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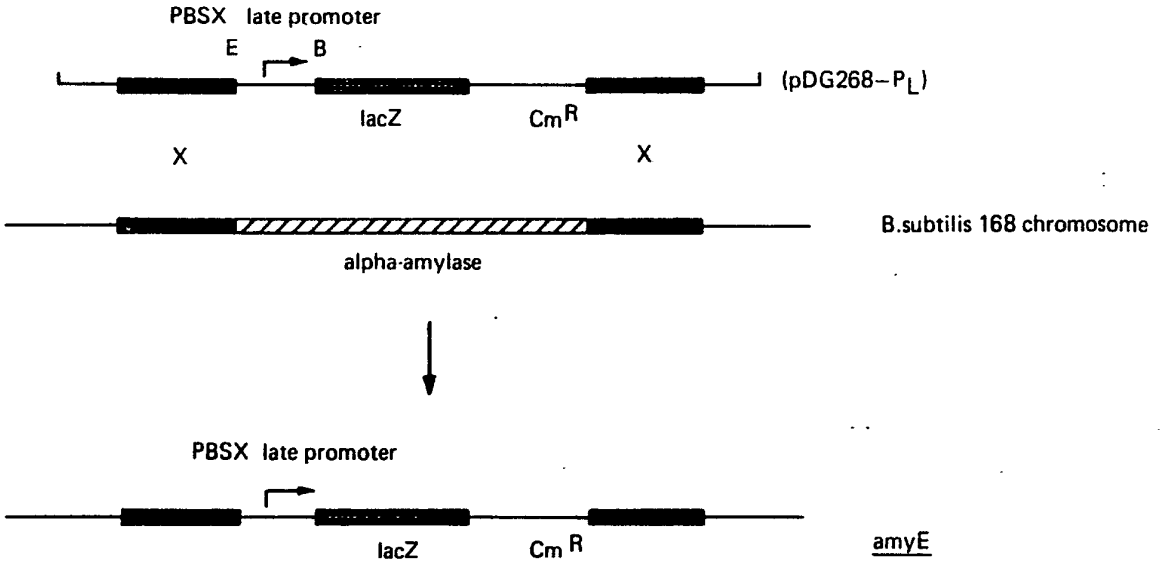

(57) Abstract

An expression system which comprises a phage-like bacteriocin ("phibacin") or a mutant thereof, or a gene or a mutant of a phibacin having a function in gene expression, such as a repressor gene, is used to transform bacterial hosts for the production of proteins, in particular in gram-positive bacteria.

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A Gene Expression System

This invention relates to expression systems which are suitable for use in a Gram positive bacterium, particularly *Bacillus* species.

The ability of cells to produce non-infectious phage-like particles is a widespread occurrence throughout bacterial species. Many of these particles have been noted for their bacteriocidal properties (6, 14, 25, 47). 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* var. vulgatus and *B. subtilis* strains 168, S31 and W23 respectively (44, 46), and PBSV found in *B. licheniformis* (23). Morphologically PBSX and related defective phage particles differ in tail length and in the number of cross-striations in the tail (44). The phage particles do not contain a complete phage genome and hence are unable to infect any known host (2, 19, 33, 34, 45). Defective phages of similar morphology and serologically related to PBSX, are also produced by all analysed strains of *B.amyloliquefaciens and B.pumulis.* (23, 44). It has been suggested that the phages may have evolved from a common ancestral prophage, lysogenic in a common bacterial ancestor (46).

The widespread occurrence of the PBSX-like defective phages throughout these *Bacillus* species, and the failure to isolate strains cured of PBSX, suggests that their continued maintenance is advantageous, if not essential for the host strain (5, 16, 46). One possible ecological advantage that these phages confer upon the host cell is the ability to adsorb to and kill cells containing a heterologous, but not the homologous, phage (44, 46). In these respects these phages appear to share characteristics of both temperate bacteriophages and bacteriocins (33, 4).

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 (4, 15, 16). Upon induction, replication of the phage genome extends into the host chromosome as seen by the 5-10 fold enrichment of genetic markers in the vicinity of the PBSX prophage (2, 16, 49). The phage particle, which consists of a small head and long contractile tail is composed of at least 26 polypeptides (29). A further 7 PBSX specific proteins have been identified in induced cells, leading to an estimation of the genome size of approximately 54kb. (29). However, the phage packages DNA fragments of only 13kb. in length which is derived largely and apparently randomly from the host chromosome (2, 18, 33, 34). Although the phage particle adsorbs to the cell wall of a sensitive cell, this DNA is not injected (33). Thus a combination of factors appears to contribute to the defective nature of this phage.

These phage-like particles have generally been referred to as "defective prophages". However, they have lost the ability to act as phages and in some respects are very similar to bacteriocins. They have, therefore, sometimes been referred to as "particulate bacteriocins". The distinctions between bacteriocins, defective phages and particulate bacteriocins have not been clearly drawn in the literature and for this reason the present inventors have suggested the use of the term "phage-like bacteriocins" or "phibacins" to denote non-infectious, phage-like particles which otherwise have the properties of bacteriocins. The term phibacin is also used to describe the phibacin genome as appropriate in the text.

According to the present invention there is provided an expression system comprising a phibacin or a mutant thereof, or a gene or a mutant gene of a phibacin having a function in gene expression. The phibacin may be a phibacin isolated from *Bacillus* species, particularly from *Bacillus subtilis*. The phibacin may be selected from the phibacins PBSW, PBSX, PBSY and PBSZ of *Bacillus subtilis*.

In particular the invention relates to an expression system comprising the phibacin deposited with the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, on 6th September 1989 under the accession no. NCIMB 40205, isogenic

derivatives thereof, and phibacins which are substantially similar thereto, particularly phibacins having at least 50% homology therewith, the said derivatives and similar phibacins having a function in gene expression.

Particularly preferred are expression systems comprising mutants of the phibacins which do not lyse the host cell on induction. Such mutants may be created by insertional mutagenesis. In particular the phibacin may carry the xhi1479 mutation. The expression system may comprise the mutant phibacin deposited with the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, on 6th September 1989 under the accession no. NCIMB 40206, isogenic derivatives thereof and phibacins which are substantially similar thereto, particularly phibacins having at least 50% homology therewith, the said derivatives and similar phibacins having a function in gene expression and being non-lysogenic on induction.

The invention also provides an expression system comprising a repressor gene, a promotor and at least one operator, isolated from a phibacin. The repressor gene may be the orf1 gene encoded by the phibacin PBSX. The repressor gene may have the DNA sequence shown in Fig. 8, or a sequence which is substantially similar thereto, particularly a sequence having at least 50% homology therewith, and encoding repressor activity. The promotor may have the DNA sequence shown in Fig. 8, or a DNA sequence substantially similar thereto, particularly a sequence having at least 50% homology therewith, and encoding promotor activity. The operator may have the sequence of any one of the operators 01, 02 and 03 shown in Fig. 8, or a DNA sequence substantially similar thereto, particularly a sequence having at least 50% homology therewith, and encoding operator activity.

The expression system may further comprise a gene encoding a positive control factor isolated from a phibacin. The positive control factor-encoding gene may be the orf2 gene encoded by the phibacin PBSX. The positive control factor-encoding gene may have the sequence shown in Fig. 16, or a sequence which is substantially similar thereto, particularly a sequence having at least 50% homology therewith and encoding positive control factor activity.

Advantageously, the expression system comprises a gene encoding a temperature-sensitive repressor so that product expression is heat-inducible. The gene encoding the temperature-sensitive repressor may be the xhi1479 allele of orf1. The temperature-sensitive repressor may have the DNA sequence shown in Fig. 9, or a sequence which is substantially similar thereto, particularly a sequence having at least 50% homology therewith, and encoding temperature-sensitive repressor activity.

In a further aspect the invention provides a repressor gene isolated from a phibacin, particularly from PBSX, and genes which are substantially similar thereto, particularly genes having at least 50% homology therewith, and encoding repressor activity. The repressor gene may be the orf1 gene of PBSX. In particular the invention provides a repressor gene having the DNA sequence shown in Figure 8 or a sequence which is substantially similar thereto, particularly a sequence being at least 50% homologous therewith, and the said gene encoding repressor activity. An example of a repressor gene is contained in the phibacin which was deposited with the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland on 6th September 1989 under the accession no. NCIMB 40205.

The invention also provides a temperature-sensitive repressor gene isolated from a phibacin, particularly from PBSX, and genes which are substantially similar thereto, particularly genes having at least 50% homology therewith, and encoding temperature-sensitive repressor activity. The temperature-sensitive repressor gene may be the xhi1479 allele of the orf1 gene of PBSX. In particular the temperature- sensitive repressor gene may have the sequence shown in fig. 9, or a sequence which is substantially similar thereto, particularly a sequence having at least 50% homology therewith, the said sequence encoding temperature-sensitive repressor activity. An example of a temperature-sensitive repressor gene is contained in the mutant phibacin which was deposited with the National Collection of Industrial

Bacteria, Torry Research Station, Aberdeen, Scotland on 6th September 1989 under the accession no. NCIMB 40206.

In a still further aspect the invention provides a gene encoding a

positive control factor isolated from a phibacin, particularly PBSX, and genes which are substantially similar thereto, particularly genes having at least 50% homology therewith and encoding positive control factor activity. In particular the invention provides a repressor gene having the DNA sequence shown in Figure 16 or a sequence which is substantially similar thereto, particularly a sequence being at least 50% homologous therewith, and the said gene encoding positive control factor activity. An example of a positive control factor gene is contained in the phibacins which were deposited with the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland on 6th September 1989 under the accession nos. NCIMB 40205 and NCIMB 40206.

The invention also relates to bacterial hosts containing one or more of the expression systems or genes as defined above. The invention also provides plasmids carrying one or more of the genes defined above.

The invention also provides all of the above materials in biologically pure or isolated form.

The invention therefore provides a method of producing a gene product in which the gene in question is inserted into an expression system as defined above and the expression system is introduced into a bacterial host to express the gene product. This method is suitable for producing proteins in Gram positive bacteria.

Also provided is a method of integrating a gene into a bacterial chromosome in which a segment of phibacin DNA is inserted into a plasmid carrying the desired gene, the plasmid is introduced into a bacterial cell carrying on the chromosome at least a portion of phibacin DNA having the same or a substantially similar DNA sequence as that of the phibacin DNA segment contained on the plasmid, and recombination events between the plasmid and the phibacin DNA in the bacterial chromosome, which integrate the desired gene into the chromosome, are selected for by methods known in the art. Any segment of phibacin DNA would be suitable to drive integration.

The term "expression system" as used herein includes vehicles or vectors for the expression of a gene in a host cell as well as

vehicles or vectors which bring about stable integration of a gene into the host chromosome.

The term "substantially similar" as used herein means sequences which have sufficient sequence identity or homology to the deposited phibacins, or genes contained in the deposited phibacin to hybridize therewith and to retain a function in gene expression, or phibicin or gene activity.

The advantage of using an expression system effective in *Bacillus* strains is that *Bacillus* is an effective secretor of proteins and using the system it would be possible to substitute *Bacillus* for *E. coli* or yeasts in processes for the production of genetically-engineered proteins to get an enhanced secretion of the protein in question. *Bacillus* strains are also "Generally Recognised As Safe" or are "GRAS" micro-organisms . *Bacillus* has long been used in the food and drink industry and in the production of antibiotics. It has the advantage that it does not contain pyrogenic substances or produce toxins. There is extensive industrial experience of using *Bacillus* in fermentations such as in the production of detergent proteases and alpha-amylase. It is also more difficult to use proteins made in *E. coli* in the food industry since this organism is not considered to be safe. Further advantages of the expression vehicles disclosed above are that there are strong ribosome binding sites and promoters linked to these phibacins, particularly PBSX, which further enhance protein production. In addition, the phibacins disclosed above have the advantage that they are stably integrated into the bacterial chromosome.

By using an expression system which is heat-inducible it is possible to grow host cells to a high cell density and then to shift the temperature to induce expression of the gene and protein production. The expression of the product can thus be achieved in a short period of time. A further advantage of this system is that simultaneously with heat-induced expression of the product, it is possible to get heat-induced amplification of the expression system thus amplifying the gene(s) encoding the product in question which in turn can substantially increase production of the product.

One embodiment of the present invention ultilizing PBSX will now be

described with reference to the accompanying drawings in which:-

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 alpha-amylase gene, isolated from pSL5 (36). The resulting plasmid contains unique restrictions sites for EcoR1, Sac1, Sma1 and BamH1 immediately 5' to the promoterless alpha-amylase gene.

FIG. 2.- Restriction map of the 33kb. cloned region of PBSX. Fragments used to direct integration of pWD3 are indicated below. Where known, the fragment end which is fused to the promoterless alpha-amylase gene is indicated by a symbol: (⟶), transcription proceeds in direction of arrow; (⊢——), no transcription in this direction (see Fig.3). Restriction sites: B, BamH1; E,EcoR1; S, Sac1; A,Sal1.

FIG. 3.- Plate tests demonstrating thermoinducible production of alpha-amylase when integrated in one orientation into the chromosome of strain 1A4201 at the PBSX locus. Plates A and C show strains with the alpha-amylase gene integrated in the direction metA-metC. Plates B and D show strains with the alpha-amylase gene integrated in the direction metC-metA (see Fig. 2). Colonies were grown on LB agar containing starch, at 30°C for 18 hours (plates A and B), or at 30°C for 12 hours followed by 48°C for 6 hours (plates C and D). Plates were subsequently stained with iodine.

FIG. 4.- Linkage of the Cm^r gene to the xhi1479 locus and adjacent chromosomal markers in two integrative strains: 1A4201::pWD316 and 1A4201::pWD312. Values are presented as percentage co-transduction using PBS-1. Numbers of transductants tested are given in parenthesis. The arrows point towards the selected marker in each case.

FIG. 5.- One dimensional SDS-polyacrylamide gel (12.5%), of [^{35}S]-methionine labelled 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. The strains and growth temperature at which the labelling

was carried out are as follows: lane 1: L8508 (37°C). 2: L8508 xhi$^+$ (48°C). 3: L8508 (48°C). 4: L8508::pWD316 (48°C). 5: L8508::pWD38 (48°C). 6: L8508::pWD37 (48°C). 7: L8508::pWD35 (48°C), 8: L8508::pWD32 (48°C). 9: L8508::pWD312 (48°C). 10: L8508::pWD39 (48°C). PBSX proteins are labelled according to the nomenclature of Mauel and Karamata (1984),(29), where X denotes a PBSX structural protein and P denotes a protein found only in cells induced for PBSX.

FIG. 6.- One dimensional SDS-polyacrylamide gel (12.5%) of [^{35}S]-methionine labelled proteins from *E coli* maxicells. Lane 1: *B. subtilis* L8508 (48°C), 2: *E. coli* CSR603, 3: *E. coli* CSR603/pWD3, 4: CSR603/pWD32 5: CSR603/pWD35, 6: CSR603/pWD37, 7: PBSX phage particle proteins. Proteins corresponding in size to PBSX proteins are indicated with arrows. Nomenclature is as in Fig. 5. The protein labelled X22 may correspond to protein X22 or X21 described by Mauel and Karamata (29).

FIG. 7.- 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 position of these genes is 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 (29).

FIG. 8.- Sequence of a segment of PBSX DNA showing the repressor gene orf1 and three putative operators, O1, O2 and O3. The figure also shows putative promoters and ribosome binding site (rbs). The putative operator recognition sequence is also shown.

FIG. 9.- Sequence including the region of DNA encoding the xhi1479 allele of orf1 isolated from the heat - inducible strain 1A4201. Where this differs from the wild-type sequence, the wild-type nucleotide has been indicated below.

FIG. 10.- The lysis negative phenotype observed upon integration of

pWD35 compared to wild type lysis proficient phenotype generated upon integration of pWD38. The plasmids carry a promoterless gene for heat stable alpha amylase (HT alpha amylase) from *Bacillus licheniformis* (37,36).

FIG. 11.- The heat inducible expression of the gene for heat stable *Bacillus licheniformis* alpha amylase after heat induction of PBSX in 1A420:pWD35 and comparison with a non-inducible strain and a strain harbouring a multicopy recombinant plasmid pSA33 containing the same alpha amylase gene.

FIG. 12a.- Growth curves of IA4201::pWD35 thermoinduced rapidly or gradually.

FIG. 12b.- Copy number of the CAT gene relative to the β-glucanase gene as a function of time, on both rapid and gradual induction showing that an increase in the copy number of genes integrated in the PBSX genome takes place on heat induction.

Fig. 13.- Construction of the PBSX late promoter-lacZ transcriptional fusion. The 1.3kb EcoR1-BamH1 fragment which contains the late promoter was ligated to EcoR1-BamH1 cut pDG268. The resulting plasmid was linearised using Xbal before transformation of B. subtilis. Alpha-amylase negative transformants were used for subsequent experiments.

