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Integrable α-Amylase Plasmid for Generating Random Transcriptional Fusions in *Bacillus subtilis*

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An integrable plasmid, pOK4, which replicated independently in *Escherichia coli* was constructed for generating transcriptional fusions in vivo in *Bacillus* DNA. It did not replicate independently in *Bacillus subtilis*, but it could be made to integrate into the chromosome of *B. subtilis* if sequences homologous to chromosomal sequences were inserted into it. It had a selectable marker for chloramphenicol resistance and carried unique sites for *Eco*RI and *Sma*I just to the 5′ side of a promoterless alpha-amylase gene from *Bacillus licheniformis*. When *B. subtilis* DNA fragments were ligated into one of these sites and the ligation mixture was used to transform an alpha-amylase-negative *B. subtilis* strain, chloramphenicol-resistant transformants could be isolated conveniently. Many of these were alpha-amylase positive, owing to the fusion of the plasmid amylase gene to chromosomal operons. In principle, because integration need not be mutagenic, it is possible to obtain fusions to any chromosomal operon. The site of each integration can be mapped, and the flanking sequences can be cloned into *E. coli*. The alpha-amylase gene can be used to detect regulated genes. We used it as an indicator to detect operons which are DNA-damage-inducible (*din*), and we identified insertions in both SPβ and PBSX prophages.

Gene fusions are powerful tools for the analysis of gene expression in both procaryotic and eucaryotic organisms. Genetic regulatory mechanisms are frequently difficult to study because the gene products under their immediate control cannot be conveniently assayed. If the control sequences of a gene of interest are fused to an indicator gene whose products can be easily detected and assayed but which lacks its own control sequences, then the activity of the control sequences under study can be assayed by monitoring the expression of the indicator gene product. Such fusions can be used, with minimal perturbation of the system, to assay responses to environmental stimuli, to follow developmental programs, or to examine the effects of different genetic backgrounds on gene expression.

It is an advantage to be able to obtain sets of strains in which an indicator gene has been inserted randomly in a genome. Such sets of gene fusions can be screened for responses to a variety of signals, facilitating the identification and characterization of particular regulated genes. Fusions have been particularly useful in mapping and cloning genes and in isolating mutations affecting the regulation of genes into which the insertions have been made.

In bacteria, randomly generated gene fusions are usually isolated by using transposons. Natural transposons have been adapted for this purpose so that they carry an indicator gene at one end which can be expressed only by readthrough from adjacent sequences after insertion in the target DNA. Several transposons, e.g., Mu, Tn*3*, and Tn*5*, have been adapted for this purpose in *Escherichia coli* and other gram-negative bacteria, e.g., *Salmonella typhimurium*, *Vibrio parahaemolyticus*, *Caulobacter crescentus*, and *Agrobacterium tumefaciens* (2, 5, 11, 22, 40). In gram-positive bacteria, the transposon Tn*917* of *Streptococcus faecalis* (44) has been transferred to *Bacillus subtilis* (50) and adapted so that gene fusion technology can be applied to the latter organism (for a review, see reference 51). It has already proved valuable in analyzing the processes of sporulation and competence (23, 51).

We investigated the use of homolgous recombination rather than transposition in the generation of gene fusions in *B. subtilis*. This was done by using integrating plasmids, which cannot replicate autonomously in *B. subtilis*, but which can integrate into the chromosome if they carry a fragment with homology to a chromosomal sequence. Such integrating plasmids have been previously used in *B. subtilis* analogously to transposons in a number of ways, e.g., for insertional mutagenesis (12, 31), gene cloning (31), and gene mapping (16, 33). They can be made to integrate at a specific chromosomal site, and they have been used to create specific translational fusions between *lacZ* and genes on the *B. subtilis* chromosome (4, 54).

