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

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The d gene from the *Bacillus subtilis* temperate bacteriophage SPβ was isolated. When introduced into an SPβ-sensitive strain of *B. subtilis*, the cloned d gene directed the synthesis of a 22-kilodalton protein and conferred on the host immunity to SPβ phage. A frameshift mutation, designated d2, was introduced into the cloned d gene, and it was subsequently crossed back into the SPβ phage genome. The resulting SPβ 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 SPβ d2 and various SPβ c mutants. The nucleotide sequence of the 1,033-base-pair *PstI*-to-*EcoRI* 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 *PstI* 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 SPβ (18). This omnipresence of the SPβ 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 SPβ (c+ ) phage can be induced by mitomycin C; it replicates at temperatures below 50°C and forms cloudy plaques on sensitive bacteria (16). The SPβ phage carrying the thermoinducible repressor gene (SPβ c2 ) 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 SPβ c1 are unable to lysogenize sensitive *B. subtilis* strains and therefore form clear plaques. Double lysogens containing both the c1 and c2 prophages can be heat induced, while those containing c+ and c2 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 an 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 molecular 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 SPβ c2 was prepared from strain CU1147 by the heat induction method essentially as described by Rosenthal et al. (13). Wild-type SPβ phage was prepared either by mitomycin C (0.8 μg/ml) induction or, as for SPβ c10 (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 trichloride [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 VT180 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 Trischloride [pH 8.0], 1 mM EDTA) buffer-saturated phenol followed by two chloroform extractions and ethanol precipitation.

**Isolation of clear-plaque SPβ c10.** During the preparation of SPβ c+ phage from the SU + 3 (SPβ) strain, a spontaneous clear-plaque phage (designated SPβ c10) was fortuitously identified. In all the tests performed, SPβ c10 was indistinguishable from SPβ c1; they were both used in these studies.

**Construction of SPβ c2 phase 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. BamHI and BglII doubly digested SPβ c2 DNA (2.8 μg) was ligated to BamHI-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...
per ml. Of 520 Amp\(^{c}\) colonies tested for their sensitivity to tetracycline (15 \(\mu\)g/ml), about 19% of the transformants were sensitive; presumably they contained inserts in the BamHI 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 plasmid-sensitive strain CU1050 and transformed with the pooled plasmid DNAs. The transformants were then tested for immunity to SP\(\beta\) 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'-GATATATTTCTAGCTGAAATCAG-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 dideoxynucleotide chain termination method (14, 15) with [\(\alpha\]35S]dATP obtained from New England Nuclear Corp. (Boston, Mass.). A synthetic 17-mer with the sequence 5'-GGTCAATCTGTCAGC-3', designed according to the preliminary sequencing data, was subsequently used as an additional sequencing primer to allow sequencing across the \(ClaI\) site at position 666 within the \(d\) gene.

**Tests for SP\(\beta\) lysogeny and immunity.** To look for the presence of SP\(\beta\) 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\(\beta\), 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\(\beta\) by streaking transformant colonies across a vertical line along which 1 \(\times\) 10^8 to 2 \(\times\) 10^8 SP\(\beta\) c10 phage had been applied on a TBAB plate containing 5 \(\mu\)g of chloramphenicol per ml (19). CU1050 cells carrying only the vector pLP1201 were lysed on plates after infection by SP\(\beta\) c10 phage and thus served as the negative control; they grew only on the first half of the cross streak. CU1050(PSB c2) cells carrying pLP1201 served as the positive control which grew across the entire streak.

**Crossing the \(d2\) frameshift mutation back into the SP\(\beta\) phage genome.** Cells of CU1147, a strain lysogenic for SP\(\beta\) c2 phage, were made competent and then transformed with pJM172, the plasmid containing the \(ClaI\)-to-\(NruI\) +2 frameshift mutation in \(d\). One of the transformants was used to inoculate 5 ml of TB containing 5 \(\mu\)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\(\beta\) phage was between 10^6 and 10^7 PFU/ml. Of approximately 10^4 plaques screened visually, 2 clear plaques were picked. One of these was plated purified through three plating cycles. The clear-plaque phage isolated was then grown up (300 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 \(NruI\) site.

