Control of Lysogeny and Immunity of *Bacillus subtilis* Temperate Bacteriophage SP β by Its *d* Gene

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The *d* gene from the *Bacillus subtilis* temperate bacteriophage SP β was isolated. When introduced into an SP β -sensitive strain of *B. subtilis*, the cloned *d* gene directed the synthesis of a 22-kilodalton protein and conferred on the host immunity to SP β phage. A frameshift mutation, designated *d*2, was introduced into the cloned *d* gene, and it was subsequently crossed back into the SP β phage genome. The resulting SP β phage grew lytically and formed clear plaques on sensitive bacteria. Although the cloned *d* gene confers immunity to the host, we could not detect complementation of the *d* gene by mixed infection with SP β *d*2 and various SP β *c* mutants. The nucleotide sequence of the 1,033-base-pair *Pst*I-to-*Eco*RI fragment containing the *d* gene was determined; it includes an open reading frame that could potentially encode a protein of 227 amino acids. The gene was mapped within the *Pst*I H fragment on the phage genome, which positions the *d* gene about 25 kilobases from the right end of the phage genome. It is transcribed from right to left.

Bacillus subtilis 168 and most of its derivatives are lysogenic for the temperate bacteriophage SP β (18). This omnipresence of the SP β prophage implies the existence of an efficient mechanism for the establishment and maintenance of lysogeny. The available data indicate that the repressor protein encoded by the *c* gene plays a central role in the establishment of lysogeny by turning off lytic-cycle genes (16, 18). Wild-type SP β (*c*⁺) phage can be induced by mitomycin C; it replicates at temperatures below 50°C and forms cloudy plaques on sensitive bacteria (16). The SP β phage carrying the thermoinducible repressor gene (SP β *c*2) also forms cloudy plaques under normal growth temperature, but when in the prophage state it can be induced by a brief heat shock of the lysogens at 50°C (13). In contrast, clear-plaque mutants such as SP β *c*1 are unable to lysogenize sensitive *B. subtilis* strains and therefore form clear plaques. Double lysogens containing both the *c*1 and *c*2 prophages can be heat induced, while those containing *c*⁺ and *c*2 cannot (13). Thus, the *c* gene encodes a *trans*-acting repressor protein involved in the establishment and maintenance of lysogeny. All the clear-plaque mutants so far tested fall within the same complementation group (18). In our attempt to clone the repressor gene from the 126-kilobase (kb) phage genome, we uncovered a new genetic element involved in controlling phage lysogeny and immunity. The biochemical and biological characterization of the cloned *d* gene is reported here.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains, phages, and plasmids used are listed in Table 1. The liquid media used were TB (1% tryptone [Difco Laboratories, Detroit, Mich.], 0.5% NaCl) and modified M (1% tryptone [Difco], 0.5% yeast extract, 1% NaCl, 5 mM CaCl₂, 5 mM MgSO₄, 0.02 mM MnCl₂) as described previously (12). Modified M medium was used for growing *B. subtilis* cells and preparing phage lysates. TBA soft agar (TB containing 0.7% agar) was used when determining phage titers. Tryptose blood agar base (TBAB; Difco), containing 5 µg of chloramphenicol per

ml when needed, was used as a plating medium for phage and bacteria.

Phage growth and DNA preparation. Phage SP β *c*2 was prepared from strain CU1147 by the heat induction method essentially as described by Rosenthal et al. (13). Wild-type SP β phage was prepared either by mitomycin C (0.8 µg/ml) induction or, as for SP β *c*10 (described below), by growing phages lytically by the method of Warner et al. (16) with the following modifications: (i) the adsorption period at 37°C was 15 to 20 min; (ii) the centrifugation step to remove unadsorbed phage was omitted; and (iii) infected cells were diluted 30- to 100-fold into prewarmed (37°C) modified M medium.

Purified phage particles were prepared by the method of Fink et al. (3) with several modifications. Polyethylene glycol 6000 was added to 10% (wt/vol) to the phage suspension along with NaCl to 0.5 M to precipitate most of the phage particles. Polyethylene glycol-precipitated phage pellets were suspended in CsCl (1.497 g/ml, in a 10 mM tris(hydroxymethyl)aminomethane [pH 7.4]–2 mM MgSO₄–10 mM CaCl₂ buffer) without attempting to remove the trace amount of polyethylene glycol and centrifuged to equilibrium with a Beckman Ti 70.1 rotor at 24,500 rpm for 28 h or with a VTi80 rotor at 60,000 rpm for 6.5 h at 18°C. After collection and dialysis of the phage band, phage suspensions were concentrated, and the phage DNA was extracted with TE (10 mM Tris(hydroxymethyl)aminomethane [pH 8.0], 1 mM EDTA) buffer-saturated phenol followed by two chloroform extractions and ethanol precipitation.

