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Characterization of PBSX, a Defective Prophage of *Bacillus subtilis*

HEATHER E. WOOD,* MICHAEL T. DAWSON, KEVIN M. DEVINE, AND DAVID J. McCONNELL

Department of Genetics, Trinity College, Dublin 2, Ireland

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PBSX, a defective *Bacillus subtilis* prophage, maps to the *metA-metC* region of the chromosome. DNA (33 kilobases) from this region of the chromosome was cloned and analyzed by insertional mutagenesis with the integrating plasmid pWD3. This plasmid had a promoterless α-amylase gene (*amyL*) that provided information on the direction and level of transcription at the site of integration. Transcription under the control of the PBSX repressor proceeded in the direction *metA* to *metC* over a distance of at least 18 kilobases. Electrophoretic analysis of proteins produced by different integrant strains upon PBSX induction and by fragments subcloned in *Escherichia coli* allowed the identification of early and late regions of the prophage. A set of contiguous fragments directing mutagenic integration suggested that the minimum size of an operon that encodes phage structural proteins is 19 kilobases. The adaptation of PBSX transcriptional and replicational functions to a chromosomally based, thermoinducible expression system is discussed.

The ability of cells to produce noninfectious phagelike particles is a widespread occurrence throughout bacterial species. Many of these particles have been noted for their bactericidal properties (4, 16, 23, 43). Included in this category are the related defective phages of *Bacillus subtilis*, PBSW, PBSX, PBSY, and PBSZ, which are resident as prophages on the chromosomes of *B. subtilis* subsp. *vulgatus* and *B. subtilis* 168, S31, and W23, respectively (40, 42). Morphologically the phage particles differ only in tail length and in the number of cross-striations in the tail (40). The phage particles do not contain a phage genome and hence are unable to infect any known host (1, 19, 29, 30, 38). Defective phages of similar morphology and serologically related to PBSX are also produced by all analyzed strains of *Bacillus amyloliquefaciens*, *B. licheniformis*, and *B. pumulis* (21, 40). It has been suggested that the phages may have evolved from a common ancestral prophage that was lysogenic in a common bacterial ancestor (42).

The widespread occurrence of the PBSX-like defective phages throughout these *Bacillus* species and the failure to isolate strains cured of PBSX suggest that their continued maintenance is advantageous, if not essential, for the host strain (6, 14, 42). One possible ecological advantage that these phages confer upon the host cell is the ability to adsorb to and kill cells containing a heterologous phage but not cells containing the homologous phage (40, 42). In these respects these phages appear to share characteristics of both temperate bacteriophages and bacteriocins (29, 42).

Of these phages, PBSX has been the most extensively studied. Mutations in both regulatory elements and in genes involved in particle morphogenesis map between *metA* and *metC* on the *B. subtilis* 168 chromosome (5, 14, 15). The prophage is induced by treatment of the cells with agents that induce the SOB response, e.g., mitomycin C, hydrogen peroxide, UV irradiation, or thymidine starvation (18, 38, 41). Upon induction, replication of the phage genome extends into the host chromosome, as seen by the 5- to 10-fold enrichment of genetic markers in the vicinity of the PBSX prophage (1, 14, 45). The phage particle, which consists of a small head and long contractile tail, is composed of at least 26 polypeptides (26). A further seven PBSX-specific proteins have been identified in induced cells, leading to an estimation of the genome size as approximately 54 kilobases (kb) (26, 44). However, the phage packages DNA fragments of approximately 13 kb in length that are derived largely from the host chromosome (1, 18, 19, 29, 30). Although the phage particle adsorbs to the cell wall of a sensitive cell, this DNA is not injected (29). Thus a combination of factors appears to contribute to the defective nature of this phage.

Due to the noninfectious nature of the phage particle and the inability to isolate a phage genome from phage heads, PBSX cannot be investigated by classical methods of bacteriophage genetics. Instead the "prophage" must be investigated as a host genetic element. Thus, to facilitate an analysis of PBSX, we cloned DNA from the *metA-metC* region of the *B. subtilis* 168 chromosome and began a characterization of the prophage based on insertional mutagenesis and the generation of gene fusions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. Bacterial strains, plasmids, and phages are listed in Table 1. *B. subtilis* IA4201 was derived by congression, by using DNA from strain SO113 to transform strain IA420. *purA*$^+$ colonies were selected and screened for acquisition of the *amy-3* mutation. The construction of the integrating vector is shown in Fig. 1. The chloramphenicol acetyltransferase gene was removed from pBD64 on a 1.1-kb *Hpa*II fragment. pUC18 was cut with *Eco*RI. The ends of both vector and insert were filled in with Klenow fragment and ligated. The promoterless α-amylase gene *amyL* (32, 33) was removed from pSL5 on a *Bam*HI-*Hind*III fragment and inserted between the *Bam*HI and *Hind*III sites of the resulting plasmid to give pWD2. The *Eco*RI site distal to the α-amylase gene in pWD2 was removed by partial *Eco*RI digestion. Ends were filled in with Klenow fragment and religated. The resulting plasmid, pWD3, contained unique restriction sites for *Eco*RI, *Sac*I, *Sma*I, and *Bam*HI immediately 5′ to the promoterless α-amylase gene.