### TABLE 1. Strain list

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype/properties</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacillus subtilis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CU1050</td>
<td>SP(\beta) metB5 sup-3 thr leu ade (su(^{-}))</td>
<td>S. A. Zahler; 13, 16</td>
</tr>
<tr>
<td>CU1147</td>
<td>trpC2 (SP(\beta) c2)</td>
<td>S. A. Zahler; 13</td>
</tr>
<tr>
<td>BGSC no. 1L4</td>
<td>su(^{-}) (SP(\beta)); source of wild-type SP(\beta)</td>
<td>Bacillus Genetic Stock Center; 16</td>
</tr>
<tr>
<td>CU1225</td>
<td>citK(^{-}) ΔileB1 KauA1 ant(^{SP}) (SP(\beta) c2 dicitK(^{-})-1)</td>
<td>S. A. Zahler; 13</td>
</tr>
<tr>
<td>CU3082</td>
<td>ilvA2 ilvD15 leuB6 leuB16 ant(^{SP}) (SP(\beta) c2 diliD(^{-})-1)</td>
<td>S. A. Zahler</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MM294</td>
<td>thi-1 hasdR17 endA1 supE44 F^-</td>
<td>9</td>
</tr>
<tr>
<td>DG99</td>
<td>thi-1 hasdR17 endA1 supE44 lacF^I lacZΔM15 proC::TnlO; host for pUC plasmid vectors</td>
<td>D. Gelfand</td>
</tr>
<tr>
<td>DG98</td>
<td>DG99/F^I lacF^I lacZΔM15 proC^+ (Pro^+ Tet(^{-}) host for M13 phage)</td>
<td>D. Gelfand</td>
</tr>
<tr>
<td><strong>Vectors</strong></td>
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<td></td>
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<td>pLP1201</td>
<td>E. coli B. subtilis shuttle vector; Cm(^r) in B. subtilis, Ap(^r) and Tc(^r) in E. coli</td>
<td>11</td>
</tr>
<tr>
<td>pUC18</td>
<td>E. coli cloning vector with polylinker sequence; Ap(^r)</td>
<td>10</td>
</tr>
<tr>
<td>M13mp10 and M13mp11</td>
<td>Coliphage vectors for cloning and sequencing</td>
<td>10</td>
</tr>
<tr>
<td><strong>Phages</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP(\beta) c(^{-})</td>
<td>Wild-type c gene</td>
<td>16</td>
</tr>
<tr>
<td>SP(\beta) c2</td>
<td>Temperature-sensitive mutation in c gene, inducible at 50(^\circ)C, normal in permissive temperature range</td>
<td>13</td>
</tr>
<tr>
<td>SP(\beta) c10</td>
<td>Inactive c gene</td>
<td>This study</td>
</tr>
<tr>
<td>SP(\beta) c1</td>
<td>Inactive c gene, also contains del1</td>
<td>4, 16</td>
</tr>
<tr>
<td>SP(\beta)::TnlO c1</td>
<td>TnlO inserted in SP(\beta) c1</td>
<td>S. A. Zahler</td>
</tr>
</tbody>
</table>
To construct the SPβ c1 d2 double mutant, CU1050 (pJM172) cells were spotted with SPβ c1 lystate 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 SPβ c2. A gene library of SPβ 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 SPβ 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 EcoRI, PstI, and Sall, 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 BamHI and BglII, the vector was cleaved with BamHI, we checked pJM135 plasmid DNA for the presence of regenerated BamHI sites. No BamHI 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 BglII fragment in the SPβ 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 SPβ genome was determined by Southern hybridizations. The restriction maps of the SPβ genome for the AvaI, BamHI, SacI, and Sall endonucleases have been reported previously (4). SPβc2 DNA digested individually with each of these enzymes and fractionated on a gel was hybridized with ³²P-labeled Sall fragments of pJM135. The cloned SPβ DNA hybridized with the AvaI D fragment, the BamHI A fragment, the SacI F fragment, the Sall C fragment (very weakly), and the AvaI C and AvaI F fragments of SPβ (data not shown). This hybridization pattern is consistent with the assignment of the location of the d gene in the SPβ 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 EcoRV fragment (Fig. 1) had no effect on the immunity phenotype of the transformants. In contrast, transformants carrying plasmids lacking either the 3.9-kb Clal fragment [Clal(1) to Clal(2) in Fig. 1] or the 4.5-kb Clal fragment [Clal(2) to Clal(3) in Fig. 1] from pJM135 lost the immunity to phage infection. It therefore seemed likely that the d gene spans the Clal(2) site.