Isolation of clear-plaque SP β *c*10. During the preparation of SP β *c*⁺ phage from the SU+3 (SP β) strain, a spontaneous clear-plaque phage (designated SP β *c*10) was fortuitously identified. In all the tests performed, SP β *c*10 was indistinguishable from SP β *c*1; they were both used in these studies.

Construction of SP β *c*2 phage DNA library and other molecular cloning methods. Plasmid pLP1201 (11) replicates in both *Escherichia coli* and *B. subtilis*, and it was used for the construction of the phage gene library. *Bam*HI and *Bgl*III doubly digested SP β *c*2 DNA (2.8 µg) was ligated to *Bam*HI-digested pLP1201 (1 µg) DNA at a final DNA concentration of about 300 µg/ml. *E. coli* MM294 (9) was transformed with the ligation reaction mixture and plated on Penassay agar (Antibiotic Medium 2; Difco) containing 50 µg of ampicillin

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TABLE 1. Strain list

Strain	Relevant genotype/properties	Source/reference
<i>Bacillus subtilis</i>		
CU1050	SP β ^s <i>metB5 sup-3 thr leu ade</i> (su ⁺ ³)	S. A. Zahler; 13,16
CU1147	<i>trpC2</i> (SP β <i>c2</i>)	S. A. Zahler; 13
BGSC no. 1L4	su ⁺ ³ (SP β); source of wild-type SP β	Bacillus Genetic Stock Center; 16
CU1225	<i>citK1</i> ⁻ Δ <i>ilvB1 kauA1 att</i> ^{SPβ} (SP β <i>c2 dcitK</i> ⁺ ⁻¹)	S. A. Zahler; 13
CU3082	<i>ilvA2 ilvD15 leuB6 leuB16 att</i> ^{SPβ} (SP β <i>c2 dilvD</i> ⁺ ⁻¹)	S. A. Zahler
<i>Escherichia coli</i>		
MM294	<i>thi-1 hsdR17 endA1 supE44</i> F ⁻	9
DG99	<i>thi-1 hsdR17 endA1 supE44 lacI</i> ^s <i>lacZ</i> Δ M15 <i>proC</i> ::Tn10; host for pUC plasmid vectors	D. Gelfand
DG98	DG99/F' <i>lacI</i> ^s <i>lacZ</i> Δ M15 <i>proC</i> ⁺ (Pro ⁺ Tet ^r host for M13 phage)	D. Gelfand
Vectors		
pLP1201	<i>E. coli/B. subtilis</i> shuttle vector; Cm ^r in <i>B. subtilis</i> , Ap ^r and Tc ^r in <i>E. coli</i>	11
pUC18	<i>E. coli</i> cloning vector with polylinker sequence; Ap ^r	10
M13mp10 and M13mp11	Coliphage vectors for cloning and sequencing	10
Phages		
SP β <i>c</i> ⁺	Wild-type <i>c</i> gene	16
SP β <i>c2</i>	Temperature-sensitive mutation in <i>c</i> gene, inducible at 50°C, normal in permissive temperature range	13
SP β <i>c10</i>	Inactive <i>c</i> gene	This study
SP β <i>c1</i>	Inactive <i>c</i> gene, also contains <i>del1</i>	4,16
SP β ::Tn917 <i>c1</i>	Tn917 inserted in SP β <i>c1</i>	S. A. Zahler

per ml. Of 520 Amp^r colonies tested for their sensitivity to tetracycline (15 μ g/ml), about 19% of the transformants were sensitive; presumably they contained inserts in the *Bam*HI site of the tetracycline resistance gene. The transformants harboring recombinant plasmids were grown in pools of five, and the plasmid DNAs were isolated (6), digested with *Xho*II, and analyzed by agarose gel electrophoresis. On the basis of the pattern of the restriction fragments, we chose six pools of these DNA preparations for transformation into *B. subtilis*. Competent cells were prepared (1) from the phage-sensitive strain CU1050 and transformed with the pooled plasmid DNAs. The transformants were then tested for immunity to SP β *c10* phage by the cross-streak test described below.

Restriction endonucleases were purchased from either New England BioLabs, Inc. (Beverly, Mass.) or Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) and used according to the specifications of the suppliers. Preparation of plasmid (5) and chromosomal (2) DNAs was essentially as previously reported. DNA restriction fragments were separated on either polyacrylamide or agarose slab gels, depending on their size, and electroeluted as described previously (7), except that the buffers used were 0.05 $\times$ TBE for acrylamide and 0.1 $\times$ TEA (4 mM Trischloride [pH 8.0], 0.1 mM EDTA, 0.5 mM sodium acetate) for agarose gels.

Site-directed mutagenesis was carried out as described previously (20). A synthetic oligonucleotide with the sequence 5'-GATATATTTTCATAAGCTTGGAATCAGAT-3' was used to modify the *d* gene sequence around nucleotide position 405. Other methods for molecular cloning in *E. coli* were essentially as described previously (7).