We constructed an integrating plasmid system which can be used conveniently as an alternative to transposons in the creation and analysis of transcriptional fusions in *B. subtilis*. It carries chloramphenicol resistance as a selectable gene, and it also carries the alpha-amylase gene from *Bacillus licheniformis* as an indicator. The promoter of the alpha-amylase gene was removed, and two unique cloning sites were placed just to the 5′ side of the ribosome-binding site. When random *B. subtilis* chromosomal restriction fragments were ligated into one of these sites and the ligation mixture was used to transform an amylase-negative strain of *B. subtilis*, chloramphenicol-resistant transformants were recovered. These arose as a result of recombination between the *B. subtilis* chromosome and homologous sequences cloned in front of the promoterless alpha-amylase gene on the plasmid. Owing to transcriptional readthrough from the chromosomal sequences, some of the transformants expressed alpha-amylase, and this could be easily detected on plates by the very sensitive starch-iodine test. This plasmid could be used to isolate and analyze regulated genes, e.g.,

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TABLE 1. Phage and bacterial strains and plasmids

Plasmid or strain	Genotype	Source or reference
Plasmid		
pSA33	Cm^r Km^r *amy*$^+$	34
pOK33	Cm^r Ap^r	33
pUC8	Ap^r	46
pBD64	Cm^r Km^r	13a
E. coli		
5K	*supE fhuA thr leu thi hsdR*	19
B. subtilis 168		
SO113	*trpC2 amy-3*	34
SO241	*recE4 amy-3*	S. Ortlepp
BD393	*thyA thyB lys amy trpC2*	14
TSDNA-A13	*dnaA13 ilvA1 metB5*	BGSC 1A18
QB832	*catA argC4 hisA1 pha-1*	BGSC 1A151
QB858	*glyB133 metD1*	BGSC 1A84
QB934	*trpC2 metC3 glyB133 tre-12*	BGSC 1A5 (9)
YB886	*trpC2 metB5 xin-1* SPβ^s *amy*	BGSC 1A304 (49)
B. subtilis W23		
SB623	*thr*	BGSC 2A1
Bacteriophage		
PBS-1		42

genes which are induced in reponse to DNA damage (*din* [damage inducible]).

MATERIALS AND METHODS

Bacteriophage and bacterial strains and plasmids. All strains and plasmids are listed in Table 1.

Media. *E. coli* and *B. subtilis* were grown routinely at 37°C on LB broth or agar (29). When appropriate, the media contained chloramphenicol, either 5 μg/ml for *B. subtilis* or 10 μg/ml for *E. coli*. Plates contained 0.2% starch, and amylase activity was detected by the presence of an unstained halo after the plates were flooded with 0.5% I_2–1% KI. If plates were drained quickly, *E. coli* colonies survived; *B. subtilis* colonies were often washed away, necessitating duplicate plates. Duplicates were made by transferring colonies individually with a loop; replication with a velvet pad caused haloes to overlap and become less reproducible. The *catA* marker was scored by halo size on LB plates containing 1% skim milk powder (a 10% stock solution of skim milk [Oxoid Ltd., London, England] was warmed to 50°C before being added to molten LB agar which had been allowed to cool to 50°C). Auxotrophic markers were checked on SS minimal agar plates (1) supplemented with 0.4% glucose and 0.005% of appropriate amino acids or thymidine.

Construction of promoterless amylase gene and pOK4. The 3.5-kilobase (kb) *Eco*RI fragment of pSA33 (34) has an *Nde*I site just to the 5′ side of the ribosome-binding site of the *B. licheniformis* alpha-amylase gene (41). This fragment was cut with *Nde*I, treated with the Klenow fragment of *E. coli* DNA polymerase I to create blunt-ended termini, and ligated to *Bam*HI linker (5′-pCGGATCCG-3′). The resulting fragment was cut with *Bam*HI and *Hin*dIII and inserted into the multiple cloning site of pUC8, which had also been cut with *Bam*HI and *Hin*dIII, producing pSL5. The promoterless alpha-amylase gene was excised from pSL5 as an *Eco*RI-*Hin*dIII fragment and ligated to the large *Eco*RI-*Hin*dIII fragment of pOK33, which carries an *E. coli* origin of replication (from pBR322) and the Cm^r gene of pC194 (33); the resulting plasmid, pOK4 (Fig. 1), had a Cm^r Amy$^+$ phenotype in *E. coli*. The Amy$^+$ phenotype was probably due to readthrough from a plasmid promoter. The 5′ end of the promoterless alpha-amylase gene was sequenced. It had the sequence expected from the construction, with *Eco*RI, *Sma*I, and *Bam*HI sites and an in-phase stop codon just to the 5′ side of the ribosome-binding site (Fig. 2).