Fig. 14.- Restriction digests of recombinant plasmids which encode the factor required for transcription from the late promoter. DNA from the PBSX early region was digested with HindIII and cloned into the HindIII site of pEB112. Plasmids were isolated from four β-galactosidase producing transformants (plasmids 1-4), and transformed into E. coli. Plasmid DNA from the E. coli transformants was digested with HindIII and restriction fragments separated on an agarose gel. Lanes 1 & 2: plasmid 1; lanes 3 & 4: plasmid 2; lanes 5 & 6: plasmid 3; lane 7: plasmid 4; lane 8: 1kb size ladder.

Fig. 15.- Nucleotide sequence of the 1.2-kb fragment which complements the xhi1479 mutation. Differences in the nucleotide sequence which occur in the temperature-inducible strain, IA4201, are

indicated above the sequence. The amino-acid sequence of Xre is indicated. Start codons for other potential reading frames are boxed. Direct repeats 01, 02, 03 and 04 are indicated in upper-case letters, while inverted repeats are indicated with facing arrows above the nucleotide sequence. -35 and -10 consensus sequences for possible promoters for transcription of xre (1a and 1b), and diverging transcription (2a and 2b), are underlined. The nucleotide sequence was determined by the method of Sanger et al.(44). These nucleotide sequences have been submitted to Gen Bank with accession Nos. M36478 and M36477.

Fig. 16.- Sequence from the left-hand end of the 1.5kb HindIII fragment to the right hand end of fragment 38. The putative amino-acid sequences in each of the three possible reading frames is also shown in conventional abbrevation.

FIG. 17. Diagramatic representation of the early region of PBSX, not drawn to scale.

Table 1:- Bacterial strains, plasmids and bacteriophage (BGSC: Bacillus Genetic Stock Center).

Table 2:- Mutagenic properties of the indicated plasmids when integrated into *B. subtilis* L8508. Presence or absence of killing of induced cells on a lawn of *B. subtilis* W23. Presence or absence of cell lysis was determined by monitoring optical density.

Table 3:- Presence or absence of the major PBSX induced proteins in cells containing the indicated integrated plasmids. Data are accumulated from 10% and 13.5% SDS-polyacrylamide gels.

Table 4:- Bacterial strains and plasmids used in assessment of positive control factor.

Table 5:- β-galactosidase production in late promoter-lacZ fusion strains in the presence or absence of mitomycin C(MMC).

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, using DNA from strain SO113 to transform IA420. PurA$^+$ colonies were selected and screened for acquisition of the amy-3 mutation. The construction of the integrating vector is shown in figure 1. The chloramphenicol acetyl-transferase gene was removed from pBD64 on a 1.1 kb. HpaII fragment. This was treated with Klenow fragment and ligated to EcoRI and Kenlow treated pUC18. The promoterless alpha-amylase gene (36, 37) was removed from pSL5 on a BamHI-HindIII and inserted between the BamHI-HindIII sites. The EcoRI site distal to the alpha-amylase gene in the resulting plasmid was removed by a partial EcoRI digestion, treatment with Klenow fragment, followed by religation. The resulting plasmid pWD3 contains unique restriction sites for EcoRI, SacI, SmaI and BamHI immediately 5' to the promoterless alpha-amylase gene.

Enzymes, chemicals and materials

Restriction enzymes, Klenow fragment and T4 DNA ligase were purchased from Boehinger Mannheim Biochemicals (Indianapolis Ind.). Nick translations were performed using an Amersham kit N5500. [^{32}P]dCTP and L-[^{35}S] methionine were also obtained from Amersham (Amersham Corp. Arlington Heights, Ill). Acrylamide, ammonium persulphate 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 New England Nuclear Corp. (Boston Mass.). X-ray film (RX) was from Fuji; X-ray developer (LX-24) and fixer (FX-40) 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 Sau3A and size fractionated on a sucrose gradient. Fragments of 14-22kb. were pooled and ligated to lambda EMBL3 digested with BamHI in a vector to insert ratio of 3:1. Recombinant plaques were

selected on a lawn of *E. coli* Nm539. Plaques were lifted onto a Pall Biodyne nylon membrane (Pall Corp., Glen Cove, N.Y.), and hybridised according to the manufacturer's instructions.

Media

B. subtilis and *E. coli* were routinely grown on Luria broth or agar. When appropriate, media contained chloramphenicol (3µg/ml) for selection in *B. subtilis* or ampicillin (50µg/ml) for selection of plasmids in *E. coli*. Alpha-amylase activity was detected by adding starch (0.2%) to the media and subsequently staining the plates with a solution of 0.5% I_2, 1% Kl.

Transformation and transduction

Transformation of *E. coli* and *B. subtilis* was carried out as described (9). PBS-1 transductions were performed as described (35). Transductants were selected either on media containing chloramphenicol, or on agar containing SS+0.4% glucose supplemented with appropriate amino acids (0.005%), and nucleosides (0.01%) and sub-cultured to appropriate media to determine linkage. Alkaline phosphatase was assayed according to the plate test described (39).

Test of PBSX killing activity

PBSX killing activity was determined by spotting lysates unto a lawn of the sensitive strain *B. subtilis* W23 as previously described (4). When cell lysis did not occur after PBSX induction, cells were sonicated before spotting supernatants unto the lawn.

DNA preparation

Small scale preparations of plasmid were obtained as described previously (3). Large scale plasmid preparations were further purified by CsCl density gradient centrifugation (28).

Large and small scale preparations of lambda DNA were prepared using either liquid culture or plate lysates (28).

Radioactive labelling of proteins

PBSX structural proteins were labelled essentially by the method described (29), except that cells of strain L8508 were grown in SS + 0.05% glucose at 37°C. Phage were induced at a cell density of 4 x 10^7 by shifting to 48°C. At 30 minutes after the temperature shift L-[^{35}S] methionine (15µCi/ml) was added and lysis allowed to continue for 1.5 hours. Phage particles were purified over a CsCl gradient.

To label proteins in cells induced for PBSX, overnight cultures were diluted to a cell density of 6 x 10^6 in SS + 0.05% glucose and grown to a cell density of 3 x 10^7. PBSX was induced by shifting the growth temperature to 48°C. At 30 min and 40 min post-induction 1ml aliquots were withdrawn and incubated with continued shaking in the presence of L-[^{35}S] methionine. After 5 min., cold methionine was added (0.33ml of 50mg/ml). Cell pellets were stored at -20°C and processed for electrophoresis as described (31). Labelling of plasmid encoded proteins in *E. coli* was performed according to the method of Sancar et al. (41).

SDS-PAGE

Labelled proteins (10^6cpm of each sample) were separated on 8%, 10% and 12.5% SDS-PAGs by the method described (26). A ^{14}C methylated protein mixture (14.3kD to 200kD) was co-electrophoresed as molecular weight markers. Gels were treated with En^{3}Hance according to the manufacturer's instructions, dried and autoradiographed for 12-48 hours.

RESULTS

Isolation of PBSX DNA

PBSX does not package its own genome (19, 34). Therefore, in order to isolate DNA coding for phage functions, a *B. subtilis* 168 chromosomal bank, (constructed in lambda EMBL3), was screened with pOK411C, a plasmid which was known to contain DNA from the PBSX prophage (36). By successive cycles of screening, approximately 33kb. of DNA from the PBSX region of the chromosome were isolated in

four overlapping lambda clones. A restriction map of the cloned region is presented in Figure 2. Hybridisation of selected fragments to chromosomal DNA digests by Southern blotting (43), indicated that no rearrangements had occurred (data not shown).

Analysis of transcriptional activity within the cloned region

With a view to analysing the transcriptional activity across this region, the cloned DNA was used to direct integration of a promoterless alpha-amylase gene into the chromosome. A series of fragments were subcloned into the integrating vector pWD3, using unique restriction sites immediately 5' to the promoterless alpha-amylase gene (fig. 2). Each plasmid was transformed into *B. subtilis* 1A4201, with selection for the acquisition of chloramphenicol resistance. In each of six cases checked, integration appeared to have occurred by a Campbell type mechanism, resulting in the plasmid sequences being flanked by direct repeats of the chromosomal DNA which directed integration (data not shown) (12, 36). Expression of the alpha-amylase gene in each of the fusion strains now provided a convenient method to assay transcriptional activity across the cloned DNA.

Four independent integrations, covering a distance of approximately 18 kb, showed low levels of alpha-amylase production 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 xhi1479 background (4), (Fig 3). However, when the alpha-amylase gene was integrated in the opposite orientation, little or no expression of the gene was observed at either growth temperature. Thus it appears that transcription of the prophage proceeds predominantly in one direction across at least 18 kb. The proportion of this induction that is attributable to gene amplification or increased transcription has not been estimated.

Position and orientation of the cloned DNA on the B. subtilis chromosome

In order 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,

1A4201::pWD312 and 1A4201::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 neighbouring chromosomal markers (fig. 4). The chloramphenicol resistance marker in strain 1A4201::pWD312 was closely linked to phoS, a mutation which results in constitutive alkaline phosphatase expression (39). In strain 1A4201::pWD316 the chloramphenicol resistance gene had integrated close to the xhi1479 allele which confers the heat-inducible phenotype (4). The data suggest the order of markers as shown in Figure 4. 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 (4, 15, 16).

The cloned DNA is of PBSX origin.

Thermo-induction of alpha-amylase expression in the fusion strains carrying the xhi1479 allele, together with the mapping data provided strong evidence that the cloned DNA was derived from the PBSX region of the chromosome, but did not exclude the possibility that much of the DNA could lie outside the PBSX genome itself. Indeed, replication of PBSX DNA is thought to extend into flanking host DNA (2, 16, 49). In order to distinguish between these possibilities the mutagenic properties of integrating plasmids were exploited: If integration is mediated by an internal fragment of an operon, then the integration event will disrupt functions of this operon (32, 40).

Production of PBSX particles can be detected by their bacteriocidal activity on a PBSX sensitive strain *B. subtilis* W23 (33,45,46). The plasmid pOK411C, isolated by O'Kane *et al.* (36), was shown to contain a fragment of PBSX origin by its ability when integrated to abolish this PBSX killing function. Each fragment as shown in figure 2 was used to direct integration of pWD3 into *B. subtilis* L8508, and the resulting strains tested for production of killing activity. Of 11 strains tested, 9 showed a marked reduction in killing activity when compared to 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 sub population of cells).

Furthermore, the same integrant strains which showed reduced killing activity failed to show the characteristic pattern of PBSX induced cell lysis when grown in liquid media (table 2). These strains presumably resulted from integration events which disrupt a PBSX operon thus preventing production of factors required for killing activity and cell lysis.

Production of PBSX proteins in strains containing integrated plasmids

At least 12 polypeptides under PBSX control have been identified in induced cells (19). The results presented in table 2 suggested that the full complement of PBSX proteins was not being produced in many of the integrant strains. To examine this in more detail, the proteins produced by a representative sample of integrant strains were radioactively labelled and separated by SDS-PAGE (fig. 5). The accumulated data from 10% and 13% gels are summarised in table 3.

Fragment 316 is derived from the extreme left hand end of the map (Fig.2). Strain L8508::pWD316 fails to show induction of any detectable phage proteins. Integration of plasmid pWD37 prevents the synthesis of a number of phage proteins, including a major head protein and its proposed precursor X35 and P36 (29), several tail proteins, X76 and X19, and non-structural proteins P32 and P31. Proteins P36/X35, X19 and P18 are observed in strains L8508::pWD35, L8508::pWD32 and L8508::pWD312, although these strains lack X76, P32 and P31. Strains L8508::pWD38 and pWD39 produce all detectable PBSX proteins, which is consistent with the non-mutagenic nature of integration directed by these two fragments.

Expression of Phage Proteins in E. coli

Integration of plasmids pWD37, pWD32, pWD35 and pWD312 prevented production of some, but not all of the phage structural proteins, which suggested that the respective fragments might be derived from a late operon of the prophage. In order to examine if these fragments contained the genes for any known phage proteins, plasmids pWD37, pWD32 and pWD35 were transformed into *E. coli* CSR603. Plasmid encoded proteins produced in maxi-cells were labelled and separated on 8% and 12.5% SDS-PAGs (Fig. 6). In the strain containing pWD35 proteins were observed which corresponded in size to previously

identified phage proteins X58, X19 and P17. In addition this fragment encoded a protein which corresponded in size to a newly identified phage protein which we have named X59. It can be seen when phage particle proteins are separated on low percentage gels. Plasmid pWD32 encoded a protein which corresponded in size to a phage tail protein, (X22), while pWD37 gave rise to a protein which 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 (7). In each case peptides of similar size to those of the phage proteins were produced, thus confirming their identity (data not shown).

Generation of a functional map of the PBSX prophage

The information obtained from the expression of proteins in the *B. subtilis* strains and from the expression of proteins in *E. coli* have been combined to create a functional map of the cloned region (fig. 7). The early region of the prophage has been so called as the production of any detectable phage proteins is prevented by integration directed by fragment 316. This suggests an integration event into an operon whose expression is required for expression of all late phage genes. Furthermore, this fragment is closely linked to the xhi1479 allele which is presumably within the prophage repressor gene.

The late operon indicated is thought to be at least 18 kb in length. The restriction map suggests that fragments within the region 8 kb to 26 kb direct mutagenic integration, indicating that each is 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. Within this proposed operon, genes have been assigned to regions based on the assumption that in each strain transcription will proceed only to the end of the fragment which directs integration in each case, i.e. the 3' end of the operon, downstream from the integrated plasmid sequences in each case will not be transcribed. Thus, a protein absent in one integrant strain, but present in a strain containing the plasmid integrated further downstream, can be assigned to the intervening region. (e.g. Protein X19 is absent in strain L8508::pWD37, but present in L8508::pWD35 and hence the gene for this protein has been

tentatively assigned to fragment 35.)

Cloning of a PBSX repressor gene.

The xhi1479 allele renders *Bacillus subtilis* thermoinducible for PBSX. A strain which contains this allele, grows normally at 37°C, but upon shifting the growth temperature to 48°C, PBSX is induced leading to cell lysis. This phenotype is presumed to be coded by a gene which encodes a PBSX repressor protein (4).

Fragments of DNA from the PBSX region of the chromosome were cloned into a replicating plasmid pRP22, and tested for their ability to allow the strain *B. subtilis* 1A4201, which is thermoinducible for PBSX, to grow at the non-permissive temperature. A clone containing a 1.2kb fragment was isolated which when cloned into pUB110 was capable of complementing the xhi1479 allele. This fragment was therefore expected to encode the wild-type repressor protein.

Determination of the nucleotide sequence.

Nucleotide sequences from the 1.2kb fragment was determined by the dideoxy chain termination method (42). The 1.2kb EcoR1 fragment was cloned in both orientations into the EcoR1 site of M13mp18. This allowed the determination of the sequence of both ends of the fragment. Further sequencing was carried out by generating a set of deletions using Bal31 exonuclease. In each case, the nucleotide sequence was determined using an M13 universal primer. Where overlapping sequences were not obtained, oligonucleotides with complementarity to the known sequence were used as primers. The nucleotide sequence was determined for both strands and is shown in Fig. 8, for bases 220-1195.

Sequence Analysis

1. Homology to known sequences

As an initial step in the analysis of the sequence, both strands of the 1200 bp sequence were searched for open reading frames of greater than 90 nucleotides in length. In total 13 such open reading frames were found of which orf1 is indicated in Fig. 8.

In order to see if the sequence was homologous to any known DNA sequence, the Genbank database (release no. 58) was searched with both strands of the DNA sequence using the methods of Lipman and Pearson (27). No significant homology was found.

The sequence was then translated into all six reading frames, ignoring stop codons, and each one was used to search the NBRF/PIR protein database. This method was employed for convenience and also has the advantage that frame shift errors in the sequence will not affect significantly the result of the homology search. The translated product of one open reading frame showed weak homology when compared with bacteriophage P22 c2 repressor, the dicA gene product from *E. coli*, phage P1/P2 C repressor, the phi105 c1 repressor, and the phi105 orf 3 gene product. The region of homology in each case was found to correspond to the open reading frame (orf1) which has the potential to code for a protein of 113 amino acids (nucleotides 397-736).

Each of the proteins with homology to orf1 is either known, or postulated to be a DNA-binding protein with a repressor function. An alignment of these proteins produced by the method of Higgins and Sharp (22) indicates that homology between these proteins is restricted to the N-terminal regions. The homologous region includes the proposed DNA binding domain of each of the previously characterised proteins and strongly suggests that a protein encoded by orf1 is also a DNA-binding protein with and α-helix, turn, α-helix motif. The putative DNA-binding domain of the orf1 protein is shown in Figure 8.

Using the methods of Dodd and Egan (11), for the detection of 'cro-like' DNA-binding domains, the proposed DNA-binding domain received a score of 1911, whereas the scores of the proteins in the master set ranged from 1684 to 2968. The authors found that in a search of the PIR protein database containing 2560 sequences, no proteins were found with a score of greater than 1700 that were not thought to be DNA-binding proteins. The score obtained suggests that orf1 encodes a 'cro-like' DNA-binding protein.