Determination of DNA sequence. Appropriate DNA fragments were cloned into the M13 phage vectors mp10 and mp11 (10). Single-stranded DNA templates were prepared, and the DNA sequence was determined on both strands by the dideoxyribonucleotide chain termination method (14, 15) with [α -³⁵S]dATP obtained from New England Nuclear

Corp. (Boston, Mass.). A synthetic 17-mer with the sequence 5'-GGTCAAATCTGTACACGC-3', designed according to the preliminary sequencing data, was subsequently used as an additional sequencing primer to allow sequencing across the *Cla*I site at position 666 within the *d* gene.

Tests for SP β lysogeny and immunity. To look for the presence of SP β prophage, we tested the *B. subtilis* strains by the method described by Rosenthal et al. (13) for their immunity to clear-plaque mutants of SP β , their ability to release phage particles, or their ability to produce betacin. Transformants of CU1050 carrying various plasmids were tested for their immunity to SP β by streaking transformant colonies across a vertical line along which 1 $\times$ 10⁸ to 2 $\times$ 10⁸ SP β *c10* phage had been applied on a TBAB plate containing 5 μ g of chloramphenicol per ml (19). CU1050 cells carrying only the vector pLP1201 were lysed on plates after infection by SP β *c10* phage and thus served as the negative control; they grew only on the first half of the cross streak. CU1050(SP β *c2*) cells carrying pLP1201 served as the positive control which grew across the entire streak.

Crossing the *d2* frameshift mutation back into the SP β phage genome. Cells of CU1147, a strain lysogenic for SP β *c2* phage, were made competent and then transformed with pJM172, the plasmid containing the *Cla*I-to-*Nru*I +2 frameshift mutation in *d*. One of the transformants was used to inoculate 5 ml of TB containing 5 μ g of chloramphenicol per ml and grown for 5 to 6 h. The titer of the supernatant containing phage particles was determined on a lawn of CU1050(pLP1201) cells. Spontaneous release of SP β phage was between 10⁶ and 10⁷ PFU/ml. Of approximately 10⁴ plaques screened visually, 2 clear plaques were picked. One of these was plaque purified through three plating cycles. The clear-plaque phage isolated was then grown up (500 ml) and gradient purified as described above. Phage DNA was extracted, and the presence of the *d2* allele was confirmed by analysis of restriction endonuclease digestions for the presence of the diagnostic *Nru*I site.

To construct the SP β *c1 d2* double mutant, CU1050 (pJM172) cells were spotted with SP β *c1* lysate on a plate. After overnight incubation, phage from the cleared zone were collected and plated on CU1050 indicator cells. Cloudy, but distinctively smaller, plaques appeared at a frequency of about 10^{-3} . These phage were plaque purified, and the presence of the *d2* allele on the genomes was confirmed as described above.

RESULTS

Molecular cloning of the *d* gene from SP β *c2*. A gene library of SP β *c2* genome cloned into the bifunctional plasmid pLP1201 was constructed (see Materials and Methods). Competent *B. subtilis* CU1050 cells were transformed with plasmids prepared from the pooled cultures. Individual transformants were then tested for SP β *c10* immunity by the cross-streaking test. Of 234 transformants tested, 15 showed immunity. Plasmid DNA from six of these was prepared and analyzed. Three of the six appeared to have identical restriction patterns for *Eco*RI, *Pst*I, and *Sal*I, while the other three differed but appeared to be related clones that had suffered deletions presumably outside of the cloned immunity gene sequence. The restriction map of one of the undeleted candidates, designated pJM135, is shown in Fig. 1. Since the phage genome was digested with *Bam*HI and *Bgl*II and the vector was cleaved with *Bam*HI, we checked pJM135 plasmid DNA for the presence of regenerated *Bam*HI sites. No *Bam*HI recognition sites were present at either junction with pLP1201 DNA. Therefore, most likely the cloned fragment in pJM135 was derived from a 5.9-kb *Bgl*II fragment in the SP β *c2* genome. The cloned gene that confers immunity was designated the *d* gene.

Mapping of the *d* gene fragment. The approximate location of the 5.9-kb fragment on the SP β genome was determined by Southern hybridizations. The restriction maps of the SP β genome for the *Ava*I, *Bam*HI, *Sac*I, and *Sal*I endonucleases have been reported previously (4). SP β *c2* DNA digested individually with each of these enzymes and fractionated on a gel was hybridized with 32 P-labeled *Sal*I fragments of pJM135. The cloned SP β DNA hybridized with the *Ava*I D fragment, the *Bam*HI A fragment, the *Sac*I F fragment, the *Sac*I B fragment (very weakly), and the *Sal*I C and D fragments of SP β (data not shown). This hybridization pattern is consistent with the assignment of the location of the *d* gene in the SP β genome as shown in Fig. 2.