Transduction and transformation. Transductions with PBS-1 were performed as previously described (33). *B. subtilis* competent cells were prepared and transformed as previously described (8), except that cells were not quick-frozen before being stored at −70°C, and they were plated on LB-chloramphenicol plates within 30 min of the addition of transforming DNA in order to favor the recovery of independent transformants. *E. coli* competent cells were prepared and transformed as described before (6), except that cultures were grown in LB broth to an optical density at 550 nm of 0.3 before being made competent.

DNA preparation. Small-scale preparations of plasmids for restriction or transformation were obtained essentially as described previously (3), except that a phenol extraction and a chloroform-isoamyl alcohol (24:1) extraction were included before the second ethanol precipitation. Large-scale preparations of plasmids required for cloning purposes were further purified by CsCl-ethidium bromide density gradient centrifugation (25).

B. subtilis chromosomal DNA was prepared from log-phase cells essentially by the method of Rodriguez and Tait (36), except that, after ethanol precipitation, proteinase K (0.1 mg/ml) was added to the solution to aid the dissolution of the DNA.

Enzymes. Restriction enzymes and T4 DNA ligase were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind., and used as recommended. When the digestion of certain DNA preparations was difficult, the preparations were incubated on ice for 5 min with 4 mM spermidine before enzyme was added. In ligations, 2 μg of chromosomal DNA were reacted to approximately 2 μg of linearized plasmid in a 50-μl volume. Nick translations were performed with an Amersham kit (Amersham Corp., Arlington Heights, Ill.).

Southern blotting. DNA was transferred from a 0.5% agarose gel to a Pall Biodyne nylon membrane (Pall Corp., Glen Cove, N.Y.), and hybridizations were performed as recommended by the manufacturer essentially by the method of Southern (39).

Amylase assays and mitomycin C induction. Cells from an overnight culture of the strain to be assayed were centrifuged for 1 min at 12,000 × *g* and resuspended in an equal volume of fresh LB broth. Samples (2 ml) of this suspension were used to inoculate 100 ml of fresh medium in duplicate. When both cultures were growing exponentially (optical density at 600 nm, 0.2 to 0.5), 0.1 μg/ml of mitomycin C was added to one of the cultures. Samples (1.5 ml) were removed from both cultures immediately and at 2-h intervals thereafter, and the supernatants were stored on ice for up to 24 h before being assayed for alpha-amylase.

High-temperature alpha-amylase was assayed by measuring the production of reducing sugars from starch at 90°C, always in duplicate. Assay tubes contained 1 ml of 0.1 M Na_2HPO_4–NaH_2PO_4 buffer (pH 6.0), 1 ml of 1% Lintner starch, and 0.2 ml of 0.5 M NaCl preheated to 90°C. Culture supernatant (0.5 ml; diluted in culture medium if necessary) was added to each tube. The tubes were incubated at 90°C for 30 min. They were cooled quickly, and the amount of reducing sugar was assayed by using dinitrosalicylic acid