When the sequence of the fragment from the strain carrying the xhi1479 allele (Figure 9) is compared with the wild type sequence

(Figure 8) it can be seen that 14 nucleotide substitutions occur within orf1. Only three of these lead to amino acid changes: glycine to serine at position 4, alanine to valine at position 19, and leucine to valine at position 78. The alanine to valine change lies within the proposed DNA-binding domain. Valine is rarely found at this position in other 'cro-like' DNA-binding domains, and the change reduces the 'Dodd and Egan' score from 1911 to 1597, which is outside the range which these authors observed for the master set of proteins (1684-2968). This observation is consistent with the proposal that orf1 encodes a "cro-like" DNA-binding protein. The evidence suggests that the xhi1479 repressor is temperature sensitive because of one or more of the amino acid substitutions identified from the comparison of the orf1 sequences shown in Figures 8 and 9.

2. Transcriptional and Translational signals

The sequence was searched for translational and transcriptional signals. The region 5' to each of the open reading frames indicated was examined for sequences which resemble ribosome binding sites (rbs) i.e. sequences which show complementarity to the 3' end of the *B. subtilis* 16S rRNA. The rbs 5' to orf1 is underlined in Fig. 8. The free energy of binding of this sequence (delta G) with the 3' end of the 16S rRNA was calculated to be -18.2 kcal mol^{-1}. Previously reported delta G values for ribosome binding sites of gram positive genes range from -11.8 to -22 kcal mol^{-1} (20). The sequence upstream from orf1 was examined for potential promoter signals related to the -35, (TTGACA), and -10, (TATAAT), consensus sequence for sigma ^{43} recognised promoters, which are usually separated by 17 or 18 base pairs (30). Two possible promoter sequences were found, P1a and P1b, and are indicated in Fig. 8.

The sequence was also examined for potential transcriptional termination sequences, which are often characterised by inverted repeats, thought to be capable of forming particular secondary structure. No typical terminator sequences were found.

3. Potential repressor binding sites.

Many phage and cellular repressors are autoregulatory, and as such are preceded by binding sites for the repressor protein. The region

preceding the gene for the repressor was examined for sequences showing characteristics of protein binding sites. Although showing little sequence homology, protein binding sites have some common features; the sequences are usually around 20 bp in size and they often contain internal dyad symmetry. The region immediately 5' to orf1 was found to contain three direct repeats of 19 bp each, which have provisionally been named O1, O2 and O3. O1 and O3 are identical over the 19 bp, and contain an internal palindromic sequence of 6 base pairs. O2 differs from these in 2 of the 19 positions, and is flanked on either side by a further inverted repeat sequence of 7 base pairs in length. The region containing these operator sequences overlaps with the proposed promoter elements for transcription of orf1 suggesting that transcription of this gene may be regulated by the protein or proteins. We predict that the orf1 gene product protein interacts at O1, O2 and O3 and controls the production of itself autogenously.

The DNA sequence shown in Fig. 8 provides good evidence that orf1 encodes a sequence specific DNA binding protein, with a typical helix-turn-helix binding domain. The DNA sequence of Fig. 8 also contains sites likely to be involved in the control of expression of orf1. These sites include putative promoters, ribosome binding sites and operators. It is hypothesised that the operator-like sequences are recognised by the product of orf1. A product of the sequence in Fig. 8, probably orf1, complements the xhi1479 allele.

The homologous 1.2kb fragment from the heat inducible strain *B.subtilis* IA4201 was also cloned and sequenced. It shows a total of 67 differences when compared to the equivalent sequence from the wild-type strain *B.subtilis* SO113. These differences include 65 nucleotide substitutions, an insertion of 2 bp at position 222 of the wild-type sequence, and a deletion of 1 bp at position 830. The sequence corresponding to the sequence of Figure 8 is shown in Figure 9.

On the basis of this evidence we claim that we have identified a PBSX repressor gene (orf1) which ultimately controls the expression of the late genes of PBSX, and any genes expressed from late PBSX promoters, and that we have identified operator sequences recognised by this repressor.

Cell lysis and induction and expression of alpha-amylase.

Tests for lysis, and for induction and expression of alpha-amylase, in *B. subtilis* strains, carrying various plasmids integrated in the PBSX genome were performed in the absence of antibiotics. Overnight cultures were inoculated into 200ml of Luria Broth in a side-arm flask and grown in an orbital incubator (Gallenkamp) at 200 rpm. Optical density, as a measure of cell numbers and therefore of growth and lysis, was measured on a Klett-Summerson photoelectric colorimeter with a green filter number 54. For thermoinduction experiments cultures were split in two and one half shifted to 48°C, the other half maintained at 37°C. The time of induction of each culture is shown in appropriate figures (10-12). In the case of rapid thermoinduction flasks were placed in a shaking waterbath for 8 minutes at 48°C, for gradual induction they were placed directly into an orbital incubator at the same temperature. Aliquots were removed at intervals prior to and post induction and centrifuged at 3,000g for 15 minutes, the pellets were retained for chromosomal DNA preparation and alpha amylase assays were performed on the supernates.

Fig. 10 shows growth curves for 1A4201::pWD35 and 1A4201::pWD38 and the effect of temperature induction at 2 hours. After heat induction the latter strain lyses, but the former does not. This shows that certain integrants, of which 1A4201::35 is an example, are deficient in lysis after induction of PBSX. This is due to mutagenesis caused by integration of derivatives of the non-replicative plasmid pWD3 carrying segments of the PBSX genome, in this case pWD35. The strain carrying pWD38 is not defective in lysis. This experiment establishes that integration at certain sites in the PBSX genome blocks lysis. Table 2 is a summary of the data for various integrants.

Expression of an heterologous gene inserted in the PBSX genome is heat inducible in a strain carrying the xhi1479 allele.

Fig. 11 shows that on raising the temperature of the culture to 48°C at time zero the strain 1A4201::pWD35 which carries the heat inducible PBSX mutation xhi1479 begins to express the HT amylase gene from pWD35, which is integrated in the PBSX genome. In a control

strain not carrying the xhi1479 allele HT alpha amylase expression was not expressed at a detectable level. In a second control strain LA2, which carries the HT amylase gene on a plasmid pSA33 (37) where it is expressed under the control of its own promoter, the level of expression of the HT gene is less and is not heat inducible.

This experiment establishes that a promoterless heterologous gene can be integrated in the PBSX genome, and that expression of this gene can be induced by heat in an xhi1479 background.

The DNA sequence of a fragment carrying the xhi1479 allele was determined as described above for the 1.2 kb fragment of PBSX and is shown in Fig. 9.

Copy number estimation

The objective of these experiments was to measure the copy number of the DNA sequences integrated in the PBSX genome before and after heat induction of a strain carrying the xhi1479 mutation.

Plasmid pJG14 (Devine et al, J. Bacteriol. 1989., 171, 1166) was used as a ^{32}P radiolabelled probe. This carries the CAT gene which is also part of all the derivatives of pWD3, and a segment of the chromosomal β-glucanase gene. It can therefore be used to measure the copy number of the pWD35 sequences integrated in the PBSX genome relative to the copy number of chromosomal sequences not linked to PBSX. Chromosomal DNA preparations from 1A4201::pWD35 were made from cells harvested at different times before and after heat induction. Heat induction was carried out under regimes as described above, rapid induction and gradual induction. The DNA was digested with EcoR1 and electrophoresed on a 0.8% agarose gel. Southern blot and hybridisation on Biodyne membranes was performed as advised by the manufacturers. Autoradiography was performed on probed filters. Copy number was estimated by comparing the intensity of the signal produced by DNA within the PBSX region to DNA from another part of the chromosome (β -glucanase). Signal intensity was measured on a DESAGA Chromatogram Densitometer CD50.

Fig. 12a shows the growth curves of 1A4201::pWD35 thermoinduced rapidly or gradually after 2 hours. Item Fig. 12b shows the copy

number of the CAT gene relative to the β-glucanase gene in both cultures as a function of time. It can be seen that the copy number of CAT rises after heat induction to peak values of 5-8 per chromosomal copy of β-glucanase. It falls later for unknown reasons.

This experiment establishes that heat induction of the PBSX system causes an increase in the copy number of genes integrated in the PBSX genome.

Transcription of the PBSX late operon requires a positive control factor.

Transcription of the PBSX late operon can be detected only after induction of the prophage. The promoter for this operon has been localised by insertional mutagenesis to lie within a 1.3kb EcoR1-Sac1 fragment, 38 (Wood et al., in press). The mechanisms whereby transcription from this promoter is controlled were examined.

For the isolation and characterisation of factors involved in transcriptional regulation from this promoter, a genetic background lacking the PBSX prophage was desirable. Although attempts to cure B. subtilis 168 of the PBSX prophage failed, strains which contain large deletions in the PBSX region of the chromosome have been isolated (5). Hybridisation analysis of one of these strains B. subtilis RB1081, indicated that the deleted region includes the late operon of the prophage and at least some of the early region, including the previously cloned repressor gene (Wood et al., manuscript submitted). Thus, this strain provides a useful background for the analysis of transcriptional control of the late operon.

Construction of late promoter-lacZ fusion strains

Bacterial strains and plasmids are listed in table 4.

Transcription from the late promoter was monitored by using a transciptional fusion to the β-galactosidase structural gene. The PBSX late promoter P_L, was removed from pWD38 on a 1.3kb EcoR1-BamH1 fragment and cloned between EcoR1 and BamH1 sites of pDG268, immediately 5' to a promoterless lacZ gene. The resulting

plasmid was linearised using XbaI and used to transform B. subtilis strains RB1081 and 1A420. Integration of the plasmid into the chromosome by a double recombination event results in disruption of the α-amylase coding sequence by the integrated P_L-lacZ fusion (see Fig.13). This resulted in strains RB1081[P_L-lacZ] and 1A420[P_L-lacZ]. In each case the late promoter-lacZ fusion is integrated at the amylase locus which is quite distinct from the PBSX prophage.

Expression of the late operon is positively regulated.

β-galactosidase production in each of these strains was examined in the absence and presence of MMC (table 5). In the case of RB1081[P_L-lacZ], no expression of β-galactosidase was observed. In 1A420[P_L-lacZ], expression of β-galactosidase was observed only after induction of the resident prophage by addition of MMC to the media. These results confirmed the presence of a promoter on this fragment and furthermore suggested that transcription from the late promoter is not directly controlled by the repressor (as no repressor gene is present in RB1081), but rather is positively controlled by a PBSX encoded factor.

Isolation of a fragment which encodes a positive control factor.

In order to isolate the gene encoding this factor, DNA from a plasmid which contains a 15kb insert spanning the PBSX early region (pHV1435h), was digested with HindIII. Fragments were ligated to the E.coli - B. subtilis shuttle vector, pEB112(cut with HindIII), and transformed into B. subtilis RB1081[P_L-lacZ]. Kanamycin resistant transformants were screened for production of β-galactosidase. Four such transformants were isolated, each of which was noted to grow poorly, and when restreaked on fresh plates, segregated white colonies. Each of the recombinant plasmids contains a 1.5kb HindIII fragment (Fig.14). One of these plasmids was named pWH15. It is not known whether the observed instability is due to the cloned insert; the vector, pEB112, has previously been reported to be unstable in B. subtilis.

This fragment may enclose a novel sigma factor such as is found in the B. subtilis lytic phage SP01 (reviewed by Losick and Pero, (53);

a factor which is required in addition to the host holoenzyme such as the gene 4 protein of ϕ29 (54); or an anti-termination factor, analogous to the Q protein of λ (reviewed in Hendrix et al., (21).

Conclusion

A molecular genetic analysis of the PBSX prophage has been carried out 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. The relative positions of these regions within the cloned region 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) (4, 15, 49).

It is suggested that many of the late phage genes are contained within a large operon of between 18 and 30 kb. in length and transcribed in the direction metA-metC. Such polycistronic operons are characteristic of other phage genomes eg. lambda (21). The coding capacity of this operon may be sufficient to produce all the phage structural and late proteins (29). However the existence of another late operon cannot be excluded as ambiguity surrounds the location of the genes coding for the proteins P35/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 20 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 (2, 18, 19, 34). The cloning of an origin of replication from the PBSX region of the chromosome has been reported (1).

By integrating the promoterless alpha-amylase gene at the PBSX locus the potential for using PBSX functions as the basis of a thermoinducible expression vehicle has been demonstrated. Plasmid based thermoinducible expression systems have been described for *B. subtilis* which exploit the controlling elements of early region of bacteriophage phi105 (10, 38). The system presented here

demonstrates that a foreign gene can be integrated on the *B. subtilis* chromosome under the control of an inducible prophage promotor. Furthermore the foreign gene is located within a structure reported to the capable of undergoing stable gene amplification (24). Indeed stable maintenance of both single and amplified copies of the alpha-amylase gene have been observed when integrated at this locus.

It is convenient to obtain heat-inducible expression by integrating the gene within PBSX carrying the xhi1479 allele. Depending on the site of integration the system may be manipulated so that on induction it shows an increase in copy number and/or it does not lyse and/or it shows induced expression of the integrated gene. These integrants are stable at low temperature.

After induction it has been shown that the copy number of the DNA integrated in PBSX increases. The repressor gene (orf1) has been cloned and sequenced. Operator and promotor sequences have been identified. The xhi1479 allele can be used as the basis of a heat inducible expression system to obtain heat inducible expression of heterologous genes transcribed from promoters which are controlled by operators of the type shown in O1,O2 and O3.

Restriction mapping the sequence data from the early region of PBSX:

Fig. 17 is not drawn to scale. but reading left to right it shows:

(i) A 1.2kb EcoR1 fragment which carries open reading frames. One orf1 is the repressor gene. The second of 69 condons reads in the opposite orientation and is called orf13. The sequence between orf1 and orf13 has four related inverted repeats. These overlap four putative promoters for sigma 37 RNA polymerase, two facing into orf1 and two facing into orf13. The inverted repeats thus resemble operators of the type O_R of phage lambda. Orf13 may be another controlling gene.

Additional information that orf1 encodes the repressor function on the 1.2kb EcoR1 fragment: a Bcl1 - Nae1 fragment of 0.73 kb carries orf1 but not orf13. This fragment encodes repressor activity as judged by its ability to confer temperature-insensitivity on 1A420l.

(ii) A 0.9 kb EcoR1 fragment.

(iii) An EcoR1 fragment called 316. Inserts at 316 block induction of PBSX.

(iv) A 0.8 kb EcoR1 fragment.

(v) An EcoR1 - Sac1 fragment called 38 which carries the late phage promoter. This has been sequenced and the sequence is shown in Fig. 16. It has three long open reading frames of unknown function.

(vi) A Sac1 - EcoR1 fragment called 340.

(vii) An EcoR1 - EcoR1 fragment called 37. This has been sequenced. This fragment is expected to contain one or more late genes.

(viii) A HindIII 1.5kb fragment which overlaps 316 and 38 and contains the small 0.8 kb EcoR1 fragment. The fragment encodes a positive control function which stimulates transcription from the late promoter on fragment 38. The HindIII fragment has been sequenced. One ORF called orf2 encodes a sequence resembling a helix-turn-helix motif and therefore may encode a DNA-binding protein. This ORF is slightly homologous to sigB of B. subtilis. A deletion of BclI fragment which overlaps the 5' end of orf2 eliminates the positive control activity of the HindIII fragment. These data suggest that orf2 encodes the positive control factor.

A current working model of the system is as follows:-

There is a single transcription unit which extends from the 1.2kb EcoR1 fragment at the left into the 1.5kb HindIII fragment. The first gene in this fragment is orf13.

The gene orf1 (now called xre) encodes the PBSX repressor.

The repressor binds to the operators between orf1 and orf13.

At low repressor concentration the expression of orf13 is repressed by Xre, the repressor encoded by orf1.

At high repressor concentration the expression of orf1 is repressed by Xre.

On induction the repressor concentration is greatly reduced allowing transcription into orf13 and into the positive control factor gene on the 1.5kb HindIII fragment which is believed to be encoded by orf 2.

The positive control factor causes transcription from the late promoter on fragment 38.

Due to the genetic and morphological similarities between PBSX and the related phibacins PBSW, PBSY and PBSZ, these phibacins would also be expected to be suitable for use as expression systems as dicussed above.