Enzymes, chemicals, and materials. Restriction enzymes, Klenow fragment, and T4 DNA ligase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Nick translations were performed with kit no. N5500 from Amersham Corp. (Arlington Heights, Ill.). [^{32}P]dCTP and L-[^{35}S]methionine were also obtained from Amersham.

* Corresponding author.

TABLE 1. Bacterial strains, plasmids, and bacteriophage

Plasmid, strain, or bacteriophage	Description	Source or reference
Plasmids		
pBD64	Cm^r	17
pUC18	Ap^r	46
pSL5	Ap^r, promoterless *amyL*	32
pOK411C	Cm^r, promoterless *amyL*	32
pWD3	Cm^r Ap^r, promoterless *amyL*	This study
E. coli		
DH5α	F^- *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* λ^- d^- *recA1 gyrA96 relA1* φ80d *lacZ*ΔM15 Δ(*lacZYA-argF*) U169	Bethesda Research Laboratories
NM539	*supF hsdR* (P2 *cox3*)	Promega Biotec (13)
CSR603	F^- *thr-1 leuB6 proA2 phr-1 recA1 argE3 thi-1 uvrA6 ara-14 lacY1 galK2 supE44 mtl-1 rpsL31 tsx-33 xyl5* λ^-	R. L. Rodriquez
B. subtilis 168 strains		
SO113	*trpC2 amy-3*	33
IA420	*ilvA1 metB5 purA16 xhi-1479 xki-1479*	BGSC[a] (5)
IA4201	*ilvA1 metB5 xhi-1479 xki-1479 amy-3*	This study
L8508	*xhi-1479 lyt-2*	D. Karamata
SL345	*phoS5 leuA8 rif-2 spoIIE64*	R. Buxton
IA78	*metC3 pyrA xtl-1*	BGSC
IA158	*metA*	BGSC
B. subtilis W23 strain SB623	*thr* (PBSZ)	BGSC
Bacteriophages		
Lambda EMBL3		Promega Biotec (13)
PBS-1		BGSC

[a] BGSC, Bacillus Genetic Stock Center, Columbus, Ohio.

Acrylamide, ammonium persulfate, and *N,N,N',N'*-tetramethylenediamine were obtained from BDH Chemicals Ltd. (Poole, England). *N,N'*-Methylbisacrylamide was purchased from Sigma Chemical Co. (St. Louis, Mo.). En^{3}Hance autoradiography enhancer was obtained from Dupont, NEN Research Products (Boston, Mass.). X-ray film (RX) was from Fuji; X-ray developer (LX-24) and fixer (FX-40) were from Eastman Kodak Co. (Rochester, N.Y.).

Construction and screening of a *B. subtilis* 168 chromosomal bank. Chromosomal DNA from *B. subtilis* SO113 was partially digested with *Sau*3A and size fractionated on a sucrose gradient. Fragments ranging in size from 14 to 22 kb were pooled and ligated to *Bam*HI-digested lambda EMBL3 with a vector/insert molar ratio of 3:1. Recombinant plaques were selected on a lawn of *Escherichia coli* NM539. Plaques were lifted onto a Pall Biodyne nylon membrane (Pall Corp., Glen Cove, N.Y.) and hybridized according to the manufacturer's instructions.

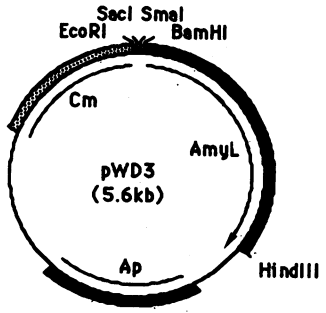

FIG. 1. Map of the integrating vector, pWD3, constructed as described in Materials and Methods. The Cm^r gene from pBD64 was subcloned into the multiple cloning site of pUC18. This was followed by insertion of the promoterless α-amylase gene (*amyL*) isolated from pSL5 (32). The resulting plasmid contained unique restriction sites for *Eco*RI, *Sac*I, *Sma*I, and *Bam*HI immediately 5' to the promoterless α-amylase gene.