To localize the *d* gene within the cloned phage DNA, we constructed various subclones. A deletion of the 1.2-kb *Eco*RV fragment (Fig. 1) had no effect on the immunity phenotype of the transformants. In contrast, transformants carrying plasmids lacking either the 3.9-kb *Cla*I fragment [*Cla*I₍₁₎ to *Cla*I₍₂₎] in Fig. 1] or the 4.5-kb *Cla*I fragment [*Cla*I₍₂₎ to *Cla*I₍₃₎] in Fig. 1] from pJM135 lost the immunity to phage infection. It therefore seemed likely that the *d* gene spans the *Cla*I₍₂₎ site.

In vitro construction of frameshift mutations in the *d* gene. Mapping data revealed the presence of multiple *Sca*I and *Pst*I sites in the cloned SP β fragment in pJM135 (Fig. 1). The 2.9-kb *Pst*I₍₁₎ to *Sca*I₍₂₎ fragment (Fig. 1) generated by partial *Pst*I digestion and complete *Sca*I digestion was cloned into *Pst*I- and *Sma*I-digested pUC18 plasmid (10). The resulting plasmid was designated pJM154. This plasmid has a unique *Cla*I restriction site within the SP β DNA insert (Fig. 1). After *Cla*I digestion and repair with *E. coli* DNA polymerase Klenow fragment, these molecules were ligated and used for transforming *E. coli* MM294. The transformants were screened for the generation of a *Nru*I site that would result

FIG. 1. Restriction map of the plasmid containing the *d* gene. The shaded region is the SP β *c2* DNA insert in the pLP1201 vector. Marks at 1-kb intervals are shown inside the circle. Some restriction enzyme sites are numbered with subscripts to facilitate identification. The location for the chloramphenicol resistance (Cm) and the ampicillin resistance (Ap) genes as well as the replication origins for *E. coli* (E) and *B. subtilis* (B) are marked.

from the ligation of completely repaired *Cla*I ends. The majority of the plasmids had lost the *Cla*I site and gained an *Nru*I site; one of these clones was designated pJM169. The minority of the resulting plasmids had neither a *Cla*I nor an *Nru*I site; one of these was designated pJM158. The 1,033-base-pair (bp) *Pst*I-*Eco*RI fragment from each of these plasmids (pJM154, pJM158, and pJM169) was purified by electroelution from 4% acrylamide gels and ligated to *Pst*I- and *Eco*RI-digested pLP1201 to replace the small *Pst*I-*Eco*RI fragment carrying most of the pBR322 β -lactamase gene. Ap^r Tc^r transformants were screened by restriction digestion to ensure the presence of the SP β DNA insert, and the resulting plasmids were designated pJM160 (*d*⁺; with *Cla*I site), pJM170 (*d*⁻; without *Cla*I), and pJM172 (*d*⁻; with *Nru*I replacing *Cla*I).

B. subtilis CU1050 transformants carrying pJM160 were immune to SP β *c*⁺ as well as SP β *c10* and other SP β *c*⁻ phage. In contrast, CU1050(pJM170) and CU1050(pJM172) did not confer immunity to these phage. From these data we conclude that the *d* gene resides within the 1-kb *Pst*I-*Eco*RI fragment and that the *Cla*I site is located within the *d* gene.

DNA sequence of the *Pst*I-*Eco*RI fragment of SP β phages. The sequencing strategy used to determine the DNA sequence of the SP β *Pst*I-*Eco*RI fragment is shown in Fig. 2. The 1,033-base sequence of the antisense strand of this fragment isolated from SP β *c2* phage is shown from the 5'-end *Pst*I site to the 3'-end *Eco*RI site in Fig. 3. A long open reading frame exists from position 135 through 815 within which is located the *Cla*I site (position 666). Four AUG (Met) and one GUG (Val) codons could be the initiation site for the *d* gene protein (Fig. 3).

FIG. 2. Map location of the cloned *d* gene on the SP β phage genome. *Pst*I and *Sal*I restriction maps of SP β DNA are taken from published work (4). The order of the *Pst*I K, H, and T fragments was determined experimentally. The shaded region is the 5.9-kb *Bgl*II fragment cloned in pJM155. The arrows represent the direction of transcription of the *d* gene. The *Eco*RI-*Pst*I fragment containing the *d* gene is expanded; the positions of the restriction sites within this fragment are indicated according to the DNA sequence shown in Fig. 3. The sequencing strategy is depicted at the bottom.

In addition to the sequence of the *d* gene shown in Fig. 3, the sequence of the insertion mutants at the *Cla*I site was also determined. A synthetic 17-mer oligonucleotide was used as the sequencing primer to determine the nucleotide sequence surrounding the *Cla*I site regions in pJM154, pJM158, and pJM169. The sequence across the *Cla*I site in pJM154 was 5'...TAT,ATC,GAT,TTT,CTC...3' (Fig. 3). The corresponding sequences for pJM158 and pJM169 were 5'...TAT,ATC,CGA,TTT,TCT,C...3' and 5'...TAT,ATC,GCG,ATT,TTC,TC...3', respectively.