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Table 1

Plasmid, strain or bacteriophage	Genotype	Source or reference
Plasmid:		
pBD64	Cm^R	(16)
pUC18	Ap^R	(44)
pSL5	Ap^R, -amy structural gene	(31)
pOK411C	Cm^R, -amy structural gene	(31)
pWD3	Cm^R Ap^R, -amy structural gene.	This study.
E. coli:		
DH5∝	F⁻endA1 hsdR17(r_K^-,m_K^+) supE44 thi-1 lambda⁻ recA1 gyrA96 relA1 o80dlacZΔM15	Bethseda Research Laboratories.
Nm539	supF hsdR (P2cox3)	(12), Promega Biotec.
CSR603	F⁻ thr-1 leuB6 proA2 phr-1 recA1 argE3 thi-1 uvrA6 ara-14 lacY1 galK2 supE44 mtl-1 rpsL31 tsx-33 xyl5 lambda⁻	R.L. Rodriquez.
B. subtilis 168:		
SO113	trpC2 amy-3	(32)
IA420	ilvA1 metB5 purA16 xhi1479 xki1479	(4), BGSC.
IA4201	ilvA1 metB5 xhi1479 xki1479 amy-3	This study.
L8508	xhi1479 lyt-2	D. Karamata.
SL345	phoS5 leuA8 rif-2 spoIIE64	R. Buxton.
IA78	metC3 pyrA xtl-1	BGSC.
IA158	metA	BGSC.
B. subtilis W23:		
SB623	thr (PBSZ)	BGSC.
Bacteriophage:		
Lambda EMBL3		(12), Promega Biotec.
PBS-1		BGSC.

Table 2

Integrated Plasmid	Killing Activity on *B.subtilis* W23	Cell Lysis upon PBSX Induction
None	+	+
316	-	-
38	+	+
37	-	-
35	-	-
314	-	-
313	-	-
32	-	-
31	-	-
311	-	-
312	-	-
39	+	+

Table 3

PROTEIN (m.wt.)	INTEGRATED PLASMID						
	316	38	37	35	32	312	39
X76 (Tail)	-	+	-	-	-	-	+
P70	-	+	+	+	+	+	+
P36	-	+	-	+	+	+	+
X35 (Head)	-	+	-	+	+	+	+
P32	-	+	-	-	-	-	+
P31	-	+	-	-	-	-	+
X19 (Tail)	-	+	-	+	+	+	+
P18	-	+	-	+	+	+	+
p14	-	+	+	+	+	+	+

TABLE 4

Strains	Description	Reference/Source
B. subtilis RB1081	*pro(AB) pyrX*ΔPBSX	Ref 5
B. subtilis 1A420	*ilvA1 metB5 purA16 xhi 1479 xki 1479*	Ref 4
Plasmids:		
pWD38	pWD3 with 1.3kb *EcoR*1-*Sac*1 fragment containing PBSX late operon promoter (P_L)	Inventors Lab.
pDG268	Cm^R, integrating vector. Contains promoterless *lacZ amy* E	Inventors Lab.
pEB112	*E. coli*-*B. subtilis* shuttle/expression vector (Ap^R,Kn^R)	Ref 52.
hHV1435h	hHH1435 with 15kb of PBSX DNA from clone C	Inventors Lab.

TABLE 5

	β-galactosidase activity	
	-MMC	+MMC
B. subtilis RB1081[P_L-lacZ] (PBSX)	-	-
B. subtilis 1A4201[P_L-lacZ] (PBSX)	-	+
B. subtilis RB1081[P_L-lacZ]/pEB112	-	n.e.
B. subtilis RB1081[P_L-lacZ]/pWH15*	+	n.e.

β-galactosidase production in B. subtilis 168 strains containing transcriptional fusions of the PBSX late promoter (P_L), to the β-galactosidase structural gene. (β-galactosidase production was scored by observing blue colouration of colonies when grown on media containing X-gal).

*Plasmid pWH15 contains a 1.5kb HindIII fragment from the early region of PBSX, cloned into the unique HindIII site of pEB112.

n.e. not examined.

CLAIMS

1. An expression system comprising a phibacin or a mutant thereof, or a gene or a mutant gene of a phibacin having a function in gene expression.

2. An expression system as claimed in claim 1 wherein the phibacin is a phibacin isolated from *Bacillus* species.

3. An expression system as claimed in claim 2 wherein the phibacin is isolated from *Bacillus subtilis*.

4. An expression system as claimed in claim 3 wherein the phibacin is selected from the phibacins PBSW, PBSX, PBSY and PBSZ of *Bacillus subtilis*.

5. An expression system as claimed in claim 1 comprising the phibacin deposited with the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, on 6th September 1989 under the accession no. NCIMB 40205, isogenic derivatives thereof, and phibacins which are substantially similar thereto, having a function in gene expression.

6. An expression system as claimed in claim 1 comprising a mutant of a phibacin which does not lyse the host cell on induction.

7. An expression system as claimed in claim 6 wherein the mutant is created by insertional mutagenesis.

8. An expression system as claimed in claim 6 wherein the phibacin carries the xhi1479 mutation.

9. An expression system as claimed in claim 6 comprising the mutant phibacin deposited with the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, on 6th September 1989 under the accession no. NCIMB 40206, isogenic derivatives thereof and phibacins which are substantially similar thereto, the said phibacins having a function in gene expression and being non-lysogenic on induction.

10. An expression system as claimed in claim 1 comprising a repressor gene, a promotor and at least one operator, isolated from a phibacin.

11. An expression system as claimed in claim 10 wherein the repressor gene is the orf1 gene encoded by the phibacin PBSX.

12. An expression system as claimed in claim 10 wherein the repressor gene has the DNA sequence shown in Fig. 8, or a sequence which is substantially similar thereto encoding repressor activity.

13. An expression system as claimed in claim 10 wherein the promotor has the DNA sequence shown in Fig. 8, or a DNA sequence substantially similar thereto, encoding promotor activity.

14. An expression system as claimed in claim 10 wherein the operator has the sequence of any one of the operators 01, 02 and 03 shown in Fig. 8, or a DNA sequence substantially similar thereto and encoding operator activity.

15. An expression system as claimed in claim 10 comprising a temperature-sensitive repressor gene so that product expression is heat-inducible.

16. An expression system as claimed in claim 15 wherein the temperature-sensitive repressor gene is the xhi1479 allele of the orf1 gene of PBSX.

17. An expression system as claimed in claim 15 wherein the temperature-sensitive repressor gene has the DNA sequence shown in Fig. 9, or a sequence which is substantially similar thereto encoding heat-inducible repressor activity.

18. An expression system as claimed in any of claims 10 to 17 which also comprises a gene encoding a positive control factor isolated from a phibacin.

19. An expression system as claimed in claim 18 wherein the positive control factor-encoding gene is the orf2 gene encoded by the phibacin PBSX.

20. An expression system as claimed in claim 18 wherein the positive control factor-encoding gene has the sequence shown in Fig. 16, or a sequence which is substantially similar thereto encoding positive control factor activity.

21. A repressor gene isolated from a phibacin.

22. A repressor gene as claimed in claim 21 which is isolated from PBSX.

23. A repressor gene as claimed in claim 22 comprising the orf1 gene of PBSX.

24. A repressor gene as claimed in claim 21 comprising a repressor gene having the DNA sequence shown in Figure 8 or a sequence which is substantially similar thereto the said gene encoding repressor activity.

25. A repressor gene as claimed in claim 21 wherein the repressor gene is a temperature-sensitive repressor gene isolated from a phibacin.

26. A repressor gene as claimed in claim 25 which is isolated from PBSX.

27. A repressor gene as claimed in claim 26 wherein the temperature-sensitive repressor gene is the xhi1479 allele of the orf1 gene of PBSX.

28. A repressor gene as claimed in claim 25 wherein the temperature-sensitive repressor gene has the sequence shown in fig. 9, or a sequence which is substantially similar thereto the said sequence encoding heat-inducible repressor activity.

29. A gene encoding a positive control factor isolated from a phibacin.

30. A gene as claimed in claim 29 which is the orf2 gene encoded by the phibacin PBSX.

31. A gene as claimed in claim 29 which has the sequence shown in Fig. 16, or a sequence which is substantially similar thereto encoding positive control factor activity.

32. A gene as claimed in claim 29 contained in the phibacins deposited

with the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland on 6th September 1989 under the accession nos. NCIMB 40205 and NCIMB 40206, or a gene substantially similar thereto encoding positive control factor activity.

33. Bacterial hosts containing one or more of the expression systems as claimed in any one of claims 1 to 20 or one or more of the genes as claimed in any one of claims 21 to 32.

34. Plasmids carrying one or more of the genes as claimed in any one of claims 21 to 32.

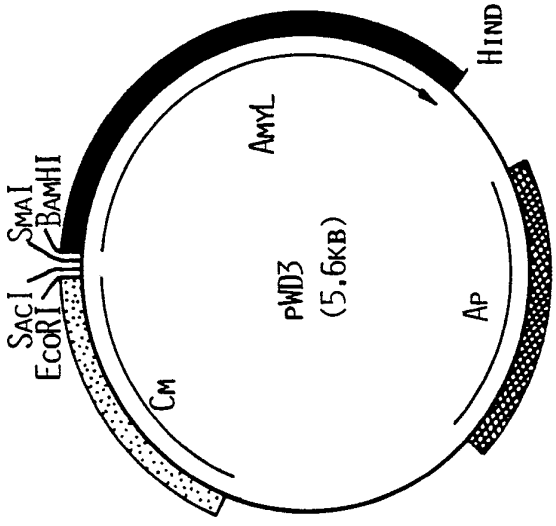
35. Expression systems as claimed in claims 1 to 20, genes as claimed in claims 21 to 32, bacterial hosts as claimed in claim 33 and plasmids as claimed in claim 34 in biologically pure or isolated form.

36. A method of producing a gene product in which the gene in question is inserted into an expression system as claimed in any of claims 1 to 20 defined above and the expression system is introduced into a bacterial host to express the gene product.

37. A method of integrating a gene into a bacterial chromosome in which a segment of phibacin DNA is inserted into a plasmid carrying the desired gene, the plasmid is introduced into a bacterial cell carrying on the chromosome at least a portion of phibacin DNA having the same or a substantially similar DNA sequence as that of the phibacin DNA segment contained on the plasmid, and recombination events between the plasmid and the phibacin DNA in the bacterial chromosome, which integrate the desired gene into the chromosome, are selected for by methods known in the art.

38. An expression system substantially as described herein with reference to the accompanying drawings.

FIG. 1


SmaI
BamHI
SacI
EcoRI
Cm
pWD3
(5.6kb)
AmyL
Ap
Hind III

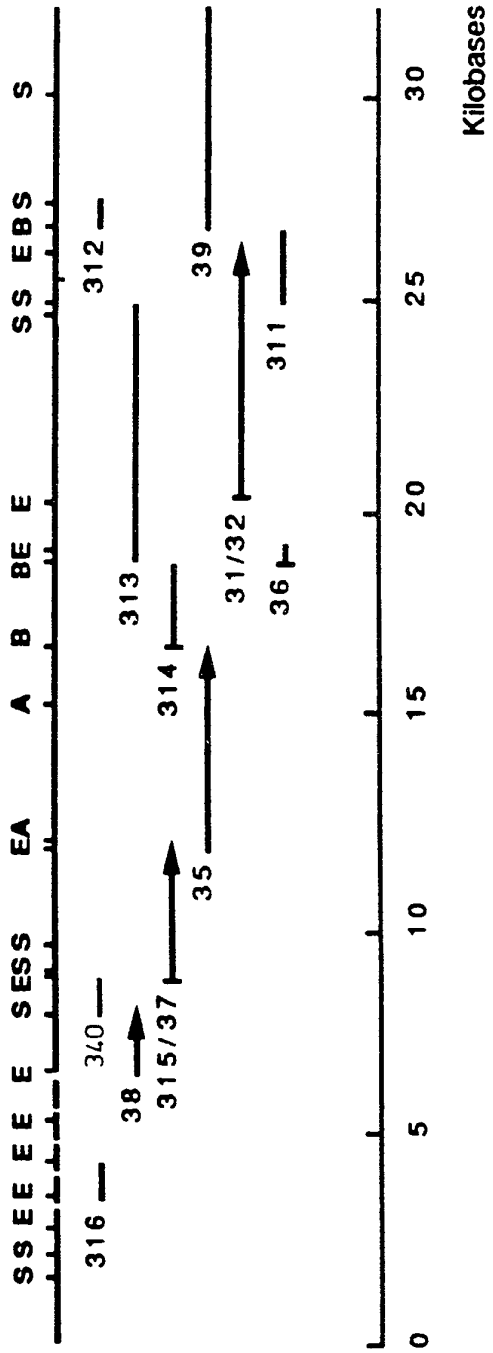

S S E E E E S E S S E A B B E E S S E B S S
316
340
38
315/37
35
314
313
312
31/32
36
39
311
0
5
10
15
20
25
30
Kilobases

FIG. 2

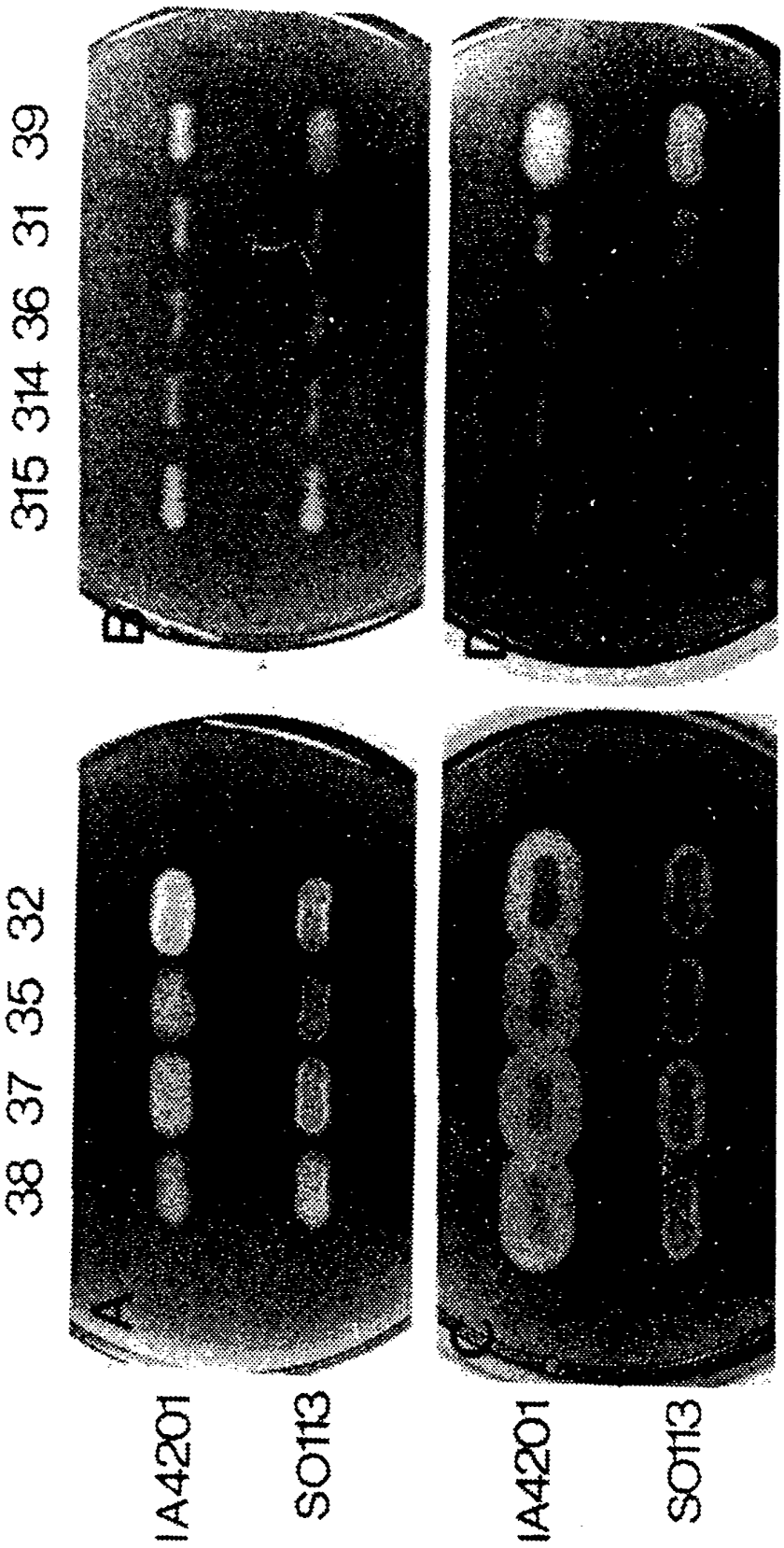


FIG. 3

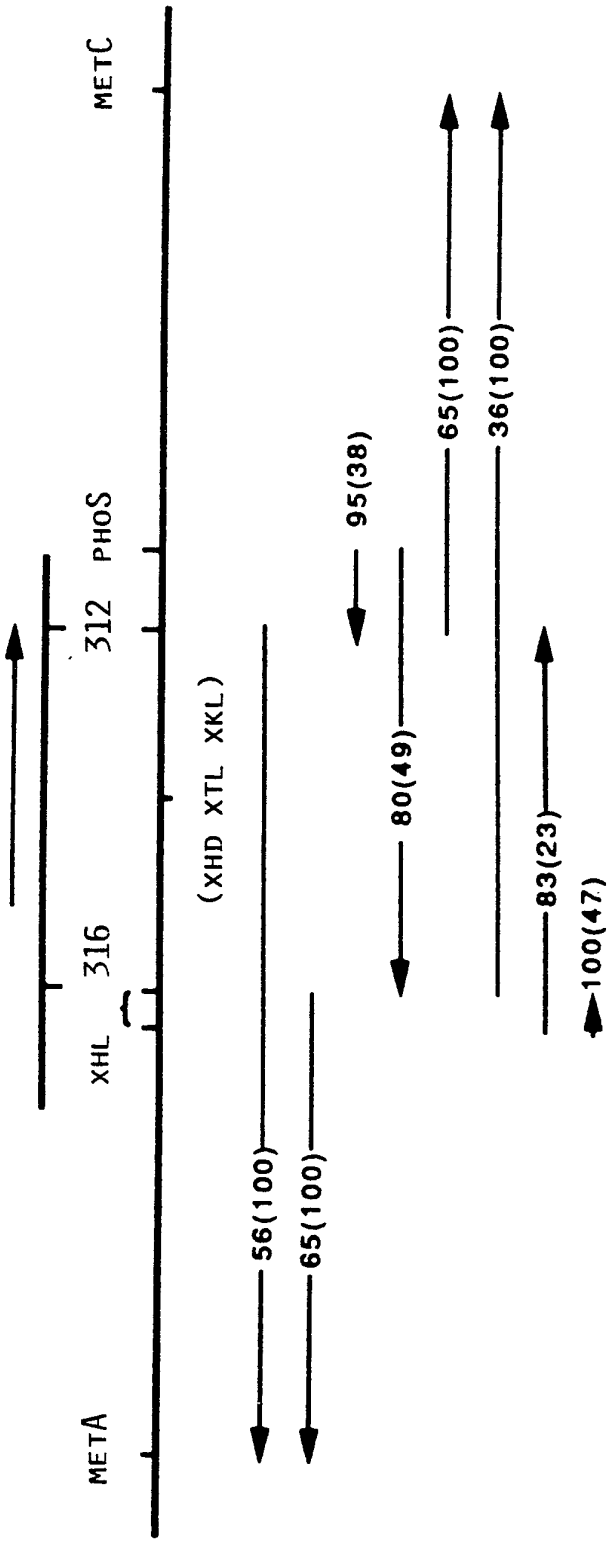

METC
PHOS
312
316
XHL
(XHD XTL XKL)
META
95(38)
80(49)
65(100)
36(100)
56(100)
65(100)
83(23)
100(47)