Media. *B. subtilis* and *E. coli* were routinely grown on Luria broth or agar. When appropriate, media contained chloramphenicol (3 μg ml^{-1}) for selection in *B. subtilis* or ampicillin (50 μg ml^{-1}) for selection of plasmids in *E. coli*. α-Amylase activity was detected by adding starch (0.2%) to the media and then staining the plates with a solution of 0.5% I_2–1% KI.

Transformation and transduction. Transformations of *E. coli* and *B. subtilis* were carried out as described previously (8, 9). PBS-1 transductions were performed as described previously (31). Transductants were selected either on LB agar containing chloramphenicol (3 μg ml^{-1}) or on minimal medium supplemented with appropriate amino acids (0.005%) and nucleosides (0.01%). Transductants were subcultured to appropriate media to determine linkage. Linkage to *phoS* was determined by assaying alkaline phosphatase activity by a plate test (36). Linkage to *xhi-1479* was determined by subculturing transductants at 48°C. Cells containing this mutation fail to grow at this temperature (5).

Test of PBSX killing activity. PBSX killing activity was determined by spotting lysates from thermally induced cultures onto a lawn of the sensitive strain *B. subtilis* W23 as previously described (5). When cell lysis did not occur after PBSX induction, cells were sonicated before supernatants were spotted onto the lawn.

DNA preparation. Small-scale preparations of plasmid

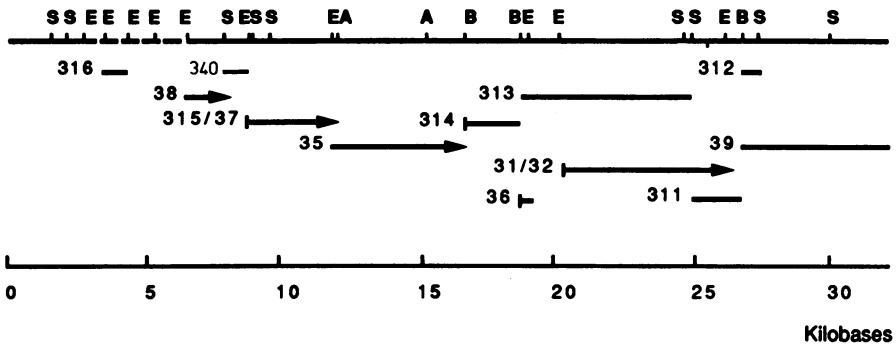

FIG. 2. Restriction map of the 33-kb cloned region. Fragments used to direct integration of pWD3 are indicated below. Where known, the fragment end that is fused to the promoterless α-amylase gene is indicated by a symbol: (→) transcription proceeds in the direction of the arrow, (—|) no transcription in the indicated direction (see Fig. 4). Restriction sites: B, *Bam*HI; E, *Eco*RI; S, *Sac*I; A, *Sal*I.

were obtained as described previously (3). Large-scale plasmid preparations were further purified by CsCl density gradient centrifugation (25). Large- and small-scale preparations of lambda DNA were prepared by using either liquid culture or plate lysates (25).

Radioactive labeling of proteins. PBSX particle proteins were labeled essentially as described previously (26). *B. subtilis* L8508 was grown in minimal medium at 37°C. Phage were induced at a cell density of approximately 4×10^7 ml^{-1} by shifting to 48°C. At 30 min after the temperature shift, L-[^{35}S]methionine (15 μCi ml^{-1}) was added, and lysis was allowed to continue for 1.5 h. Phage particles were purified over a CsCl gradient.

Proteins produced in cells after induction of PBSX were labeled as follows. Overnight cultures were diluted to a cell density of 6×10^6 ml^{-1} in minimal medium and grown to a cell density of 3×10^7 ml^{-1}. PBSX was induced by shifting the growth temperature to 48°C. At 30 and 40 min postinduction, 1-ml samples were withdrawn and incubated with continued shaking in the presence of L-[^{35}S]methionine. After 5 min, cold methionine was added (0.33 ml of a solution of 50 mg ml^{-1}). Cell pellets were stored at −20°C and processed for electrophoresis as described previously (27).

Labeling of plasmid-encoded proteins in *E. coli* was performed by the method of Sancar et al. (37).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Labeled proteins (10^6 cpm of each sample) were separated on 8, 10, and 12.5% sodium dodecyl sulfate–polyacrylamide gels as described previously (24). A ^{14}C-labeled methylated protein mixture (14.3 to 200 kilodaltons) was coelectrophoresed for molecular mass markers. Gels were treated with En^{3}Hance according to the manufacturer's instructions, dried, and autoradiographed for 12 to 48 h.