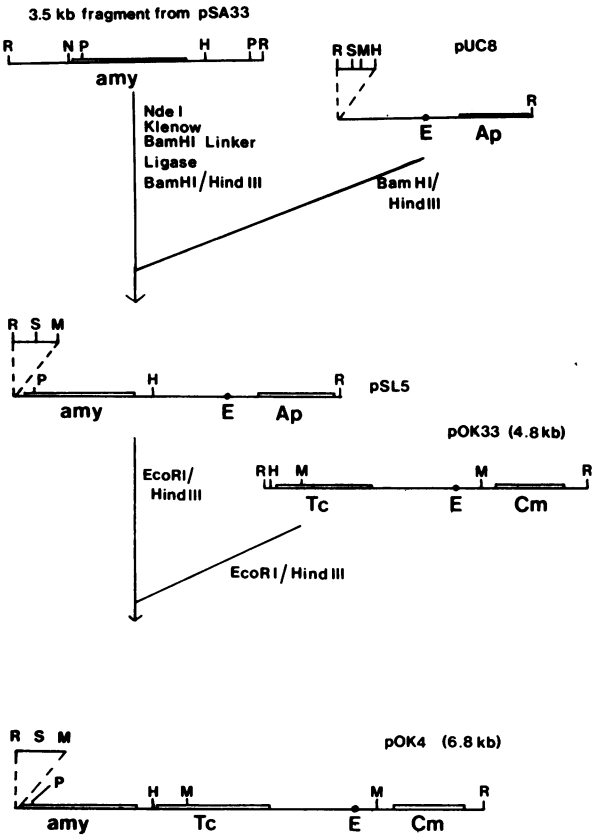

FIG. 1. Construction of pOK4. The alpha-amylase gene from pSA33 (34) was separated from its promoter and ligated to pUC8, as described in Materials and Methods, to give pSL5. The promoterless alpha-amylase from pSL5 was then inserted into pOK33 (33). Restriction sites: R, *Eco*RI; N, *Nde*I; P, *Pst*I, H, *Hin*dIII; S, *Sma*I; M, *Bam*HI; C, *Cla*I. E, Origin of replication of pBR322.

(28). One unit of enzyme activity was defined as the amount of enzyme producing 0.15 μmol of reducing sugar in 30 min in the standard assay.

RESULTS

Generation of transcriptional fusions using pOK4. The plasmid pOK4 (Fig. 1) used for generating transcriptional fusions carried the Cm^r gene from pC194, which can be selected in both *E. coli* and *B. subtilis* (18). It could replicate autonomously in *E. coli* but not in *B. subtilis*. It carried the structural gene for the high-temperature alpha-amylase from *B. licheniformis* (34), with only 30 base pairs remaining of

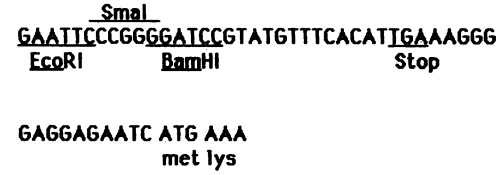

FIG. 2. Sequence of the 5′ end of the promoterless alpha-amylase gene. The sequence at the 5′ end of the promoterless gene was determined by the dideoxy method (37). The restriction sites shown are from the multiple cloning site of pUC8. These are separated from the start of the alpha-amylase gene by 30 base pairs which contain the ribosome-binding site sequence and a stop codon in the same frame as the start codon.

TABLE 2. Transformants from DNA from different restriction digests

Enzyme	Size (kb)[a]	Cm^r transformants[b]	Auxotrophs (%)[c]	Amy$^+$ (%)[d]
*Alu*I	0.5	22	0	68
*Hae*III	0.5–2	49	2.0	69
*Rsa*I	0.5–2	166	3.0	72
*Hin*dII	1–15	501	1.8	73

[a] Determined by electrophoresis on agarose gels.
[b] A 1-μg amount of ligation mixture was used in each of the four transformations.
[c] Colonies were classified as auxotrophs by inability to grow on SS medium supplemented with tryptophan.
[d] Determined by the presence of a halo after overnight growth on LB-chloramphenicol-starch plates.

the upstream 5′ flanking region. This upstream region included the amylase ribosome-binding site and an in-phase stop codon (Fig. 2) (41) and was, in turn, preceded by unique *Eco*RI and *Sma*I cloning sites. The stop codon should prevent the formation of translational fusions. The *B. lichenformis* amylase gene does not have enough homology to the *B. subtilis* amylase gene to allow pOK4 to integrate into the latter gene; when pOK4 was used to transform competent cells of *B. subtilis*, no chloramphenicol-resistant transformants were obtained.

