configuration), or the prophage in the normal attachment site (P configuration). We determined the location of the transducing phage DNA in 14 double lysogens by showing linkage of the wild-type *ilvB* gene carried by the phage to *pheA* (near *ilvBC-leu*) or to *metB* (near the normal attachment site). We showed that SPβ d*leuD*-1 is in the B configuration in seven double lysogens and in the P configuration in five. Our result with the two remaining double lysogens was surprising; the *ilvB*$^+$ allele was linked to both *pheA* and *metB*. Although we have not yet characterized these lysogens further, it is possible that a gene conversion event resulted in the conversion of *ilvB2* to *ilvB*$^+$ in these strains. Alternatively, two copies of SPβ d*leuD*-1 may be present, one in the B configuration and one in P.

We have described manipulations to produce recombinants of SPβ d*leuD*-1 that carry mutant bacterial alleles. By these manipulations we have compiled a set of null phages. Each phage in the set carries a mutant allele for one of the *ilvBC-leu* genes. We also isolated transducing phages from CU1881 that carry less bacterial DNA than SPβ d*leuD*-1 does. Each phage carries a unique amount of genetic material from the *ilvBC-leu* region. We refer to this set as short phages.

We devised a simple complementation test using both the null and short phages to identify the gene within which any *ilv* or *leu* mutation lies. Figure 2, for example, shows the complementation pattern for the *leuD117* mutation. The *leu* mutation of the recipient strain is complemented by three of the four null phages (Fig. 2a). The noncomplementing phage, SPβ d*leuD*-1[*leuD117*], has a nonfunctional *leuD* gene; thus the mutation must be in *leuD*. We have found that genetic complementation can easily be distinguished from recombination because complementation produces the wild-type phenotype at a much higher frequency.

In agreement with these results, the phage SPβ d*leuD*-1 complemented the *leu* mutation, but the short phage, SPβ d*leuB*-11, did not (Fig. 2b); therefore the mutation must lie in *leuD*. Our collection of short phages is useful in some additional ways. First, they provide information concerning gene order. For example, SPβ d*leuD*-1 complements all *leu* mutations. SPβ d*leuB*-1 complements mutations in *leuA*, *leuC*, and *leuB*, but not in *leuD*. This indicates that *leuD* lies beyond (to the right of) *leuA*, *leuC*, and *leuB*. Our results for the gene order *ilvB-ilvC-leuA-leuC-leuB-leuD* are consistent with the published order (11). Second, the short phages provide information about the relative order of mutations within a cistron. For example, SPβ d*leuC*-2 carries bacterial DNA that ends within *leuB*. When this phage is spotted onto various *leuB* mutants, no complementation (heavy growth) is seen. When it is spotted onto a *leuB6* mutant a few colonies result, but none are produced on a *leuB16* mutant. This result suggests that, although SPβ d*leuC*-2 does not carry the entire *leuB* gene, it does carry the wild-type allele for *leuB6*; thus Leu$^+$ recombinants can be produced. Since no Leu$^+$ recombinants are produced with a *leuB16* mutant, the endpoint of SPβ d*leuC*-2 is before, or very close to, *leuB16*. Thus *leuB6* lies to the left (proximal to *leuC*) of *leuB16*). This agrees with published mapping data (11).

Similar complementation tests can be done with *ilv* mutations. *ilvB* and *ilvC* are only two of the four known *ilv* genes. *ilvA* and *ilvD* lie just to the left of the normal SPβ attachment site. Our laboratory has specialized transducing phages for these genes, and they are included in our complementation testing for *ilv* mutations (1).

We have provided strong evidence for the existence of a fourth *leu* cistron, *leuD*, which lies to the right of *leuB*. It is interesting that there are also four *leu* cistrons in *E. coli* (10) and *S. typhimurium* (6), and the *leu* gene order in *B. subtilis* is identical to the order found in those two species. Although we have not performed the enzyme assays, *leuD* probably encodes one of two polypeptide chains of IPM isomerase, as it does in the enteric bacteria.

We plan to use our transducing phages for *ilvBC-leu* to study control mutations that affect the expression of these genes. We hope to construct a restriction endonuclease map of the region and correlate it with the genetic map by using the short phages. Finally, we are using SPβ d*leuD*-1 as a source of DNA for subcloning the *ilvBC-leu* genes into plasmid vectors.

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