FIG. 4

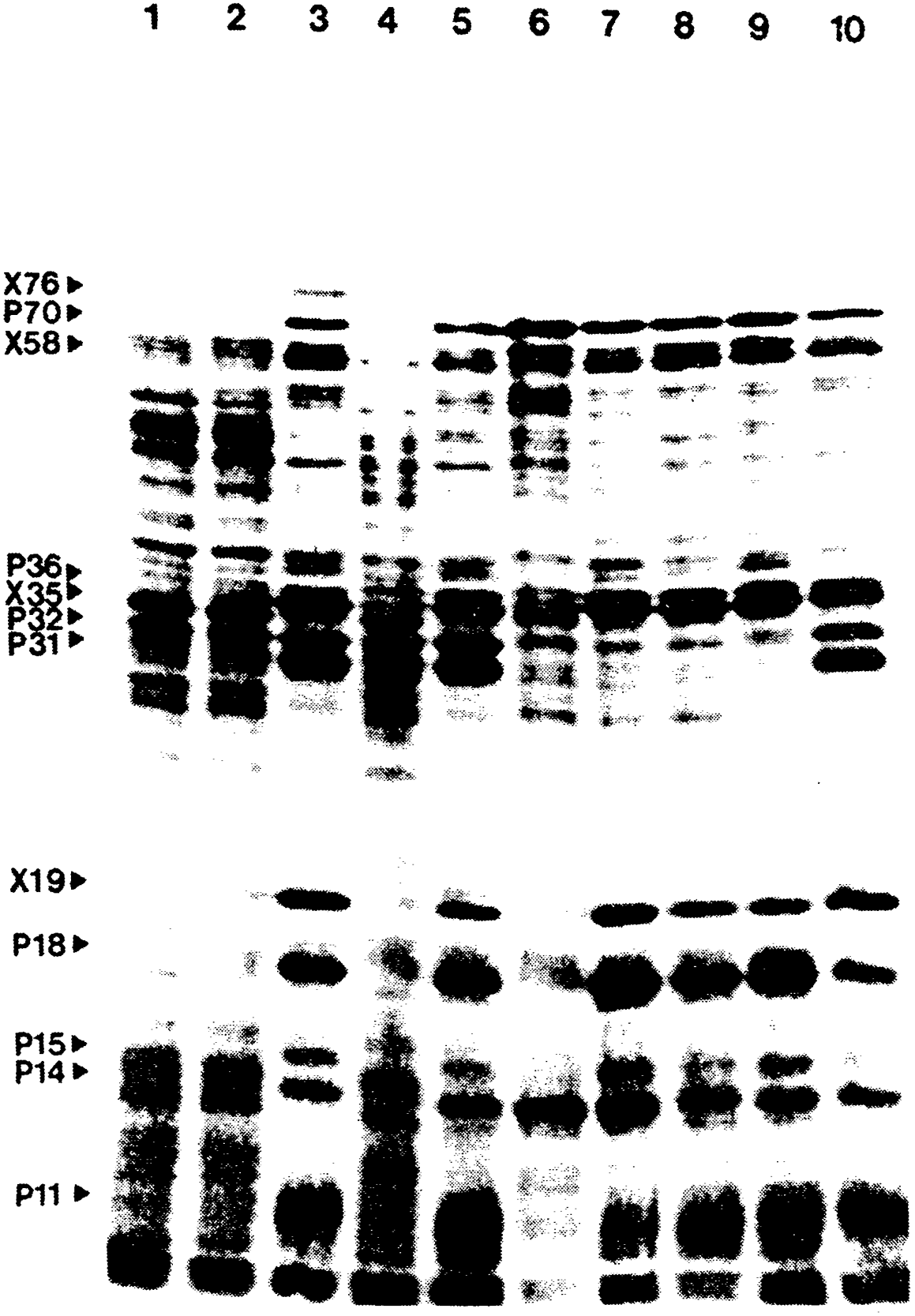


FIG. 5

REPLACEMENT SHEET

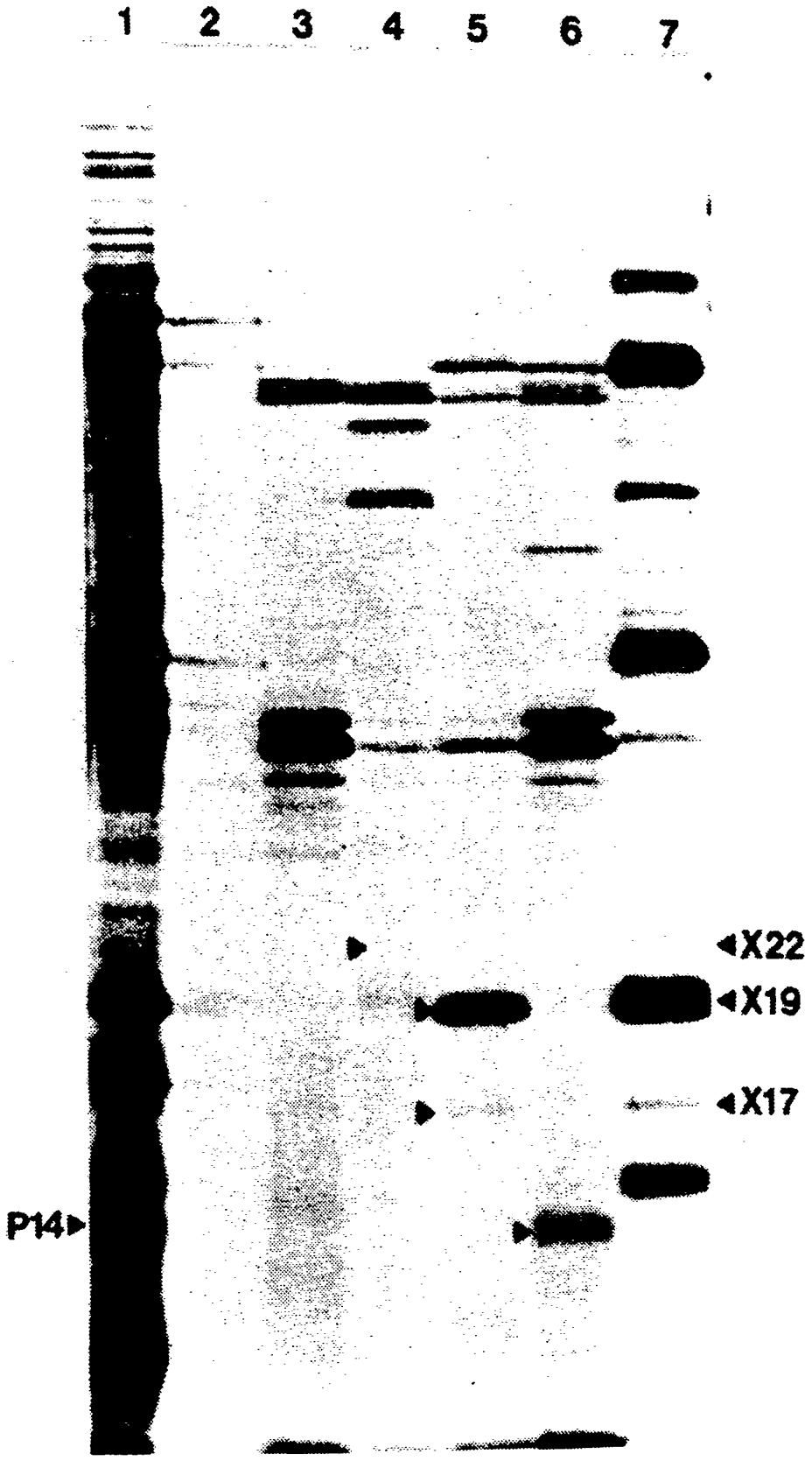


FIG. 6

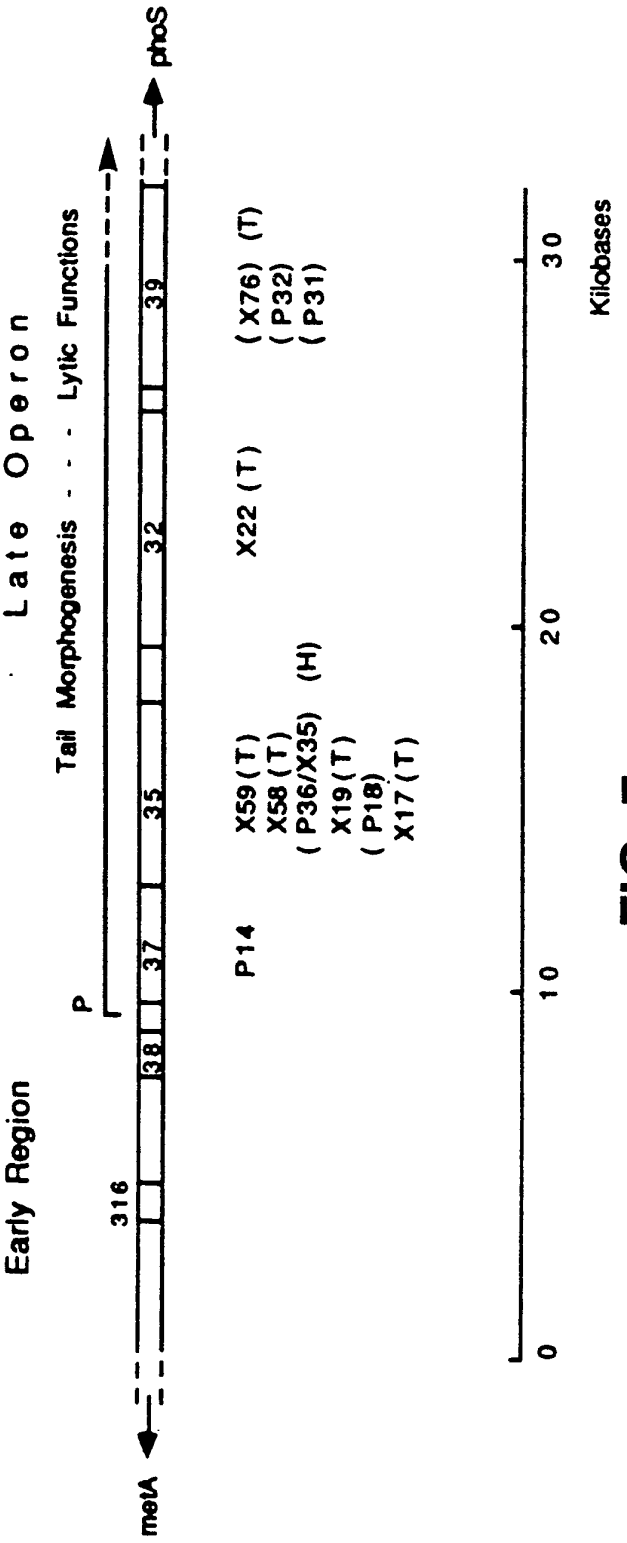

Late Operon
Tail Morphogenesis - - - Lytic Functions
Early Region
metA
316
38
37
P
35
32
39
phoS
P14
X59 (T)
X58 (T)
(P36/X35)
X19 (T)
(P18)
X17 (T)
(H)
X22 (T)
(X76) (T)
(P32)
(P31)
0
10
20
30
Kilobases

FIG. 7

THREE PUTATIVE OPERATORS
AMINO ACID SEQUENCE
START
REPRESSOR GENE
SEQUENCE
END
Repr.

FIG. 8a

ctgttctctaaaacatataaaaagtagaccgatataaagaaaaaaagtgtttatttttttaaagaaaaggga

aagatttctacactaccttccagtcctatacgggctttttctttctcgctaaaaacagaacaaaacgttcga

aagggagtattcaattgggcgattacttatcacatctggaggaatacgttaaaaatttatacggccggct

gggcatcacatccccctcatcacattgacatgctgaaaaatcgcaaaggatctggatatttgggtgcatttt

gaggatatggggagcatgatggtgaaaatacgacggcatgtacagtatcgtattgaaccaaaaaaagtcac

gggaagagcaatgggaggatttggccatgagctgtgccacgtgttaaagcatgcaggcaatcatttca

gatgaacaagctcttcagagagcttcaggaattc

FIG. 8b

```
                M
      atgtgatatcactctcctgatctgTTTTGATACTTTTCGTA  258
                --          t          a   t
TCaactgttaccaagtataaacgatacaaaactgtatcatcaagttatTTTTGATACTTTTTTTTATCataa  328
   gga            tt
ctttaTTTTGATACAGATTGTATCtataatcattagtaacttagggagtttaaaaaagagaggtcatagt  398
              tt          a     g
atgataggcagcagattgaagagtctcagagggaaaaggacacaggaagaaatcgtatctcatatcggtg  468
        g                                 c   a   c
M I G S R L K S L R G K R T Q E E I V S H I G V
      G                                   A
tgtcgcgggcacgatatcccactatgaaaacgggcgaagcgaacctgattacgacacactccaaaagct  538
      a                           c
  S R A R Y S H Y E N G R S E P D Y D T L L Q K L
ggctgattacttcaagtaacgactgattacttattaacagggaaagacaaaaaatccgatgacgatatg  608
                                    g
  A D Y F Q V T T D Y L L L T G K D K K S D D D M
ttctcagatcccgacttgcaggtagcataccgtgatatgcaggatttttccccagaaagcaaacagcagg  678
           g   c                 c t       c
F S D P D L Q V A Y R D M Q D F S P E S K Q Q A
              L
```

FIG. 9a

```
ccattgaatttatcaactatttaaaagaaaaagagaaaaaccggaaaccgaaaaataaataaatatttct  748
      c                                                    cg
   I  E  F  I  N  Y  L  K  E  K  N  R  K  P  K  N  K     orfi (ts)

ctgttctctaaaacatatgaaaaatagaccgatataaagaaaaaagtgtttatttttttaagaaaaggga  818
                  a  g                                  a

aagatttcaacacactttccagtcctattagggctttttctttctcgctaaaaacagaacacacgttcgaa  888
      t     V  c        ac                                  a
            t

agggagtattcaattgggcgattacttatcacatctggaggaatacgtaaaaaatttatacagccggctg  958
                                                  t        g

ggcatcacctccccccatcacattgacatgctgaaaatcgcaaaggatctggatatttgggttcatttttg 1028
      a     t                                                 g

aggatatggggagcatgatggttaaatacgatggcatgtacagtatcgtattgaatcaaagaaaatcacg 1098
                        g           c                 c     a     g

agaagagcaatgggaggatttggccatgaactgtgccacgtgttaaagcacgcaggcaatcatttttcaa 1168
g                       g                                 t                 g

atgaacaagctcttcagggaactgcag                                             1195
                  a  g  t
```