RESULTS

Isolation of PBSX DNA. PBSX does not package its own genome (19, 30). Therefore, to isolate DNA coding for phage functions, a *B. subtilis* 168 chromosomal bank constructed in lambda EMBL3 was screened with pOK4IIC, a plasmid which is known to contain DNA from the PBSX prophage (32). By successive cycles of screening, approximately 33 kb of DNA from the PBSX region of the chromosome were isolated in three overlapping lambda clones. A restriction map of the cloned region is presented in Fig. 2. Hybridization of selected fragments to chromosomal DNA digests by the Southern blot technique (39) indicated that no rearrangements of PBSX DNA had occurred during the cloning procedure (data not shown).

Construction and use of integrating plasmids. The organization of the PBSX prophage was studied by using a series of integrating plasmids derived from pWD3 (Fig. 1). Integrating plasmids have previously been used for the generation of gene fusions, for insertional mutagenesis and gene mapping, and in the identification of polycistronic operons (reviewed in reference 11). The major features of pWD3 are that it is unable to replicate autonomously in *B. subtilis*, it contains a promoterless α-amylase gene that can be used for the generation of transcriptional gene fusions, and it encodes both chloramphenicol acetyltransferase and β-lactamase, which can be used for selection of cells containing the plasmid in *B. subtilis* and *E. coli*, respectively. Derivatives of pWD3 were constructed by ligating the fragments shown in Fig. 2 into unique restriction sites located immediately 5′ to the promoterless α-amylase gene. The resulting plasmids were numbered according to the fragment they contain. Thus pWD3 that contained the 1.3-kb *Eco*RI-*Sac*I fragment number 38 was named pWD38.

These plasmids were used to transform *B. subtilis* IA4201, SO113, and L8508 by selecting for chloramphenicol resistance. In each of six cases checked, integration of the plasmid into the chromosome appeared to have occurred by a Campbell-type mechanism. The result of such an integration event is that the pWD3 sequences are maintained on the chromosome, flanked in each case by direct repeats of the fragment of DNA that directed the integration event (12). These derivative strains, which contained plasmids integrated at various positions within the PBSX region of the *B. subtilis* 168 chromosome, were then used in the analysis of the cloned region as described below.

Position and orientation of the cloned DNA on the *B. subtilis* chromosome. To establish the position of the cloned DNA on the chromosome and to determine its orientation, two strains with plasmids integrated at either extremity of the cloned region, IA4201::pWD312 and IA4201::pWD316, were used as donors and recipients in PBS-1 transduction. In each case the site of integration of the chloramphenicol resistance marker was mapped with respect to neighboring chromosomal markers (Fig. 3). The chloramphenicol resistance marker in strain IA4201::pWD312 was closely linked to *phoS*, a mutation which results in constitutive alkaline phosphatase expression (36). In strain IA4201::pWD316, the chloramphenicol acetyltransferase gene had integrated close to the *xhi-1479* mutation, which confers the heat-inducible

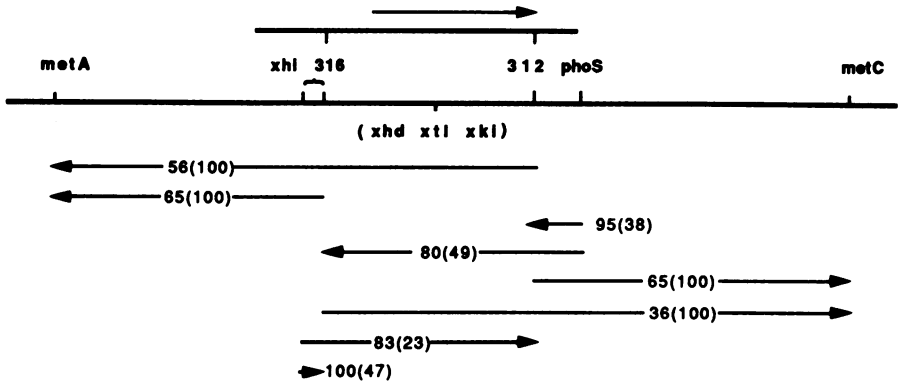

FIG. 3. Mapping of the Cm^r gene to the *xhi-1479* locus and adjacent chromosomal markers in two integrative strains: IA4201::pWD316 and IA4201::pWD312. Values are presented as percentages of cotransduction using PBS-1. Numbers of transductants tested are given in parentheses. The arrows point toward the selected marker in each case.

phenotype (5). The data suggest the order of markers as shown in Fig. 3. Although mapping studies were not carried out with respect to other PBSX markers, correlation with the known genetic map for this region suggests that the cloned DNA spans the sites of mutations within PBSX genes coding for head (*xhd*) and tail (*xtl*, *xki*) proteins (5, 14, 15).

Identification of PBSX operons. Since the consequences of integration directed by fragments lying either within or overlapping the end of an operon are different (being mutagenic or nonmutagenic, respectively), we were able to use this technique to delimit the extent of operons within the cloned PBSX DNA (28, 35).