To generate a range of operon fusions, digests of *B. subtilis* DNA were prepared by using *Alu*I, *Hae*III, *Rsa*I, and *Hin*dII, restriction enzymes which produce blunt-ended fragments. Each of the digests was ligated to pOK4 which had been linearized with *Sma*I in an approximate ratio of 1:1. A 1-μg amount of each ligation mixture was used to transform competent cells of *B. subtilis* SO113, which is *amy*$^-$, and after 30 min were allowed for expression, chloramphenicol-resistant transformants were selected by plating on LB-chloramphenicol agar. A total of 738 transformants was obtained (Table 2). More transformants were obtained when larger chromosomal fragments were used, as expected if the limiting factor in the frequency of transformation is the efficiency of homologous recombination (26).

Integration by the Campbell mechanism (Fig. 3) (10) is expected to cause mutations if the integration is mediated by a fragment which is internal to an operon, and other mechanisms may also cause mutations (31; see below). One measure of the frequency of mutation by an integrating plasmid population is the frequency with which auxotrophs are generated. Approximately 2% of all the transformants which were obtained were auxotrophs (Table 2).

Amylase expression. The 738 transformants obtained from the different ligation mixtures were screened for amylase activity by spotting the transformants directly onto LB-starch plates (Fig. 4). Some (28%) of the transformants showed no haloes after iodine staining. This is as expected if the alpha-amylase gene had integrated in the opposite orientation to the direction of transcription. The remainder (72%) displayed haloes which varied greatly in size. Some were nearly 1 cm in diameter, whereas others showed small but definite haloes which were just larger than the colony. The range of different levels of activity suggested that pOK4 had integrated at different sites, with the promoterless alpha-amylase gene coming under the control of a selection of different chromosomal promoters. That 72% of all transformants produced detectable levels of amylase after overnight growth on plates was surprising. If only one strand of the chromosomal DNA is transcribed at any one point, then no more than 50% of the transformants are expected to