FIG. 9b

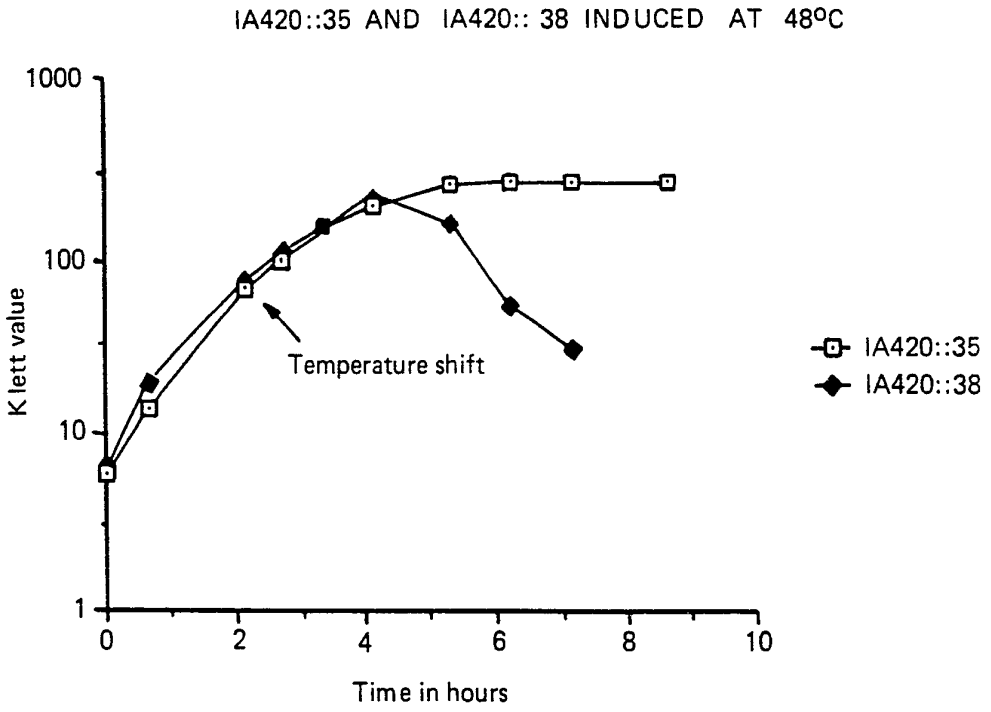

IA420::35 AND IA420:: 38 INDUCED AT 48°C
1000
100
10
1
Klett value
Temperature shift
0
2
4
6
8
10
Time in hours
IA420::35
IA420::38

FIG. 10

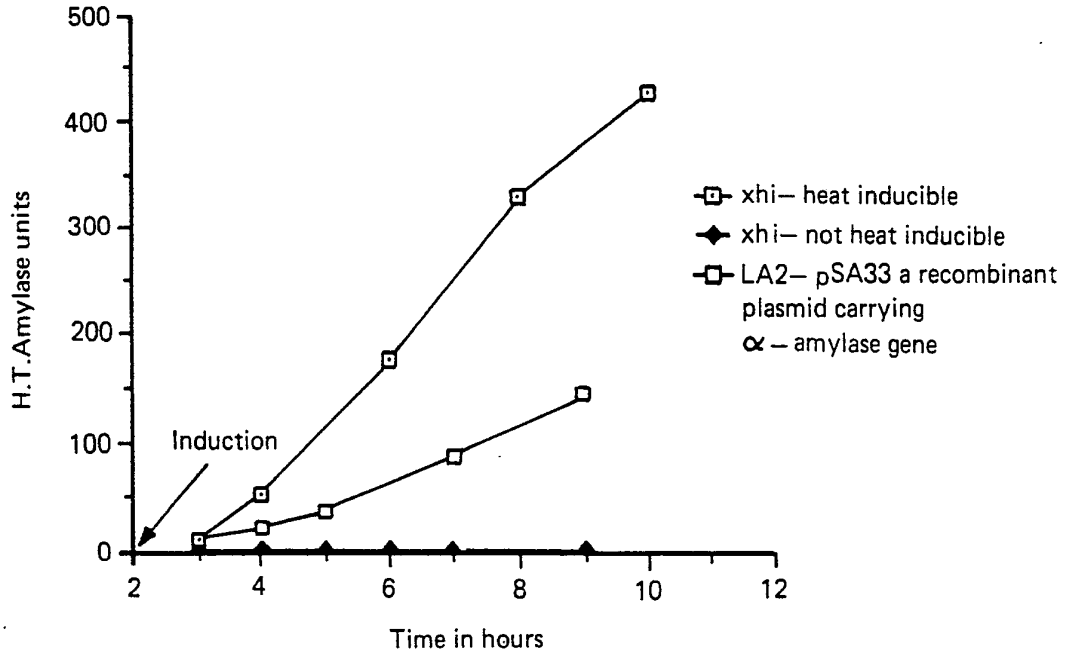

500
400
300
200
100
0
H.T.Amylase units
Induction
2
4
6
8
10
12
Time in hours
xhi– heat inducible
xhi– not heat inducible
LA2– pSA33 a recombinant plasmid carrying
α – amylase gene

FIG. 11

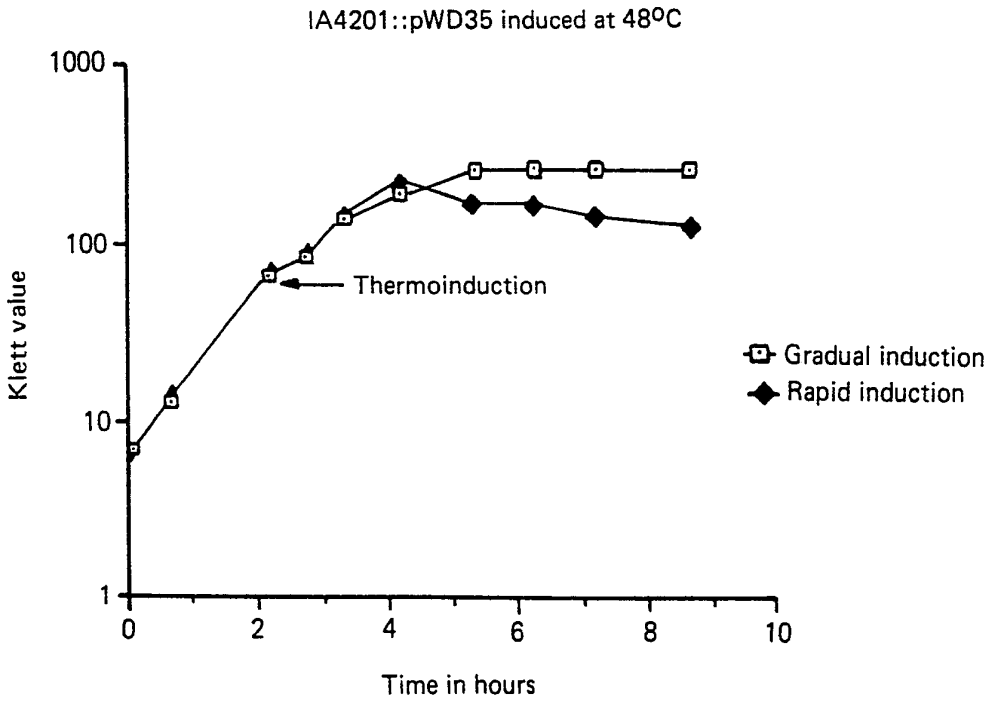


FIG. 12a

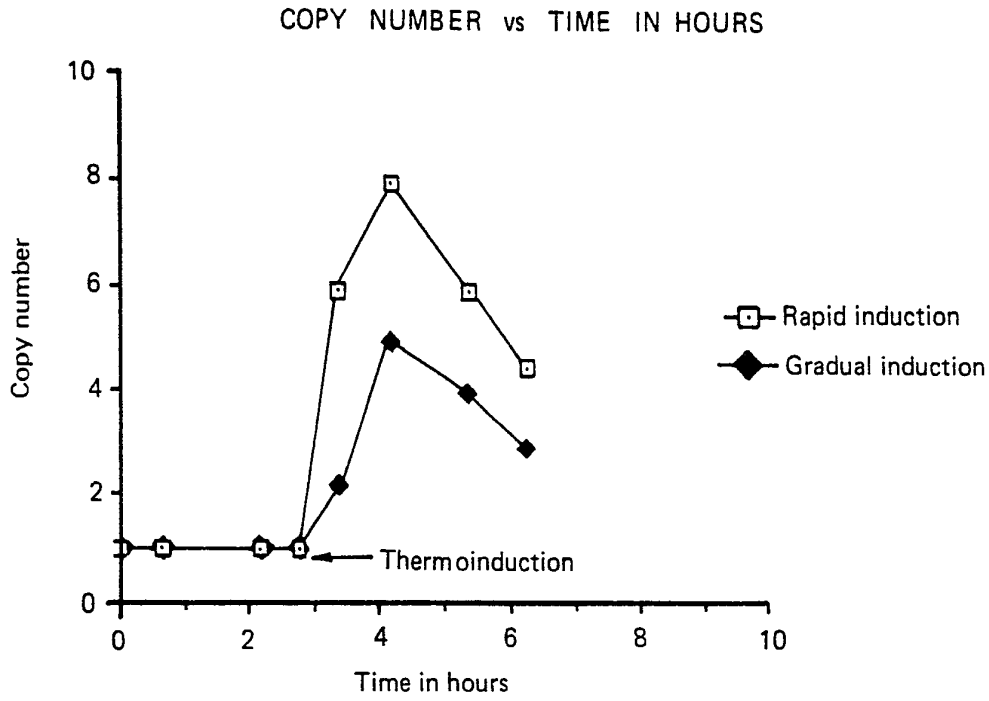


FIG. 12b

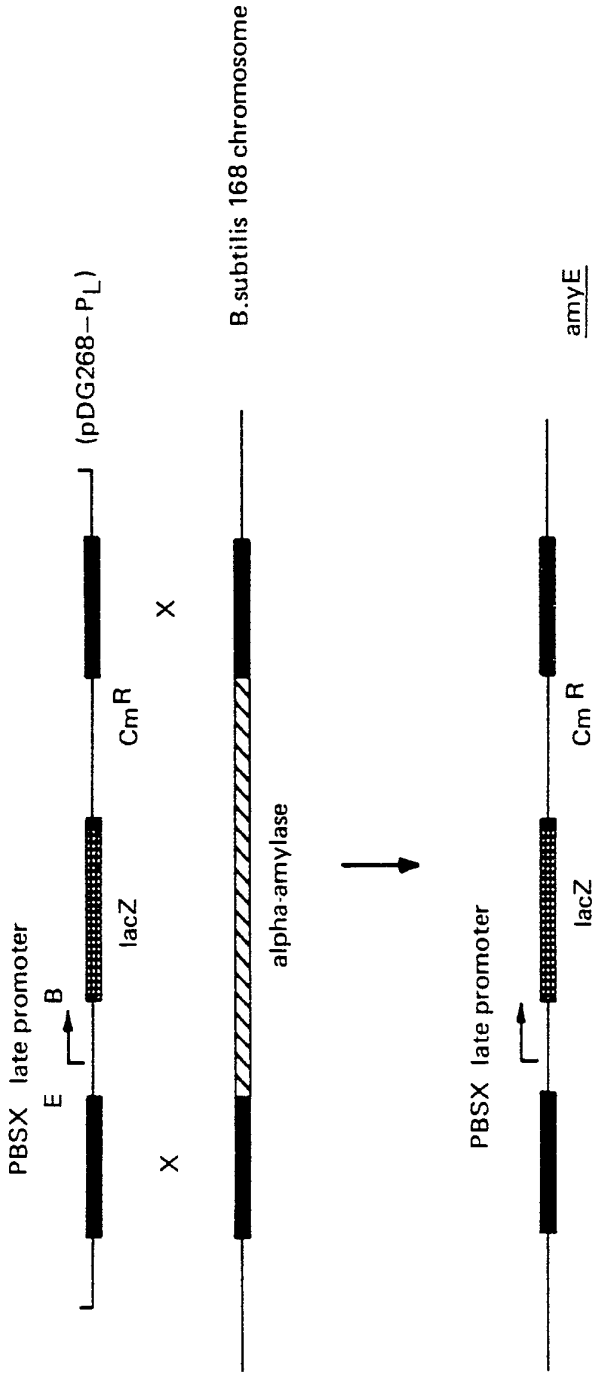

(pDG268–P_L)
B.subtilis 168 chromosome
amyE
PBSX late promoter
E
B
lacZ
Cm^R
X
X
alpha-amylase
PBSX late promoter
lacZ
Cm R

FIG. 13

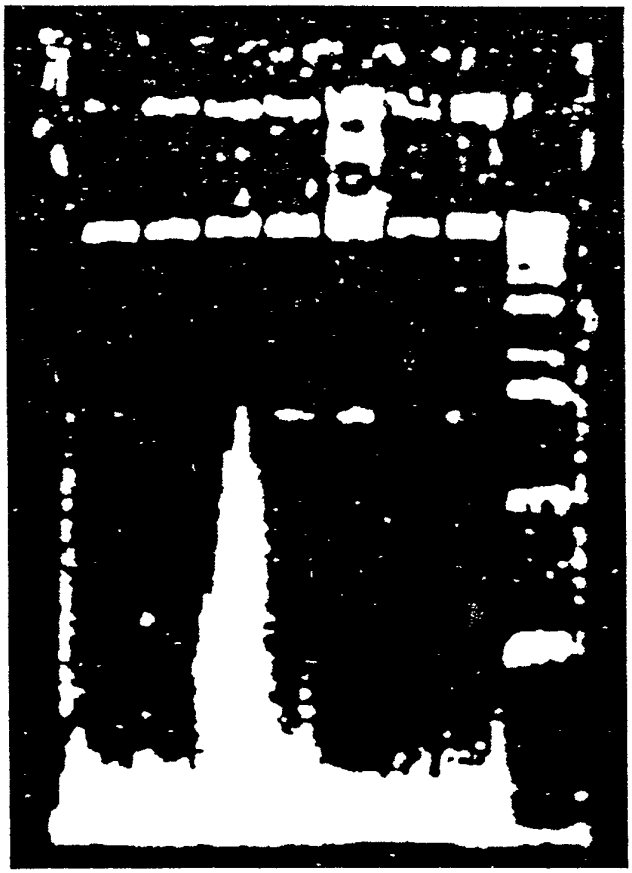


FIG. 14

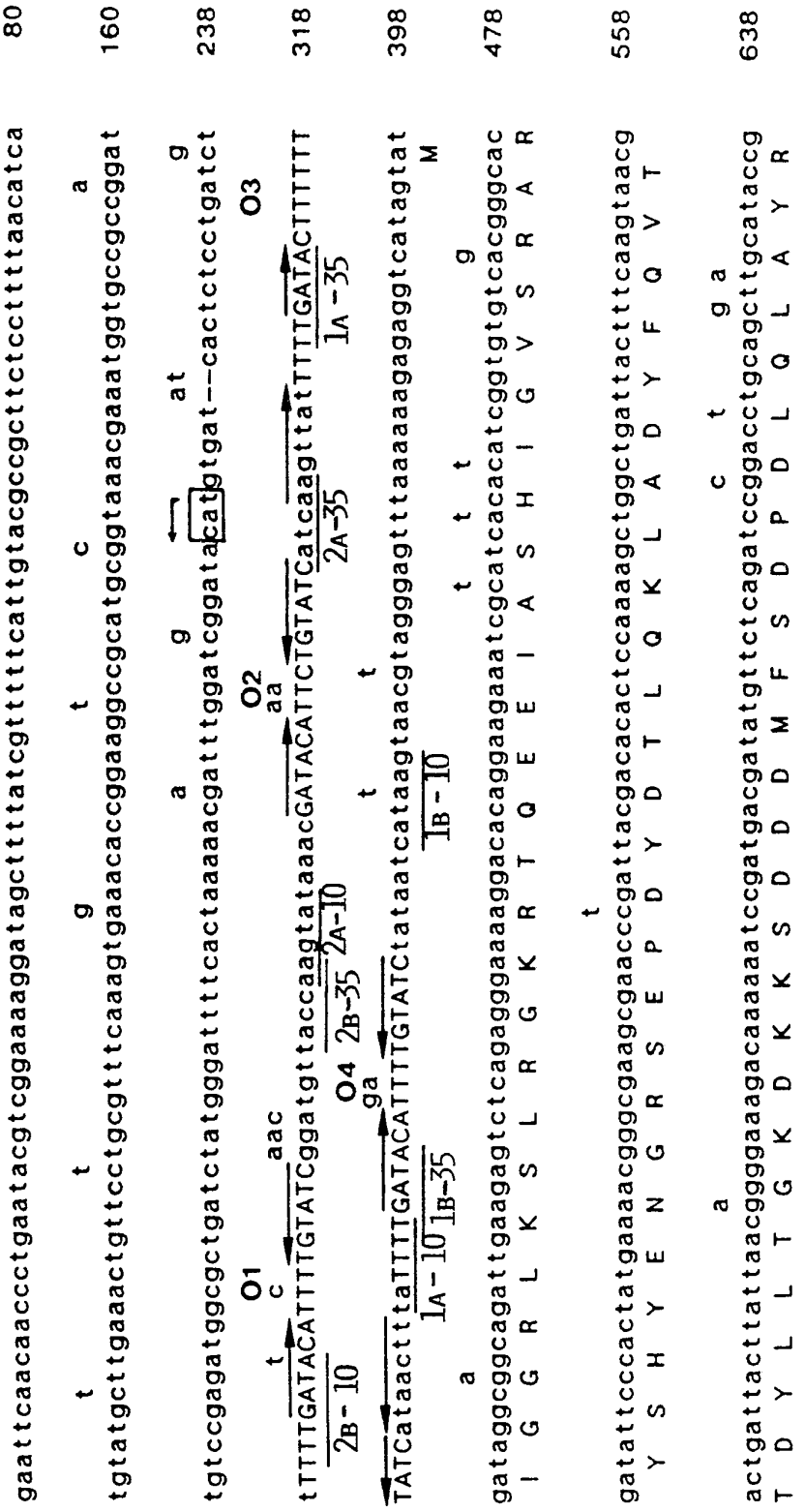


FIG.15A

```
t                                                             t
cgatatgcaggattttccccagaaagcaaacagcaggccatcgaatttatcaactatttaaaagaaaaagagaaaaacc   718
 D  M  Q  D  F  S  P  E  S  K  Q  Q  A  I  E  F  I  N  Y  L  K  E  K  E  K  N  R

 g                                      at                        g     a
gcaaaccgaaaaataaataaatcgttctctgttctctaaaacatataaaaagtagaccgatataaagaaaaaagtgttta   798
  K  P  K  N  K  <

      t                             a   -  t             ta                    c
tttttaaagaaaagggaaagatttctacactaccttccagtcctatacgggcttttctttctcgctaaaaacagaacaa    878

                                                                   a            a
acgttcgaaagggagtattcaattgggcgattacttatcacatctggaggaatacgttaaaaatttatacggccggctgg   958

       c     c                                                          t
gcatcacatcccctcatcacattgacatgctgaaaatcgcaaaggatctggatatttgggtgcattttgaggatatgggg   1038

   ->       t       t                          t    g    a     a
agc[atg][atg][gtg]aaatacgacggcatgtacagtatcgtattgaaccaaaaaaagtcacgggaagagcaatgggaggattt   1118

         a                    c                   a                      g  a  g
tggccatgagctgtgccacgtgttaaagcatgcaggcaatcattttcagatgaacaagctcttcagagagcttcaggaattc 1200
```