One assay for the production of PBSX particles is their bactericidal activity against a PBSX-sensitive strain, *B. subtilis* W23 (29, 38, 42). The plasmid pOK411C, isolated by O'Kane et al. (32), was shown to contain a fragment of PBSX origin by its ability, when integrated, to abolish this PBSX killing function. In a similar manner, 12 derivatives of *B. subtilis* L8508 were tested for production of killing activity after thermoinduction of PBSX. Of these, 10 showed a marked reduction in killing activity when compared with that of the parental strain (Table 2). Residual killing activity in these strains may be due to a low level of transcriptional readthrough from the plasmid sequences or to excision of the plasmid sequences in a subpopulation of cells. Furthermore, the same integrant strains that showed reduced killing activity failed to show the characteristic pattern of PBSX-induced cell lysis when grown in liquid medium (Table 2). These strains presumably resulted from integration events that disrupt a PBSX operon, thus preventing production of factors required for killing activity and cell lysis. Mutagenic integration events spanned a total distance of at least 22 kb, which confirms that much of the cloned DNA is of PBSX origin. Furthermore, nine of the fragments that direct mutagenic integration mapped to the region between 8 and 27 kb. Each of these fragments was derived from an internal part of an operon. If these fragments are contiguous, as the restriction map suggests, then these fragments must be derived from the one operon. This suggests that an operon of at least 19 kb spans this region, although confirmation of this will depend on sequence data from this region to ensure that small restriction fragments have not been overlooked. Integration directed by fragments 38 and 39 was apparently nonmutagenic. Combined with the results presented below, this indicates that the beginning of the operon lies within fragment 38. The end of this operon may lie within fragment 39, although it is possible that further cistrons, undetectable by the criterea used here, are located downstream.

TABLE 2. Mutagenic properties of integrating plasmids

Integrated plasmid	Killing activity on *B. subtilis* W23[a]	Cell lysis upon PBSX induction[b]
None	+	+
pWD316	−	−
pWD38	+	+
pWD340	−	−
pWD37	−	−
pWD35	−	−
pWD314	−	−
pWD313	−	−
pWD32	−	−
pWD31	−	−
pWD311	−	−
pWD312	−	−
pWD39	+	+

[a] Determined by spotting lysed or sonicated cultures on a lawn of *B. subtilis* W23.

[b] Determined by monitoring optical density.

Integration directed by fragment 316, which lies outside this operon, was also mutagenic, and therefore fragment 316 is part of a second PBSX operon.

Analysis of transcriptional activity within the cloned region. With a view to analyzing the transcriptional activity across the putative 19-kb operon, expression of the α-amylase gene in appropriate derivatives of IA4201 was examined after thermoinduction of the prophage. In each case integration of pWD3 sequences resulted in fusion of the promoterless α-amylase gene to the chromosome. Expression of the α-amylase gene in these strains reflects the transcriptional activity and the direction of transcription on the chromosome at the point of fusion.

When the α-amylase gene was integrated in the direction *metA-metC* at four positions across this region [strains IA4201::pWD38, IA4201::pWD37, IA4201::pWD35, and IA4201::pWD32], low levels of α-amylase production were observed when cells were grown at 30°C. Expression was increased when the growth temperature was shifted to 48°C, the temperature at which the prophage is induced in the *xhi-1479* background (5) (Fig. 4). However, when the α-amylase gene was integrated in the opposite orientation, little or no expression of the gene was observed at either growth temperature. Thus we conclude that transcription of

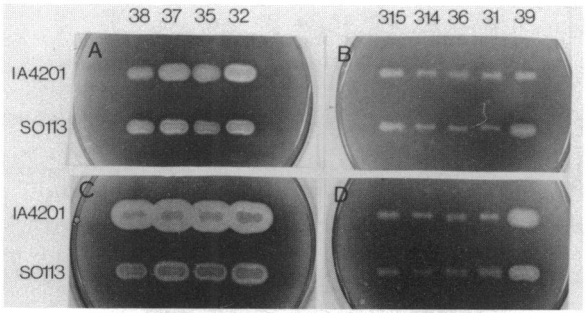

FIG. 4. Plate tests demonstrating thermoinducible production of α-amylase when integrated in one orientation into the chromosome of strain IA4201 at the PBSX locus. Plates A and C show strains with the α-amylase gene integrated in the direction *metA-metC*. Plates B and D show strains with the α-amylase gene integrated in the direction *metC-metA* (Fig. 2). Colonies were grown on LB agar containing starch at 30°C for 18 h (plates A and B) or at 30°C for 12 h followed by 48°C for 6 h (plates C and D). Plates were stained with 0.5% I_2–1% KI.

this region proceeds predominantly in the direction *metA-metC*, as shown in Fig. 2 and 5. It follows that the promoter for the operon that has been proposed to span this region is located within the 1.3-kb fragment 38.