In vitro construction of frameshift mutations in the d gene. Mapping data revealed the presence of multiple SacI and PstI sites in the cloned SPβ fragment in pJM135 (Fig. 1). The 2.9-kb PstI(2) to SacI(2) fragment (Fig. 1) generated by partial PstI digestion and complete SacI digestion was cloned into PstI- and Smal-digested pUC18 plasmid (10). The resulting plasmid was designated pJM154. This plasmid has a unique Clal restriction site within the SPβ DNA insert (Fig. 1). After Clal 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 NruI site that would result from the ligation of completely repaired Clal ends. The majority of the plasmids had lost the Clal site and gained an NruI site; one of these clones was designated pJM169. The minority of the resulting plasmids had neither a Clal nor an NruI site; one of these was designated pJM158. The 1.033-base-pair (bp) PstI-EcoRI fragment from each of these plasmids (pJM154, pJM158, and pJM169) was purified by electrophoresion from 4% acrylamide gels and ligated to PstI- and EcoRI-digested pLP1201 to replace the small PstI-EcoRI fragment carrying most of the pBR322 β-lactamase gene. Ap⁺ Te⁺ transformants were screened by restriction digestion to ensure the presence of the SPβ DNA insert, and the resulting plasmids were designated pJM160 (d⁺; with Clal site), pJM170 (d⁻; without Clal), and pJM172 (d⁻ with NruI replacing Clal).

B. subtilis CU1050 transformants carrying pJM160 were immune to SPβ c⁺ as well as SPβ c10 and other SPβ 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 PstI-EcoRI fragment and that the Clal site is located within the d gene.

DNA sequence of the PstI-EcoRI fragment of SPβ phages. The sequencing strategy used to determine the DNA sequence of the SPβ PstI-EcoRI fragment is shown in Fig. 2. The 1.033-base sequence of the antisense strand of this fragment isolated from SPβ c2 phage is shown from the 5' end PstI site to the 3' end EcoRI site in Fig. 3. A long open reading frame exists from position 135 through 815 within which is located the Clal site (position 666). Four AUG (Met) and one GUG (Val) codons could be the initiation site for the d gene protein (Fig. 3).
In addition to the sequence of the d gene shown in Fig. 3, the sequence of the insertion mutants at the Clai site was also determined. A synthetic 17-mer oligonucleotide was used as the sequencing primer to determine the nucleotide sequence surrounding the Clai site regions in pJM154, pJM158, and pJM169. The sequence across the Clai site in pJM154 was 5'...TAT,ATC,GAT,TTT,CTC,..3' (Fig. 3). The corresponding sequences for pJM158 and pJM169 were 5'...TAT,ATC,GCG,ATT,TTT,CTC,..3' and 5'...TAT,ATC,GCG,ATT,TTT,CTC,..3', respectively.

The Clai site was apparently only partially repaired, resulting in a +1 frameshift mutation in the d gene on pJM158. The properly repaired Clai ends were ligated and generated a +2 frameshift mutation and an NruI site (TCGCGA) in the d gene on pJM169. We designate the alleles with the +1 and +2 frameshift mutations d1 and d2, respectively.

The same PstI-EcoRI fragment was also isolated from the SP 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 SPβ.
c2. Although the d gene was identified by its ability to confer on its host immunity to superinfecting SPβ phage, this piece of biochemical data clearly established the fact that the cloned immunity gene is distinct from the repressor gene.

**Analyses of the d gene protein.** By comparing the sizes of the proteins produced in the *E. coli* in vivo 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 *Pl* promoter (H. C. Wong and Y. E. Tine, unpublished data). The plasmid containing this sequence was introduced into a proper *E. coli* strain and, under the conditions that induced the *Pl* promoter, a large amount of the 15-kDa protein was produced. It was isolated and used for raising rabbit antiserum.

The gene proteins encoded by the SPβ 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 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 SPβ 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 *PstI* and *Hae*III sites located in this region, but the precise location of these regulatory sequences has not been further defined.

SPβ 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 SPβ phage, we performed the following tests. SPβ 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 SPβ prophage. Of 50 isolates tested, none gave a positive result. We also used an alternative test which permits direct selection of lysogens. Phage SPβ::Tn917 c1 is a c1-derived clear-plaque phage carrying 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 SPβ::Tn917 c1 at a multiplicity of infection of 1. After absorption and incubation, 2 × 10⁸ 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 SPβ 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 SPβ c1 phages.