The *Cla*I site was apparently only partially repaired, resulting in a +1 frameshift mutation in the *d* gene on pJM158. The properly repaired *Cla*I ends were ligated and generated a +2 frameshift mutation and an *Nru*I site (TCGCGA) in the *d* gene on pJM169. We designate the alleles with the +1 and +2 frameshift mutations *d*1 and *d*2, respectively.

The same *Pst*I-*Eco*RI fragment was also isolated from the SP β c1, c10, and c⁺ genomes by cloning into the coliphage M13mp10 vector. The sequences of these fragments were analyzed. They were all identical to that isolated from SP β

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1  CTGCAGATTATCTCAAAGAACTGCTCATTGAGATAAAATCAGAAAAGAACTAATGATTTCACTTTCCATTATACAGTTCTCCATGACTGGAAACAATGGTATAATGTAGGAAATGATA
   P
121  CCGCGCGTGGTGGTGAATGGGATATAAGTTTATGGCTTATGGTGGCTATTTTATTCTGTCTTTTCTTTTGGCTAATGGACGGCTGGAGAGGCATGGGAATTTGCTTAATAATTGTAGGTT
   M G Y K F M A Y G G Y F L F C L F F L L M D G W R G M G I C L I I V G 35
241  TAGCCCTATTAGCACTTGAGCCCTATAAAATTAAGCCCAAAAAATATAGATAAACTAAAAGAAAATGCGGAACACTTAAGCACTTCGACGGTGGTTTAAATCCAGACAATTTCTTTA
   L A L L A L E P Y K I K A Q K N I D K L K E N A E T L K H F D G G F N P D N F F 75
361  ATACTTACAAAACATAAATTCATTTAAGGAATCTGATTCCTTTGTGAAATATATCAGCTTAATAAGATGAACATATTGAAGAATACACAATCCCTTTTCCAATGTTATAGAATCCG
   N T Y K T K I A F K E S D S L V K I Y Q L N K D E H I E E Y T I P F S N V I E S 115
481  AAATTGCTTTAGACAATCAAAATAATTTCTAAAGTATCAAAATCAGGAATCGTAGCTGGTGGCTGTTAGCTGGAGGAATTGGAGCTGCAATTGGAGGGTTGTCTGCCCTCTCAATACAAA
   E I A L D N Q I I S K V S K S G I V A G G L L A G G I G A A I G G L S A S S I Q 155
601  ATGAAATGGTCAAATCTGTCACGCTAAAGATTACTGTTGAAGACCTCGGTAAAGCCTATCCATTATATCGATTTTCTCCACACAGAAGTTGAAGGGTATAATATTCAGGGGTATAAAA
   N E M V K S V T L K I T V E D L G K P I H Y I D F L P T Q E V E G Y N I Q G Y K 195
   C
721  AAGATAGCAATGTCAATCAACAGCACTTACGAATGAGAATATTGGCATGGTGTATGGATGTAATTAAGAAGCAAAACAAAGTCGCTCAATAACTGAGTGGCTTTTCTTTTGTG
   K D S N V I Q Q A L T N A E Y W H G V M D V I I K K A N K V A Q * 227
841  CTCTCCCTACTGAAAGGAAGTGATTCCTACTTGAGTCAAAACCTCAAAATTATACTAACCCGCAAGCTGATACCTCATCCAAAACGTGCGAACAGTTAAATCAGCAAAATTAATCCCT
   X
961  GGAAAAGAACTCAACTCCCTCAAGCTCAATACAAACATTGATTCTACAACCTTAAAGCTCTGCAAGAAATTC
   E

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FIG. 3. DNA sequence of the 1,033-bp *Pst*I-*Eco*RI fragment of SP β c2. Restriction sites are designated by upper-case letters as follows: P, *Pst*I; C, *Cla*I; X, *Xmn*I; and E, *Eco*RI. The potential translational initiation sites (in-phase AUG and GUG codons) are underlined. The deduced amino acid sequence for the entire open reading frame is shown in single-letter code. The amino acids are numbered on the right, and nucleotide positions are numbered on the left.

c2. Although the *d* gene was identified by its ability to confer on its host immunity to superinfecting SP β phage, this piece of biochemical data clearly established the fact that the cloned immunity gene is distinct from the repressor *c* gene.

Analyses of the *d* gene protein. By comparing the sizes of the proteins produced in the *E. coli* in vitro transcription-translation system directed by plasmids pJM154, pJM158, and pJM169, a protein of 15 kilodaltons (kDa) was identified initially as the *d* protein. Sequence analysis of the amino-terminal portion of this protein (data not shown) indicated that it corresponded to the peptide initiated at the GUG (Val) codon at nucleotide position 405 shown in Fig. 3.