FIG. 15B

```
K L C Q T K K V I V E H T G I G V V F H
 S F V K Q R K S L W N I P V L E L F F I
  A L S N K E S H C G T Y R Y W S C F S S
AAGCTTTGTCAAACAAAGAAAGTCATTGTGGAACATACCGGTATTGGAGTTGTTTTTCAT
    10        20        30        40        50        60
TTCGAAACAGTTTGTTTCTTTCAGTAACACCTTGTATGGCCATAACCTCAACAAAAAGTA
L K T L C L F D N H F M G T N S N N K M
 A K D F L S L * Q P V Y R Y Q L Q K E D
  S Q * V F F T M T S C V P I P T T K * G

P C P N C R S A T D L T P V I Q K L E Q
 H V R T A G P R L T * R L S F K S W S K
  M S E L P V R D * L N A C H S K A G A N
CCATGTCCGAACTGCCGGTCCGCGACTGACTTAACGCCTGTCATTCAAAAGCTGGAGCAA
     70        80        90       100       110       120
GGTACAGGCTTGACGGCCAGGCGCTGACTGAATTGCGGACAGTAAGTTTTCGACCTCGTT
W T R V A P G R S V * R R D N L L Q L L
 M D S S G T R S Q S L A Q * E F A P A F
  H G F Q R D A V S K V G T M * F S S C I

M L T A G K A R L N I Y D * T A D C S N
 C * Q R E K R G * I S M I K Q L T A L I
  A D S G K S E A E Y L * L N S * L L * S
ATGCTGACAGCGGGAAAAGCGAGGCTGAATATCTATGATTAAACAGCTGACTGCTCTAAT
    130       140       150       160       170       180
TACGACTGTCGCCCTTTTCGCTCCGACTTATAGATACTAATTTGTCGACTGACGAGATTA
H Q C R S F R P Q I D I I L C S V A R I
 A S L P F L S A S Y R H N F L Q S S * D
  S V A P F A L S F I * S * V A S Q E L R

R F A V S G K A N R K K H * T M V * G R
 A L L F R A K R T E K N I E Q W Y K D D
  L C C F G Q S E Q K K T L N N G I R T T
CGCTTTGCTGTTTCGGGCAAAGCGAACAGAAAAAAACATTGAACAATGGTATAAGGACGA
    190       200       210       220       230       240
GCGAAACGACAAAGCCCGTTTCGCTTGTCTTTTTTTGTAACTTGTTACCATATTCCTGCT
A K S N R A F R V S F F M S C H Y L S S
 S Q Q K P C L S C F F V N F L P I L V V
  K A T E P L A F L F F C Q V I T Y P R R

R E V K V L A K T K Q A E K S P A P W R
 G K * K C W Q R Q N R Q R K A L R R G V
  G S E S V G K D K T G R E K P C A V A C
CGGGAAGTGAAAGTGTTGGCAAAGACAAAACAGGCAGAGAAAAGCCCTGCGCCGTGGCGT
    250       260       270       280       290       300
GCCCTTCACTTTCACAACCGTTTCTGTTTTGTCCGTCTCTTTTCGGGACGCGGCACCGCA
P F H F H Q C L C F L C L F A R R R P T
 P L S L T P L S L V P L S F G Q A T A H
  S T F T N A F V F C A S F L G A G H R A

A V P C G D T K P I Y I Y S A Y S E E E
 L S R A G I R N R S I F I Q L T V K K K
  C P V R G Y E T D L Y L F S L Q * R R K
GCTGTCCCGTGCGGGGATACGAAACCGATCTATATTTATTCAGCTTACAGTGAAGAAGAA
    310       320       330       340       350       360
CGACAGGGCACGCCCCTATGCTTTGGCTAGATATAAATAAGTCGAATGTCACTTCTTCTT
S D R A P I R F R D I N I * S V T F F F
 Q G T R P Y S V S R Y K N L K C H L L F
  T G H P S V F G I * I * E A * L S S S F
```

FIG. 16a

```
K E R F P Y S N G R L I A A V F D L S S
 K K D F R T Q T G G * L Q L Y L T S A L
  R K I S V L K R A A D C S C I * P Q L L
AAAGAAAGATTTCCGTACTCAAACGGGCGGCTGATTGCAGCTGTATTTGACCTCAGCTCT
     370      380      390      400      410      420
TTTCTTTCTAAAGGCATGAGTTTGCCCGCCGACTAACGTCGACATAAACTGGAGTCGAGA
F F S K R V * V P P Q N C S Y K V E A R
 L F I E T S L R A A S Q L Q I Q G * S K
  S L N G Y E F P R S I A A T N S R L E *

Y S Q K S N A S L M A A A P E L L E A S
 I R K K A M P L * W P L R L N C W K R L
  F A K K Q C L F D G R C A * I A G S V *
TATTCGCAAAAAAGCAATGCCTCTTTGATGGCCGCTGCGCCTGAATTGCTGGAAGCGTCT
     430      440      450      460      470      480
ATAAGCGTTTTTTCGTTACGGAGAAACTACCGGCGACGCGGACTTAACGACCTTCGCAGA
I R L F A I G R Q H G S R R F Q Q F R R
 N A F F C H R K S P R Q A Q I A P L T *
  E C F L L A E K I A A A G S N S S A D L

K A A V D F L K G N S I H S K E R I I Q
 K Q Q L I F * K G I L F I Q R S V S F S
  S S S * F S E R E F Y S F K G A Y H S A
AAAGCAGCAGTTGATTTTCTGAAAGGGAATTCTATTCATTCAAAGGAGCGTATCATTCAG
     490      500      510      520      530      540
TTTCGTCGTCAACTAAAAGACTTTCCCTTAAGATAAGTAAGTTTCCTCGCATAGTAAGTC
F C C N I K Q F P I R N M * L L T D N L
 L L L Q N E S L S N * E N L P A Y * E A
  A A T S K R F P F E I * E F S R I M * S

L L E K A E A S A A P K R G G N K T * F
 Y * K K L K Q A L H R K G E E I K H D S
  I R K S * S K R C T E K G R K * N M I H
CTATTAGAAAAAGCTGAAGCAAGCGCTGCACCGAAAAGGGGAGGAAATAAAACATGATTC
     550      560      570      580      590      600
GATAATCTTTTTCGACTTCGTTCGCGACGTGGCTTTTCCCCTCCTTTATTTTGTACTAAG
* * F F S F C A S C R F P S S I F C S E
 I L F L Q L L R Q V S F P L F Y F M I *
  N S F A S A L A A G F L P P F L V H N M

I R K N C C I S I P S R L R A S W R T G
 S E K T A A Y R F R H A * E P A G G R E
  P K K L L H I D S V T L K S Q L E D G K
ATCCGAAAAAACTGCTGCATATCGATTCCGTCACGCTTAAGAGCCAGCTGGAGGACGGGA
     610      620      630      640      650      660
TAGGCTTTTTTGACGACGTATAGCTAAGGCAGTGCGAATTCTCGGTCGACCTCCTGCCCT
D S F V A A Y R N R * A * S G A P P R S
 G F F S S C I S E T V S L L W S S S P F
  R F F Q Q M D I G D R K L A L Q L V P F

K S V I I V D G I K Q E A W I T E A P E
 N P S L L W T A S S K K H G S Q K R Q S
  I R H Y C G R H Q A R S M D H R S A R A
AAATCCGTCATTATTGTGGACGGCATCAAGCAAGAAGCATGGATCACAGAAGCGCCAGAG
     670      680      690      700      710      720
TTTAGGCAGTAATAACACCTGCCGTAGTTCGTTCTTCGTACCTAGTGTCTTCGCGGTCTC
F G D N N H V A D L L F C P D C F R W L
 I R * * Q P R C * A L L M S * L L A L A
  D T M I T S P M L C S A H I V S A G S C
```

FIG. 16b

```
H G K T L V E T R K G D L A R V E F E I
 M E K R S S K Q E R A I L L V W N L K S
  W K N A R R N K K G R S C S C G I * N R
CATGGAAAAACGCTCGTCGAAACAAGAAAGGGCGATCTTGCTCGTGTGGAATTTGAAATC
    730     740     750     760     770     780
GTACCTTTTTGCGAGCAGCTTTGTTCTTTCCCGCTAGAACGAGCACACCTTAAACTTTAG
M S F R E D F C S L A I K S T H F K F D
 H F F A R R F L F P R D Q E H P I Q F R
  P F V S T S V L F P S R A R T S N S I P

G Y K L N * S E N R I R P R R K A C G H
 A T N * I K A K T E Y V Q D G K P A D T
  L Q I K L K R K Q N T S K T E S L R T L
GGCTACAAATTAAATTAAAGCGAAAACAGAATACGTCCAAGACGGAAAGCCTGCGGACAC
    790     800     810     820     830     840
CCGATGTTTAATTTAATTTCGCTTTTGTCTTATGCAGGTTCTGCCTTTCGGACGCCTGTG
A V F * I L A F V S Y T W S P F G A S V
 S C I L N F R F C F V D L V S L R R V S
  * L N F * L S F L I R G L R F A Q P C Q

* S T A Q H L C V D W C P F F I C Q K *
 D Q L H S I C A L I G V R F L F A K N E
  I N C T A F V R * L V S V F Y L P K M R
TGATCAACTGCACAGCATTTGTGCGTTGATTGGTGTCCGTTTTTTATTTGCCAAAAATGA
    850     860     870     880     890     900
ACTAGTTGACGTGTCGTAAACACGCAACTAACCACAGGCAAAAAATAAACGGTTTTTACT
S * S C L M Q A N I P T R K K N A L F S
 I L Q V A N T R Q N T D T K * K G F I L
  D V A C C K H T S Q H G N K I Q W F H P

G G S * N A R L T I * I * T H A Q T N K
 E D H R M Q D L L F E Y K R T L K Q T R
  R I I E C K T Y Y L N I N A R S N K Q E
GGAGGATCATAGAATGCAAGACTTACTATTTGAATATAAACGCACGCTCAAACAAACAAG
    910     920     930     940     950     960
CCTCCTAGTATCTTACGTTCTGAATGATAAACTTATATTTGCGTGCGAGTTTGTTTGTTC
S S * L I C S K S N S Y L R V S L C V L
 L I M S H L V * * K F I F A R E F L C S
  P D Y F A L S V I Q I Y V C A * V F L F

N T I * T A R * G R * I R A L S * R A E
 I Q Y K P L A E A D E S V L S A E E L K
  Y N I N R S L R Q M N P C S Q L K S * R
AATACAATATAAACCGCTCGCTGAGGCAGATGAATCCGTGCTCTCAGCTGAAGAGCTGAA
    970     980     990    1000    1010    1020
TTATGTTATATTTGGCGAGCGACTCCGTCTACTTAGGCACGAGAGTCGACTTCTCGACTT
I C Y L G S A S A S S D T S E A S S S F
 Y L I F R E S L C I F G H E * S F L Q L
  V I Y V A R Q P L H I R A R L Q L A S P

G * K N H Q K Y D Y * S * I C N R M A *
 D K K I I R N M I T D L E Y V T E W L E
  I K K S S E I * L L I L N M * Q N G L K
GGATAAAAAAATCATCAGAAATATGATTACTGATCTTGAATATGTAACAGAATGGCTTGA
    1030    1040    1050    1060    1070    1080
CCTATTTTTTTAGTAGTCTTTATACTAATGACTAGAACTTATACATTGTCTTACCGAACT
S L F I M L F I I V S R S Y T V S H S S
 I F F D D S I H N S I K F I Y C F P K F
  Y F F * * F Y S * Q D Q I H L L I A Q F
```

FIG. 16c

```
K R K A A R H Q T G D * P A * C L P A A
 K G R Q P G I R R A I D R R D V Y Q R L
  K E G S P A S D G R L T G V M F T S G *
AAAAGGAAGGCAGCCCGGCATCAGACGGGCGATTGACCGGCGTGATGTTTACCAGCGGCT
     1090    1100    1110    1120    1130    1140
TTTTCCTTCCGTCGGGCCGTAGTCTGCCCGCTAACTGGCCGCACTACAAATGGTCGCCGA
F P L C G P M L R A I S R R S T * W R S
 F S P L G A D S P R N V P T I N V L P Q
  L F A A R C * V P S Q G A H H K G A A S

D D Q G P E N H R I I F Q R Y D V * A G
 M I K D P R I I E S F S S A M M F E P D
  * S R T R E S S N H F P A L * C L S R T
GATGATCAAGGACCCGAGAATCATCGAATCATTTTCCAGCGCTATGATGTTTGAGCCGGA
     1150    1160    1170    1180    1190    1200
CTACTAGTTCCTGGGCTCTTAGTAGCTTAGTAAAAGGTCGCGATACTACAAACTCGGCCT
I I L S G L I M S D N E L A I I N S G S
 H D L V R S D D F * K G A S H H K L R V
  S * P G S F * R I M K W R * S T Q A P R

R T G I R R R Q R * N S R S I S P V N G
 G Q V S E E D R D R I R E A L A L L T D
  D R Y Q K K T E I E F E K H * P C * R T
CGGACAGGTATCAGAAGAAGACAGAGATAGAATTCGAGAAGCATTAGCCCTGTTAACGGA
     1210    1220    1230    1240    1250    1260
GCCTGTCCATAGTCTTCTTCTGTCTCTATCTTAAGCTCTTCGTAATCGGGACAATTGCCT
P C T D S S S L S L I R S A N A R N V S
 S L Y * F F V S I S N S F C * G Q * R V
  V P I L L L C L Y F E L L M L G T L P C

Q R K G N V F A A * G R M F F L * T D R
 R E K E M F L L H K V E C F S Y E R I A
  E K R K C F C C I R * N V F L M N G S P
CAGAGAAAAGGAAATGTTTTTGCTGCATAAGGTAGAATGTTTTTCTTATGAACGGATCGC
     1270    1280    1290    1300    1310    1320
GTCTCTTTTCCTTTACAAAAACGACGTATTCCATCTTACAAAAAGAATACTTGCCTAGCG
L S F S I N K S C L T S H K E * S R I A
 S F L F H K Q Q M L Y F T K R I F P D G
  L F P F T K A A Y P L I N K K H V S R R

R S S R R K K I D S A N D D * T G E F K
 D L L G V K K S T V Q T T I K R A S L K
  I F S A * K N R Q C K R R L N G R V * R
CGATCTTCTCGGCGTAAAAAAATCGACAGTGCAAACGACGATTAAACGGGCGAGTTTAAA
     1330    1340    1350    1360    1370    1380
GCTAGAAGAGCCGCATTTTTTTAGCTGTCACGTTTGCTGCTAATTTGCCCGCTCAAATTT
S R R P T F F F D V T C V V I L R A L K F
 I K E A Y F F R C H L R R N F P R T * L
  D E R R L F I S L A F S S * V P S N L S

D A K T A G R N E S I T C L K A C H T F
 M Q R Q Q E E M N R S L A * K L V I R L
  C K D S R K K * I D H L P E S L S Y V C
GATGCAAAGACAGCAGGAAGAAATGAATCGATCACTTGCCTGAAAGCTTGTCATACGTTT
     1390    1400    1410    1420    1430    1440
CTACGTTTCTGTCGTCCTTCTTTACTTAGCTAGTGAACGGACTTTCGAACAGTATGCAAA
I C L C C S S I F R D S A Q F S T M R K
 H A L F S V L A L P F L F F H S I D S I * V K Q G R S F L A K Q D * Y V T N Q A
```