Production of PBSX proteins in strains containing integrated plasmids. The results presented above led to the identification of two PBSX operons but provided no indication as to the functions encoded by these operons. Previous studies have identified at least 12 polypeptides under PBSX control that can be detected in induced cells (26). We envisaged that analysis of the PBSX proteins produced in the integrant strains might provide information as to the functions encoded by these operons and furthermore might allow the order of genes within the large operon to be determined. Integration into the 5′ end of this operon (for example, that directed by fragment 37), will prevent transcription of all DNA located downstream from the point of integration. Integration events that place the plasmid successively further downstream (e.g., integration of pWD35, pWD32, and pWD312) will allow transcription of progressively more of the operon and will thus allow more phage proteins to be produced. Hence, a gene that encodes a protein that is absent in one integrant strain, but present in a strain containing the plasmid integrated further downstream, can be assigned to the DNA in the intervening region. The proteins that were produced by a representative sample of L8508 derivative strains were examined and compared with the proteins produced by the parental strain, L8508. Proteins were radioactively labeled with a 5-min pulse 40 min post-thermoinduction of PBSX and separated by electrophoresis on 10 to 13% sodium dodecyl sulfate–polyacrylamide gels (Fig. 6). The accumulated data, scoring the major PBSX proteins for selected derivative strains, are summarized in Table 3.

Integration of plasmid pWD37 prevented the synthesis of a number of phage proteins, including a major head protein and its proposed precursor (X35 and P36, respectively [26]), several tail proteins, X76 and X19, and nonstructural proteins P32 and P31. Strains L8508::pWD35, L8508::pWD32, and L8508::pWD312 differed from L8508::pWD37 in that proteins P36/X35, X19, and P18 were synthesized. However, these strains also lacked X76, P32, and P31. Strains L8508::pWD38 and L8508::pWD39 produced all detectable PBSX proteins, which is consistent with the nonmutagenic nature of integration directed by these two fragments. These results, which show that integration into the large operon prevents, the synthesis of a number of phage structural proteins, indicate that this operon encodes proteins required for particle morphogenesis. By analogy with characterized prophages, this has been provisionally named the late operon. By using the data in Table 3, the genes for the some of the major PBSX proteins have been assigned to regions within the late operon (Fig. 5). For example, protein X19, a major tail protein, is absent in strain L8508::pWD37 but present in L8508::pWD35; hence the gene for this protein has been tentatively assigned to fragment 35.

Integration directed by fragment 316, which defines a second PBSX operon, prevented production of any detectable PBSX proteins. We conclude from this that integration disrupts the function of an operon, the expression of which is required for expression of the late operon. Again by analogy with characterized prophages, we named this an early operon. This designation is consistent with the PBS-1 transduction data, which showed fragment 316 to be closely linked to the *xhi-1479* mutation. This mutation, which results in a thermoinducible phenotype, is presumed to lie within the prophage repressor gene (5).

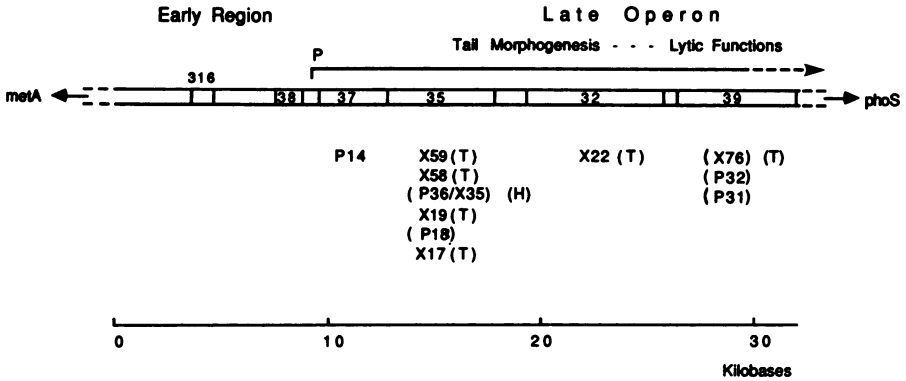

FIG. 5. Diagramatic representation of the functions of the cloned PBSX DNA as determined in this study. The early region is defined by integration of pWD316, which abolishes induction of any detectable PBSX proteins. The late operon is so called by analogy with other bacteriophage genomes; it encodes largely phage structural proteins. The proteins encoded by each region are indicated below. Parentheses indicate that the positions of these genes are tentative, based only on the analysis of integrative strains. The allocation of other genes has been confirmed by analysis in *E. coli* maxicells. H, Phage head protein; T, phage tail protein (26).