The sequence downstream from the GUG codon at nucleotide position 405 consists of an open reading frame of 137 codons. This 137-amino acid polypeptide was used as the antigen to raise antiserum. To achieve this, we carried out oligonucleotide-directed site-specific mutagenesis to convert the GUG codon at position 405 (Fig. 3) to an AUG codon and introduced a *Hind*III site immediately 5' to the ATG sequence (see Materials and Methods). The sequence between the newly created *Hind*III site and the *Xmn*I site (nucleotide position 858) beyond the termination codon was then cloned behind the coliphage lambda gene *N* Shine-Dalgarno sequence and the *p_L* promoter (H. C. Wong and Y. E. Ting, unpublished data). The plasmid containing this sequence was introduced into a proper *E. coli* strain and, under the conditions that induced the *p_L* promoter, a large amount of the 15-kDa protein was produced. It was isolated and used for raising rabbit antiserum against the *d* gene protein.

The *d* gene proteins encoded by the SP β prophage and by various plasmids were analyzed by the immunoblotting method, using the rabbit antiserum raised against the 15-kDa protein. Cell extracts were prepared from CU1050, CU1147, and CU1050 derivatives harboring pLP1201, pJM160, or pJM172. The results (Fig. 4) indicated that the in vivo-synthesized *d* gene protein is about 22 kDa. Furthermore, the *d* gene protein was only detected in CU1050(pJM160). Although the method used was sensitive enough to detect one molecule of the 15-kDa peptide per cell in a test experiment, no signal was obtained in the extracts from CU1147 that carries the SP β c2 prophage. We also did not detect immunoreactive proteins from the extracts of CU1050(pJM172). This suggests that the *d2* protein probably is unstable in vivo.

We also fused the DNA fragment encoding the amino-terminal portion of the *d* gene (positions 1 to 540; Fig. 3) to the *E. coli lacZ* coding sequence and obtained results (not shown) indicating that the fusion gene directed the synthesis of enzymatically active β -galactosidase in vivo in both *E. coli* and *B. subtilis* CU1050. The *lacZ* fusion data show that a promoter and a translation initiation signal that function in both *E. coli* and *B. subtilis* transformants are contained between the *Pst*I and *Hae*III sites located in this region, but the precise location of these regulatory sequences has not been further defined.

SP β phage does not lysogenize cells carrying the cloned *d* gene. The introduction of the two frameshift mutations into the *d* gene (as described for pJM170 and pJM172) was correlated with the loss of the immunity phenotype. To test whether the apparent immunity conferred to the host by the *d* gene is due to efficient lysogeny of the infecting SP β phage, we performed the following tests. SP β c1 lysate was spotted on a lawn of CU1050(pJM160) and incubated overnight. Although no lysis was evident, cells within the zone where phage lysate was spotted were isolated and tested for the presence of SP β prophage. Of 50 isolates tested, none gave

FIG. 4. Immunoblotting analysis of the *d* proteins synthesized in vivo. Cellular extracts prepared from *B. subtilis* CU1050 (lane b), CU1147 (lane c), CU1050(pLP1210) (lane d), CU1050(pJM160) (lane e), and CU1050(pJM172) (lane f) were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose paper, and then probed with the antiserum raised against the purified 137-amino acid peptide (lane a) derived from the *d* protein. The immunoreactions were visualized by the horseradish peroxidase-TMB-dextran sulfate method (kindly prepared and provided by W. Bloch et al. of Cetus Corporation). The molecular weight of the protein markers ($\times 10^3$) is shown on the right.

a positive result. We also used an alternative test which permits direct selection of lysogens. Phage SP β ::Tn917 c1 is a c1-derived clear-plaque phage containing the Tn917 transposon (17). Cells lysogenic to this phage are resistant to erythromycin and lincomycin because of the presence of the resistance gene on the transposon. Cells from strain CU1050(pJM160) were infected with SP β ::Tn917 c1 at a multiplicity of infection of 1. After absorption and incubation, 2×10^8 infected cells were plated on selective medium. No antibiotic-resistant colony was obtained. Assuming that the Tn917 transposon in this phage does not inactivate any SP β gene that is involved in immunity and lysogeny, these results indicate that the presence of the cloned *d* gene in the host prevents lysogeny as well as lytic growth of the infecting SP β c1 phages.

Genetic analyses of SP β phage carrying the *d2* mutation. We crossed the *d2* allele into the SP β c2 genome and studied the phenotype of the SP β c2 *d2* phage (see Materials and Methods). Two clear-plaque phages were identified among the spontaneously released phages from CU1147(pJM172) transformants. Phage DNA from one of these clones was isolated and analyzed. The presence of an additional *Nru*I site that is diagnostic of the *d2* allele confirmed that the phage indeed carried the *d2* mutation. The genome of the clear-plaque phage contained two *Nru*I sites approximately 6.6 kb apart (Fig. 5A, lane b) instead of a single site found in the c2 phage genome (Fig. 5A, lane b). Thus, the *d2* mutation causes the clear-plaque phenotype in the SP β c2 *d2* phage. We also crossed the *d2* mutation into the lytic phage SP β c1

FIG. 5. Electrophoretic analysis of SP β *c2* and SP β *c2 d2* phage DNAs. Molecular weight markers are *Eco*RI-*Hind*III-digested coliphage λ DNA (lanes c and h) and *Sal*I- and *Pst*I-digested SP β phage DNA (lanes d and i, respectively) as previously reported (4). (A) A 0.4% agarose gel fractionation of SP β *c2* and SP β *c2 d2* DNAs digested with *Nru*I (lanes a and b, respectively), with *Sal*I (lanes d and g, respectively), and with *Sal*I and *Nru*I (lanes e and f, respectively). (B) A 0.8% agarose gel fractionation of SP β *c2* and SP β *c2 d2* DNAs digested with *Pst*I (lanes h and i, respectively) and with *Pst*I and *Nru*I (lanes j and k, respectively).