**Genetic analyses of SPβ phage carrying the d2 mutation.** We crossed the d2 allele into the SPβ c2 genome and studied the phenotype of the SPβ 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 SPβ c2 d2 phage. We also crossed the d2 mutation into the lytic phage SPβ c1
(see Materials and Methods). The SPβ 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 SPβ phage since they spontaneously released phage particles and produced betaicin. 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 SPβ 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 SPβ c2 d2 phage was further tested for complementation with SPβ 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 SPβ c2 phage. In addition, of 10 independently isolated phage tested, none carried the diagnostic NruI site unique to the d2 mutation. Thus, these cells were lysogenic for SPβ 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 SPβ prophages. Southern analysis data are shown in Fig. 5. The probe reacted to the total DNA isolated from CU1050(SPβ c2) (strain CU1147). No hybridization was detected with strains carrying either SPβ c2 deinK+1 or SPβ c2 dilvD+1. It is interesting to note that the citK and dilvD markers are located on the opposite side of the attachment site for SPβ on the chromosome; thus, each of these two defective phage presumably has a different phage arm deleted. Out 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 NruI site unique to the d2 allele. The NruI site present in the SPβ c2 genome is located in the SalI D fragment. The SalI-NruI double digestion of the approximately 18-kb SalI D fragment (Fig. 5A, lane d) was cleaved into two fragments of about 9.7 and 7.5 kb (Fig. 5A, lane e). The additional NruI site in the clear-plaque mutant c2 d2 also maps in the SalI 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 SalI-NruI fragment of SPβ c2 DNA (Fig. 5A, lane e). Further mapping of the NruI sites with respect to the PstI recognition sequences indicated that the unique NruI site in the SPβ c2 genome was located in the PstI K fragment (Fig. 5B, lanes i and j). The approximately 5-kb K fragment (lane i) disappeared when doubly digested with NruI (lane j).
additional NruI site introduced in the c2 d2 mutant was located in the PstI H fragment, which was shortened to about 5 kb when double digested with PstI and NruI (Fig. 5B, lanes k and l). Since the H fragment was reported to be 6 kb in size, NruI 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 NruI and SalI-NruI restriction data made it possible to map the two NruI sites in the SPβ c2 d2 genome (Fig. 2), where the ClaI site shown in the d gene was modified to generate the second NruI site in SPβ c2 d2.

**DISCUSSION**

A 5.9-kb BglII fragment of SPβ c2 d2 phage genome was cloned into a plasmid, and it was shown to confer immunity to the SPβ c10 clear-plaque phage and to other SPβ phage. This gene, designated d, was further localized to the 1,033-bp PstI-EcoRI fragment within the BglII fragment by subcloning. The location of this 5.9-kb BglII fragment on the phage genome was determined by Southern hybridization analysis, and the mapping data are summarized in Fig. 2. The PstI T fragment and a portion of the PstI H fragment that map in this region of the phage genome (4) were contained within the cloned BglII fragment (data not shown).

By in vitro conversion of the ClaI restriction site within the d gene to an NruI site, we were able to position the d gene within the PstI H fragment and to localize precisely the d gene on the SPβ genome. These mapping data also clarified the relative order of the clustered PstI 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 PstI-EcoRI 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 340, Fig. 3) was fused to the E. coli lacZ2 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 SPβ 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 SPβ phage as was shown by using the SPβ::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 SPβ plasmid and, at least for the clear-plaque phage, it also prevents SPβ 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 SPβ c2 phage normally forms cloudy plaques, as does the wild-type phage. But SPβ c2 d2 phase 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 SPβ c2 d2 and SPβ c1, only two types of cells were recovered from the lysis zone in this experiment: SPβ-resistant cells, and cells lysogenic for the recombinant phage SPβ c2. The other recombinant product, phage SPβ c1 d2, was not obtained. Since we have shown that B. subtilis is unable to stably maintain SPβ c1 d2 at the prophage state, this result was not unexpected. On the other hand, it has been shown that mixed infection of SPβ 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 SPβ c2 d2 and SPβ c1. Our failure to obtain such double lysogens suggests that the regulation of the lysogeny and immunity processes in SPβ is complex. Possibly the d gene product has more than one regulatory role in controlling phage immunity and lysogenization.

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LITERATURE CITED