FIG. 16d

```
A T Y K * I E H D T K R L A D Q P L L *
 P P I S E * S M T L S G W L I S R F Y E
  H L * V N R A * H * A A G * S A A F M N
GCCACCTATAAGTGAATAGAGCATGACACTAAGCGGCTGGCTGATCAGCCGCTTTTATGA
     1450    1460   1470    1480    1490   1500
CGGTGGATATTCACTTATCTCGTACTGTGATTCGCCGACCGACTAGTCGGCGAAAATACT
G G I L S Y L M V S L P Q S I L R K * S
 W R Y T F L A H C * A A P Q D A A K I F
  V * L H I S C S V L R S A S * G S K H I

I N N H A G G G G D A V A * K H N S A N
 * T T M L E V A V M Q * H E N T T A R T
  K Q P C W R W R * C S S M K T Q Q R E Q
ATAAACAACCATGCTGGAGGTGGCGGTGATGCAGTAGCATGAAAACACAACAGCGCGAAC
     1510   1520   1530    1540    1550   1560
TATTTGTTGGTACGACCTCCACCGCCACTACGTCATCGTACTTTTGTGTTGTCGCGCTTG
Y V V M S S T A T I C Y C S F V V A R V
 L C G H Q L H R H H L L M F V C C R S C
  F L W A P P P P S A T A H F C L L A F L

K H * Q S I N N I K E R S Q I G R L R T
 S I S N L S T T S R K D H K S G D C G H
  A L A I Y Q Q H Q G K I T N R A I A D T
AAGCATTAGCAATCTATCAACAACATCAAGGAAAGATCACAAATCGGGCGATTGCGGACA
     1570   1580   1590    1600    1610   1620
TTCGTAATCGTTAGATAGTTGTTGTAGTTCCTTTCTAGTGTTTAGCCCGCTAACGCCTGT
L M L L R D V V D L F S * L D P S Q P C
 A N A I * * C C * P F I V F R A I A S V
  C * C D I L L M L S L D C I P R N R V C

Q S V F P R K Q S A S G K N K T N G K R
 N R C F R E N N R H L E K T R Q M E R G
  I G V S A K T I G I W K K Q D K W K E A
CAATCGGTGTTTCCGCGAAAACAATCGGCATCTGGAAAAAACAAGACAAATGGAAAGAGG
     1630   1640   1650    1660    1670   1680
GTTAGCCACAAAGGCGCTTTTGTTAGCCGTAGACCTTTTTTGTTCTGTTTACCTTTCTCC
L R H K R S F L R C R S F V L C I S L P
 I P T E A F V I P M Q F F C S L H F S A
  D T N G R F C D A D P F F L V F P F L R

R C F L R P K T N K N S A L * T T M N *
 A V F C V Q K R T K T A P Y K Q R * I K
  L F S A S K N E Q K Q R P I N N D E L N
CGCTGTTTTCTGCGTCCAAAAACGAACAAAAACAGCGCCCTATAAACAACGATGAATTAA
     1690   1700    1710   1720   1730    1740
GCGACAAAAGACGCAGGTTTTTGCTTGTTTTTGTCGCGGGATATTTGTTGCTACTTAATT
A T K Q T W F R V F V A G * L C R H I L
 S N E A D L F S C F C R G I F L S S N F
  Q K R R G F V F L F L A R Y V V I F * I

M N A S G C F A C I T S K A S M P H S Q
 * T P A A V L P V L R Q K L Q C H T V S
  E R Q R L F C L Y Y V K S F N A T Q S A
ATGAACGCCAGCGGCTGTTTTGCCTGTATTACGTCAAAAGCTTCAATGCCACACAGTCAG
     1750   1760   1770    1780    1790   1800
TACTTGCGGTCGCCGACAAAACGGACATAATGCAGTTTTCGAAGTTACGGTGTGTCAGTC
H V G A A T K G T N R * F S * H W V T L
 S R W R S N Q R Y * T L L K L A V C D A
  F A L P Q K A Q I V D F A E I G C L * C
```

FIG. 16e

```
Q S K R A I L R T A L M * R A A D S * K
 N Q S G L F S G Q R S C D G Q P T L K K
  I K A G Y S P D S A H V T G S R L L K N
CAATCAAAGCGGGCTATTCTCCGGACAGCGCTCATGTGACGGGCAGCCGACTCTTAAAAA
     1810  1820  1830  1840  1850  1860
GTTAGTTTCGCCCGATAAGAGGCCTGTCGCGAGTACACTGCCCGTCGGCTGAGAATTTTT
L * L P S N E P C R E H S P C G V R L F
 I L A P * E G S L A * T V P L R S K F F
  D R F A I R R V A S M H R A A S E * F V

T K R S L L K L D A L K K K W S M K C L
 R K G R C * N * T H * K R N G Q * N V Y
  E K V A A E I R R I K K E M V N E M F I
ACGAAAAGGTCGCTGCTGAAATTAGACGCATTAAAAAAGAAATGGTCAATGAAATGTTTA
     1870  1880  1890  1900  1910  1920
TGCTTTTCCAGCGACGACTTTAATCTGCGTAATTTTTTCTTTACCAGTTACTTTACAAAT
R F P R Q Q F * V C * F L F P * H F T *
 S F T A A S I L R M L F S I T L S I N I
  F L D S S F N S A N F F F H D I F H K N

L K R W M C C R F I S R S R L R I L R T
 * S D G C A A G L Y Q D R V C G Y Y G L
  E A M D V L Q V Y I K I A F A D I T D Y
TTGAAGCGATGGATGTGCTGCAGGTTTATATCAAGATCGCGTTTGCGGATATTACGGACT
     1930  1940  1950  1960  1970  1980
AACTTCGCTACCTACACGACGTCCAAATATAGTTCTAGCGCAAACGCCTATAATGCCTGA
Q L S P H A A P K Y * S R T Q P Y * P S
 S A I S T S C T * I L I A N A S I V S *
  F R H I H Q L N I D L D R K R I N R V I

M * P L E K K R S R L S G N R V R C L M
 C D L W K K R G P G C R E I G S A V * *
  V T F G K K E V Q A V G K S G P L F D E
ATGTGACCTTTGGAAAAAAAGAGGTCCAGGCTGTCGGGAAATCGGGTCCGCTGTTTGATG
     1990  2000  2010  2020  2030  2040
TACACTGGAAACCTTTTTTTCTCCAGGTCCGACAGCCCTTTAGCCCAGGCGACAAACTAC
H S R Q F F L P G P Q R S I P D A T Q H
 T V K P F F S T W A T P F D P G S N S S
  H G K S F F L D L S D P F R T R Q K I F

K M I I R L * R K S A L S M S K T P G S
 R * * S D Y E G N Q L C R C Q R L R A R
  D D N P I M K E I S F V D V K D S G L V
AAGATGATAATCCGATTATGAAGGAAATCAGCTTTGTCGATGTCAAAGACTCCGGGCTCG
     2050  2060  2070  2080  2090  2100
TTCTACTATTAGGCTAATACTTCCTTTAGTCGAAACAGCTACAGTTTCTGAGGCCCGAGC
L H Y D S * S P F * S Q R H * L S R A R
 S S L G I I F S I L K T S T L S E P S T
  I I I R N H L F D A K D I D F V G P E N

L M A P L * R K Q S L G K R H C H Q A C
 * W H H C N G S K A W E R G I A I K L A
  D G T I V T E A K L G K E A L P S S L Q
TTGATGGCACCATTGTAACGGAAGCAAAGCTTGGGAAAGAGGCATTGCCATCAAGCTTGC
     2110  2120  2130  2140  2150  2160
AACTACCGTGGTAACATTGCCTTCGTTTCGAACCCTTTCTCCGTAACGGTAGTTCGAACG
Q H C W Q L P L L A Q S L P M A M L S A
 S P V M T V S A F S P F S A N G D L K C
  I A G N Y R F C L K P F L C Q W * A Q L
```

FIG. 16f

```
R * N E G A * E A I L I F * F V S R S I
 D K M K A L E K L S L Y F D L F P D Q F
  I K * R R L R S Y P Y I L I C F Q I N L
AGATAAAATGAAGGCGCTTGAGAAGCTATCCTTATATTTTGATTTGTTTCCAGATCAATT
     2170   2180   2190   2200   2210   2220
TCTATTTTACTTCCGCGAACTCTTCGATAGGAATATAAAACTAAACAAAGGTCTAGTTAA
S L I F A S S F S D K Y K S K N G S * N
 I F H L R K L L * G * I K I Q K W I L K
  Y F S P A Q S A I R I N Q N T E L D I *

* T K N * K * E I E A C Q T K S G E N R
 K Q K I E N E K L K L A K Q K A E K T D
  N K K L K M R N * S L P N K K R R K Q M
TAAACAAAAAATTGAAAATGAGAAATTGAAGCTTGCCAAACAAAAAGCGGAGAAAACAGA
     2230   2240   2250   2260   2270   2280
ATTTGTTTTTTAACTTTTACTCTTTAACTTCGAACGGTTTGTTTTTCGCCTCTTTTGTCT
L C F I S F S F N F S A L C F A S F V S
 F L F N F I L F Q L K G F L F R L F C I
  V F F Q F H S I S A Q W V F L P S F L H

* Q P G A D * N Y D Q T K R A Q V M I V
 D S Q E P I E I M I K R K E R K S * L *
  T A R S R L K L * S N E K S A S H D C K
TGACAGCCAGGAGCCGATTGAAATTATGATCAAACGAAAAGAGCGCAAGTCATGATTGTA
     2290   2300   2310   2320   2330   2340
ACTGTCGGTCCTCGGCTAACTTTAATACTAGTTTGCTTTTCTCGCGTTCAGTACTAACAT
S L W S G I S I I I L R F S R L D H N Y
 V A L L R N F N H D F S F L A L * S Q L
  C G P A S Q F * S * V F L A C T M I T F

K E I N P H F E D Y V F N W E Q T Y Q F
 K K S T L I S K I T C S I G S R R T S F
  R N Q P S F R R L R V Q L G A D V P V S
AAAGAAATCAACCCTCATTTCGAAGATTACGTGTTCAATTGGGAGCAGACGTACCAGTTT
     2350   2360   2370   2380   2390   2400
TTTCTTTAGTTGGGAGTAAAGCTTCTAATGCACAAGTTAACCCTCGTCTGCATGGTCAAA
F F D V R M E F I V H E I P L L R V L K
 L F * G E N R L N R T * N P A S T G T E
  S I L G * K S S * T N L Q S C V Y W N R

L V G G Y G S S K S Y H T A L K I V L K
 L S A A T A H P K A I I P H * K S C * S
  C R R L R L I Q K L S Y R I E N R A K A
CTTGTCGGCGGCTACGGCTCATCCAAAAGCTATCATACCGCATTGAAAATCGTGCTAAAG
     2410   2420   2430   2440   2450   2460
GAACAGCCGCCGATGCCGAGTAGGTTTTCGATAGTATGGCGTAACTTTTAGCACGATTTC
K D A A V A * G F A I M G C Q F D H * L
 Q R R S R S M W F S D Y R M S F R A L A
  T P P * P E D L L * * V A N F I T S F S

L L K E K R T A L V I R E V F D T H R D
 C * R K N G R P L * S G R C S I P I G I
  A E G K T D G P C D P G G V R Y P S G F
CTGCTGAAGGAAAAACGGACGGCCCTTGTGATCCGGGAGGTGTTCGATACCCATCGGGAT
     2470   2480   2490   2500   2510   2520
GACGACTTCCTTTTTGCCTGCCGGGAACACTAGGCCCTCCACAAGCTATGGGTAGCCCTA
Q Q L F F P R G K H D P L H E I G M P I
 A S P F V S P G Q S G P P T R Y G D P N
  S F S F R V A R T I R S T N S V W R S E
```

FIG. 16g

```
S T F A L F Q E V I E E L
 R P S P C F K R * S K S S
  D L R L V S R G D R R A -
TCGACCTTCGCCTTGTTTCAAGAGGTGATCGAAGAGCTC
    2530  2540   2550
AGCTGGAAGCGGAACAAAGTTCTCCACTAGCTTCTCGAG
R G E G Q K L L H D F L E
 S R R R T E L P S R L A -
  V K A K N * S T I S S S -
```

FIG. 16h

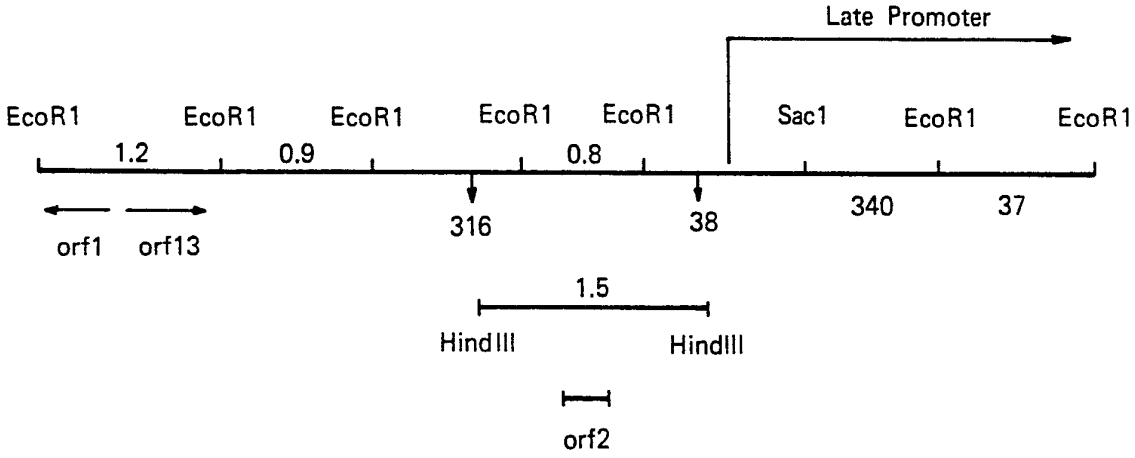


FIG. 17

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 91/00074

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 N 15/74, 15/75

II. FIELDS SEARCHED

Minimum Documentation Searched[7]	
Classification System	Classification Symbols
IPC5	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	Gene, Vol. 96, 1990 Heather E. Wood et al.: "Characterisation of a repressor gene (xre) and a temperature-sensitive allele from the Bacillus subtilis prophage, PBSX", see page 83 - page 88 --	1-38
P,X	Journal of Bacteriology, Vol. 172, No. 5, May 1990 Heather E. Wood et al.: "Characterization of PBSX, a Defective Prophage of Bacillus subtilis", see page 2667 - page 2674 --	1-38
A	Journal of Virology, Vol. 54, No. 3, June 1985 Linda M. Anderson et al.: "DNA Packaging by the Bacillus subtilis Defective Bacteriophage PBSX", see page 773 - page 780 --	1-38

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11th June 1991	1990 -06- 18
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Mikael Bergstrand

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Journal of General Microbiology, Vol. 128, 1982 J. Barrie Ward et al.: "Purification and Characterization of Two Phage PBSX-induced Lytic Enzymes of Bacillus subtilis 168: An N-Acetylmuramoyl-L-alanine Amidase and an N-Acetylmuramidase", see page 1171 - page 1178 --	1-38
A	Journal of General Microbiology, Vol. 128, 1982 P.J. Piggot et al.: "Bacteriophage PBSX-induced Deletion Mutants of Bacillus subtilis Constitutive for Alkaline Phosphatase", see page 663 - page 669 --	1-38
A	Journal of Bacteriology, Vol. 150, No. 3, June 1982 Linda M. Andersson et al.: "Isolation of an Autonomously Replicating DNA Fragment from the Region of Defectibe Bacteriophage PBSX of Bacillus subtilis", see page 1280 - page 1286 --	1-38
A	J.gen.Virol., Vol. 46, 1980 R.S. Buxton: "Selection of Bacillus subtilis 168 Mutants with Deletions ofthe PBSX Prophage", see page 427 - page 437 --	1-38

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 91/00074

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 91-04-30
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date