(see Materials and Methods). The SP β *c1 d2* phage formed smaller and cloudy plaques on a CU1050 lawn. The cells within the plaques were isolated and further characterized. They appeared to be lysogenic to SP β phage since they spontaneously released phage particles and produced betacin. However, these lysogens were very unstable. Upon several transfers by streaking on plates, cured cells appeared at high frequency. This *c1 d2* phage is phenotypically similar to the *int*⁻ SP β phage that forms abortive lysogens as described by Zahler (18). The main difference is that the *c1 d2* phage forms cloudy plaques in the absence of a functional repressor gene owing to the *c1* mutation.

The clear-plaque SP β *c2 d2* phage was further tested for complementation with SP β *c1* by mixed infection. When the mixed lysate was spotted on the CU1050 lawn and incubated overnight, the clear zone appeared to be the same as those seen with either phage lysate alone; only scattered phage-resistant colonies grew within the lysis zones. Upon further incubation for a few hours, a thin film of bacteria could be detected in the lysis zone spotted with the mixed lysate, but not with the individual lysates. These cells were picked and purified by repeated streaking. All of them spontaneously released cloudy-plaque phage morphologically similar to the SP β *c2* phage. In addition, of 10 independently isolated phage tested, none carried the diagnostic *Nru*I site unique to the *d2* mutation. Thus, these cells were lysogenic for SP β *c2* that was generated by recombination between the *c1* and the *c2 d2* phage.

Physical mapping of the *d* gene. The sequencing primer

complementary to the internal sequence of the *d* gene (nucleotide positions 608 to 624 in Fig. 3) was used to probe the genomes of strains carrying defective SP β prophages. Southern analysis data are shown in Fig. 6. The probe reacted to the total DNA isolated from CU1050(SP β *c2*) (strain CU1147). No hybridization was detected with strains carrying either SP β *c2 dcitK*⁺-1 or SP β *c2 dilvD*⁺-1. It is interesting to note that the *citK* and *ilvD* markers are located on the opposite side of the attachment site for SP β on the chromosome; thus, each of these two defective phage presumably has a different phage arm deleted. Our data suggest that one of the defective phage might carry an additional deletion previously undetected by genetic analyses.

The precise physical location of the *d* gene on the phage genome was determined by mapping the *Nru*I site unique to the *d2* allele. The *Nru*I site present in the SP β *c2* genome is located in the *Sal*I D fragment. The *Sal*I-*Nru*I double digestion resulted in the approximately 18-kb *Sal*I D fragment (Fig. 5A, lane d) being cleaved into two fragments of about 9.7 and 7.5 kb (Fig. 5A, lane e). The additional *Nru*I site in the clear-plaque mutant *c2 d2* also maps in the *Sal*I D fragment. Similar double digestion produced two fragments of about 6.6 and 3 kb (Fig. 5A, lane f) from the original 9.7-kb *Sal*I-*Nru*I fragment of SP β *c2* DNA (Fig. 5A, lane e). Further mapping of the *Nru*I sites with respect to the *Pst*I recognition sequences indicated that the unique *Nru*I site in the SP β *c2* genome was located in the *Pst*I K fragment (Fig. 5B, lanes i and j). The approximately 5-kb K fragment (lane i) disappeared when doubly digested with *Nru*I (lane j). The

FIG. 6. Detection of *d* gene sequence in the SP β prophages by hybridization. Plasmid pJM160 DNA (lane a) and chromosomal DNAs from CU1050 (lane b), CU1147 (lane c), CU1225 (lane d), or CU3082 (lane e) were digested with *Pst*I and *Eco*RI, fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and then probed with the ³²P-labeled oligonucleotide complementary to the antisense strand of the *d* gene (see Materials and Methods for the sequence of the oligonucleotide). The sizes (in kilobases) of the DNA markers are shown on the right.

additional *Nru*I site introduced in the *c2 d2* mutant was located in the *Pst*I H fragment, which was shortened to about 5 kb when double digested with *Pst*I and *Nru*I (Fig. 5B, lanes k and l). Since the H fragment was reported to be 6 kb in size (4), *Nru*I cleavage resulted in the 5-kb product replacing the cleaved K fragment and a 1-kb fragment that was not resolved in the gel analysis. These data coupled with the *Nru*I and *Sal*I-*Nru*I restriction data made it possible to map the two *Nru*I sites in the SP β *c2 d2* genome (Fig. 2), where the *Cla*I site shown in the *d* gene was modified to generate the second *Nru*I site in SP β *c2 d2*.