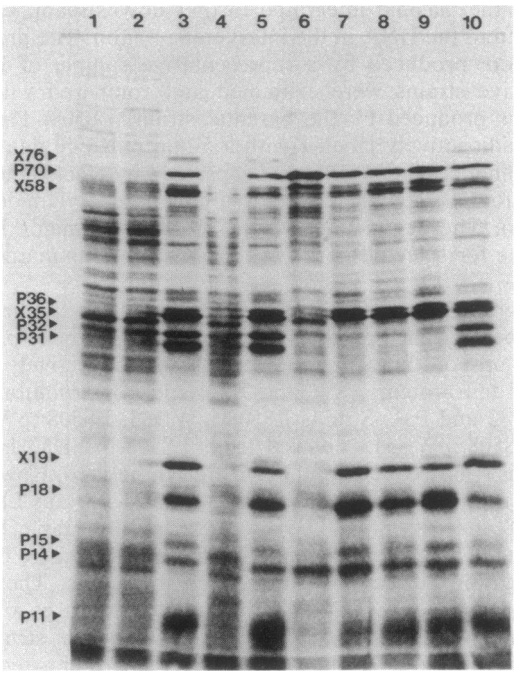

FIG. 6. One-dimensional 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled proteins from *B. subtilis* parental (L8508) and integrative strains. PBSX proteins were induced by a shift in growth temperature to 48°C as described in Materials and Methods. Proteins were labeled at 40 min postinduction unless otherwise indicated. *B. subtilis* strains in which the labeling was carried out are as follows (lanes): 1, L8508 (uninduced), 2, L8508 *xhi*$^+$; 3, L8508; 4, L8508::pWD316; 5, L8508::pWD38; 6, L8508::pWD37; 7, L8508::pWD35; 8, L8508::pWD32; 9, L8508::pWD312; 10, L8508::pWD39. PBSX proteins are labeled according to the nomenclature of Mauel and Karamata (26); X denotes a PBSX structural protein, and P denotes a protein found only in cells induced for PBSX.

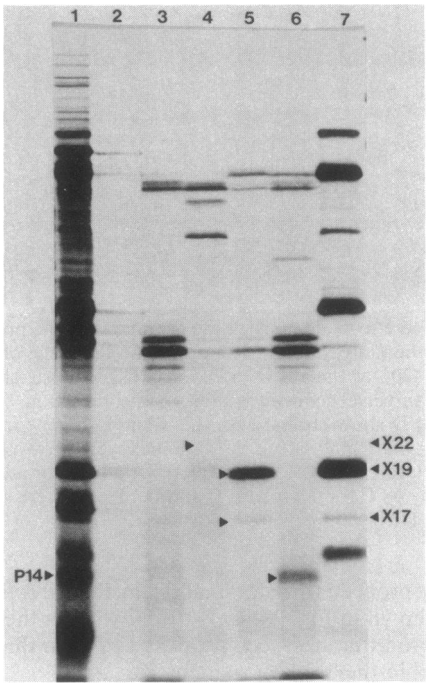

FIG. 7. One-dimensional 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled proteins from *E. coli* maxicells. Lanes: 1, *B. subtilis* L8508 (labeled after PBSX induction); 2, *E. coli* CSR603; 3, *E. coli* CSR603(pWD3); 4, *E. coli* CSR603(pWD32); 5, *E. coli* CSR603(pWD35); 6, *E. coli* CSR603(pWD37); 7, PBSX phage particle proteins. Proteins corresponding in size to PBSX proteins are indicated with arrows. Nomenclature is as in Fig. 6. The protein labeled X22 may correspond to either protein X22 or X21 described by Mauel and Karamata (26).

Expression of phage proteins in *E. coli*. The assignment of genes within the late operon, based on the proteins produced in the integrant strains, was confirmed and extended by examination of proteins produced by selected fragments in *E. coli*. In derivatives of pWD3, transcription from an unidentified promoter located within the vector sequences proceeds into the cloned insert. Plasmids pWD37, pWD32, and pWD35 were transformed into *E. coli* CSR603. Plasmid-encoded proteins produced in maxicells were labeled and separated on 8 and 12.5% sodium dodecyl sulfate-polyacrylamide gels (Fig. 7). In the strain containing pWD35, proteins were observed that corresponded in size to previously identified phage proteins X58, X19, and P17. In addition, this fragment encoded a protein that corresponded in size to a newly identified phage protein, which we named X59. This protein could be seen when phage particle proteins were separated on 8% sodium dodecyl sulfate-polyacrylamide gels. Plasmid pWD32 encoded a protein that corresponded in size to a phage tail protein (X22), whereas pWD37 gave rise to a protein that corresponded to P14, a protein of unknown function found in induced cells. Proteins X58 and X19 were produced in sufficient amounts to be subjected to limited proteolysis by the method of Cleveland et al. (7). In each case peptides similar in size to those of the phage proteins were produced, thus confirming their identity (data not shown). Several proteins were produced that did not correspond in size to any known phage-specific proteins. These may represent genuine phage products or, alternatively, may arise due to truncation of proteins or to abnormal processing in a heterologous host.

The data obtained from the analysis of proteins produced in *E. coli* are consistent with the map based on the analysis of *B. subtilis* integrant strains, and the results have been included in Fig. 5. A few ambiguities are apparent. The major head protein X35, its precursor, P36, and a further protein P18 were not produced by fragment 35 when cloned in *E. coli*. The gene for one of these proteins may span the

TABLE 3. Presence or absence of the major PBSX-induced proteins in *B. subtilis* L8508 derivatives containing the indicated integrated plasmids

Protein[a]	PBSX proteins in strain containing:						
	pWD316	pWD38	pWD37	pWD35	pWD32	pWD312	pWD39
X76 (tail)	−	+	−	−	−	−	+
P70	−	+	+	+	+	+	+
P36	−	+	−	+	+	+	+
X35 (head)	−	+	−	+	+	+	+
P32	−	+	−	−	−	−	+
P31	−	+	−	−	−	−	+
X19 (tail)	−	+	−	+	+	+	+
P18	−	+	−	+	+	+	+
P14	−	+	+	+	+	+	+

[a] Proteins X58 and P11 (Fig. 6) were not included because they could not be scored consistently over a number of samples.

junction between fragments 37 and 35. Alternatively, positive control factors may be produced by this region which allow transcription of these genes placed elsewhere within the prophage. These possibilities need further examination.

DISCUSSION

We carried out a provisional genetic analysis of the PBSX prophage by examining the effects of integrating plasmid sequences at different sites within the *metA-metC* region of the *B. subtilis* 168 chromosome. Insertional mutagenesis has allowed the identification of early and late regions of the prophage, leading to a functional map (Fig. 5). This map is consistent with proposed genetic map of this region, which suggests that mutations within a regulatory region (*xin* and *xhi*) are located to the *metA*-proximal side of mutations within genes affecting particle proteins (*xhd*, *xtl*, *xki*) (5, 15, 45).

We suggest that many of the late phage genes are contained within a large operon, at least 19 kb in length, that is transcribed in the direction from *metA* to *metC*. Such polycistronic operons are characteristic of other phage genomes, e.g., lambda (20). The coding capacity of this operon may be sufficient to produce all of the phage structural and late proteins (26). However the existence of another late operon cannot be excluded, since ambiguity surrounds the location of the genes coding for proteins P36/X35 and P18.

Although the full extent of the PBSX genome has not been determined, it is apparent from insertional mutagenesis that it is at least 22 kb. This is confirmation at the DNA level that the PBSX genome is considerably larger than the 13-kb fragments of DNA that are packaged within the phage heads (1, 18, 19, 29, 30). The cloning of an origin of replication from the PBSX region of the chromosome has been reported (2). We have not to our knowledge cloned an origin of replication of the prophage, and therefore we suspect that further uncloned regions of the prophage extend toward *metA*.

It has been suggested that PBSX and related phages are maintained by the host cell because of the ecological advantage conferred by the bactericidal activity of the phage particles (42). However, the failure to isolate strains cured of PBSX has led to speculation that the prophage may contribute another essential function to the host (5, 6, 14). To address this question, strains of *B. subtilis* with deletions of the PBSX region of the chromosome have been isolated, but the full extent of these deletions have not been determined (6). The cloning of DNA from this region of the chromosome should allow the end points of these deletions to be mapped and should also facilitate the construction of strains with deletions of all or specified parts of the prophage. Furthermore, it will be of interest to determine the genetic organization and control of this system, which appears to represent a stage in evolution between a bacteriophage and a bacteriocin.

By integrating the promoterless α-amylase gene at the PBSX locus, we demonstrated the potential for using PBSX functions as the basis of a thermoinducible expression system. Plasmid-based thermoinducible expression systems have been described for *B. subtilis*; these systems exploit the controlling elements of the early region of bacteriophage ϕ105 (10, 34). The system presented here demonstrates that a foreign gene can be integrated on the *B. subtilis* chromosome under the control of an inducible prophage promoter. Furthermore, the foreign gene is located within a structure reported to be capable of undergoing stable gene amplification (22). Indeed, stable maintenance of both single and amplified copies of the α-amylase gene have been observed when integrated at this locus (M. Dawson, unpublished results). To complement this chromosomally based system, we are currently developing a plasmid-based system that utilizes the early control regions of the prophage.

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