DISCUSSION

A 5.9-kb *Bgl*II fragment of SP β *c2* phage genome was cloned into a plasmid, and it was shown to confer immunity to the SP β *c10* clear-plaque phage and to other SP β phage. This gene, designated *d*, was further localized to the 1,033-bp *Pst*I-*Eco*RI fragment within the *Bgl*II fragment by subcloning. The location of this 5.9-kb *Bgl*II fragment on the phage genome was determined by Southern hybridization analysis, and the mapping data are summarized in Fig. 2. The *Pst*I T fragment and a portion of the *Pst*I H fragment that map in this region of the phage genome (4) were contained within the cloned *Bgl*II fragment (data not shown). By in vitro conversion of the *Cla*I restriction site within the *d* gene to an *Nru*I site, we were able to position the *d* gene within the *Pst*I H fragment and to localize precisely the *d* gene on the SP β genome. These mapping data also clarified the relative order of the clustered *Pst*I K, H, T, and A fragments (4) on the genome (Fig. 2).

The 1,033-bp *d* gene fragment contains an open reading frame (227 codons) which begins with the methionine codon AUG. Immunoblotting data indicated that the *d* gene protein is 22 kDa, an estimate based on its mobility in a gel. This does not help to identify unambiguously which one of the four clustered AUG codons is the initiation codon for the *d*

protein. The fourth Met codon (nucleotide position 213 in Fig. 3) is preceded by a sequence closely resembling the Shine-Dalgarno sequence (8), and it would encode a protein of 201 amino acids (~22 kDa). Whether this is indeed the initiation codon for the *d* gene remains to be determined.

The transcriptional initiation site for the *d* gene is likely also contained within the *Pst*I-*Eco*RI fragment. In CU1050(pJM160) cells, both biological expression of the *d* gene (by immunity test) and synthesis of the 22-kDa *d* protein were detected. In addition, the DNA fragment encoding the amino-terminal portion of the *d* gene (positions 1 to 540, Fig. 3) was fused to the *E. coli lacZ* coding sequence, and the fusion gene directed the synthesis of enzymatically active β -galactosidase in vivo in both *E. coli* and *B. subtilis* (unpublished data). These data also suggest that the *d* gene protein does not positively regulate its own expression.

The expression of the *d* gene is probably regulated in vivo. We detected a fair amount of *d* protein in CU1050(pJM160) cells by immunoblotting, but could not detect any *d* protein in CU1147, the strain carrying the SP β *c2* prophage. Either the *d* gene is not normally expressed in the lysogen or the *d* protein is synthesized but sequestered or degraded so it is not detectable under the conditions used for the preparation of the cell extracts.

Cloned *d* gene confers on its host immunity to superinfecting phages. The lack of observable lytic growth is not due to efficient lysogeny of the infecting SP β phage as was shown by using the SP β ::Tn917 *c1* phage to infect CU1050 (pJM160). In this experiment, no lysogenic clone was obtained. Thus, functional expression of the cloned *d* gene on a plasmid totally prevents superinfecting SP β from lytic growth and, at least for the clear-plaque phage, it also prevents SP β from lysogenic growth. These experiments indicate that the *d* gene protein acts in *trans* to confer immunity.

In addition to controlling immunity, the *d* gene is also involved in lysogeny. The SP β *c2* phage normally forms cloudy plaques, as does the wild-type phage. But SP β *c2 d2* phage forms clear-plaque phage on indicator cells. This suggests that either the *d* gene mutation blocks the repressor function or it is involved in the regulation of an essential step in the establishment of lysogeny.

Mixed infection experiments with *c2 d2* and other clear-plaque *c*⁻ phage suggest that there is no *d* gene complementation between these two types of clear-plaque phage, although the *d* gene confers immunity in *trans*. When CU1050 was infected with SP β *c2 d2* and SP β *c1*, only two types of cells were recovered from the lysis zone in this experiment: SP β -resistant cells, and cells lysogenic for the recombinant phage SP β *c2*. The other recombination product, phage SP β *c1 d2*, was not obtained. Since we have shown that *B. subtilis* is unable to stably maintain SP β *c1 d2* at the prophage state, this result was not unexpected. On the other hand, it has been shown that mixed infection of SP β *c1* and *c2* produces double lysogens (13). If the *d* gene product acts in *trans* and *d2* is a recessive mutation, then one would also expect to obtain double lysogens carrying both SP β *c2 d2* and SP β *c1*. Our failure to obtain such double lysogens suggests that the regulation of the lysogeny and immunity processes in SP β is complex. Possibly the *d* gene product has more than one regulatory role in controlling phage immunity and lysogenization.

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