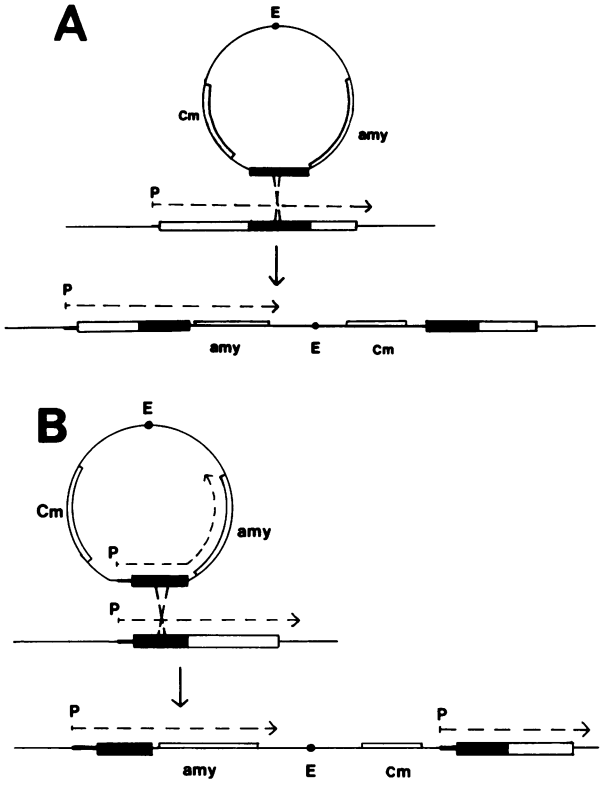

FIG. 3. (A) Mutagenic Campbell-type integration of a recombinant pOK4 into the *B. subtilis* chromosome. The recombinant plasmid derived from pOK4 is shown as closed circular and carrying a piece of *B. subtilis* DNA (▬) inserted just upstream from the promoterless alpha-amylase gene (*amy*). The homologous sequence on the *B. subtilis* chromosome is also shown (▬). Integration by homologous recombination results in disruption of the chromosomal structural gene. The integrated alpha-amylase can be transcribed (arrow shows direction of transcription) from the chromosomal gene promoter (P). E, Origin of replication of pOK4. (B) Nonmutagenic Campbell-type insertion. The fragment cloned on pOK4 is shown to contain the 5′ end and promoter (P) of a *B. subtilis* chromosomal gene. Integration does not lead to disruption of the chromosomal gene. Neither would the gene be disrupted if the fragment cloned contained the 3′ end of the gene.

produce any amylase. To confirm that the figure of 72% was not spurious, caused perhaps by picking up mixed colonies from the original plates, 110 transformants were streaked for single colonies and 73 (66%) had an Amy$^+$ phenotype. Possible reasons for the high number of Amy$^+$ transformants are considered below.

The alpha-amylase gene of *B. licheniformis* is subject to catabolite repression, both in its natural host and when cloned on a multicopy plasmid in *B. subtilis* (S. A. Ortlepp, Ph.D. thesis, University of Dublin, Dublin, Ireland, 1983). The 83 chromosomal insertions of the promoterless alpha-amylase gene which we tested are also catabolite repressed, as judged by the differences in the sizes of the haloes when the insertion strains are plated on LB and LB plus glucose (B. Laoide, C. O'Kane, and D. J. McConnell, unpublished observations). These observations need to be confirmed by more detailed studies. The observations are interesting because they imply that the promoterless amylase gene on pOK4 may retain all the *cis*-acting sequences necessary for its catabolite repression and that the repression is independent of the promoter used and of the promoter position. The mechanism of this repression is currently being investigated.

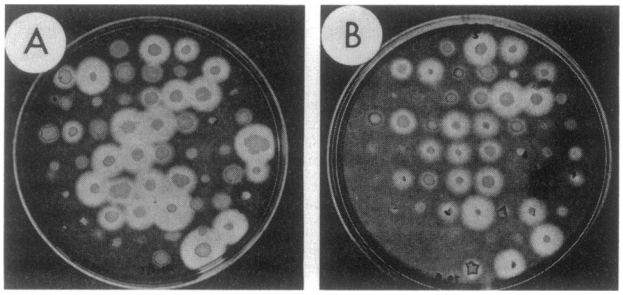

FIG. 4. Plate tests for alpha-amylase activity of colonies carrying insertions of pOK4. A total of 54 strains of *B. subtilis* carrying insertions were plated on LB-starch-chloramphenicol agar without (A) and with (B) 0.05 μg mitomycin C added per ml. After growth, the plates were stained with iodine. The photographs show that 37 of the insertion strains produced some amylase. One of the *din* insertions, SO113::pOK4/542, is shown with arrows.

Recovery of fusions to regulated operons. It is possible to use the sensitive and convenient plate test for amylase activity to screen for transformants in which the gene has been placed by fusion under a regulatory system which can be manipulated by adjusting the growth conditions on the plates. Several DNA-damaged-inducible (*din*) functions are induced during the SOS-like response of *B. subtilis* (known as the SOB response) to DNA-damaging agents such as mitomycin C (24, 48). It seemed likely that there would be one or more *din* operon fusions in the bank of transformants which had been generated.

Accordingly, the collection of 738 pOK4 insertions was screened for colonies in which amylase synthesis was induced by mitomycin C. Colonies were spotted on LB agar with or without mitomycin C (0.05 μg/ml), and two showed a marked difference in expression as judged by halo size (Fig. 4 and 5). These colonies, designated SO113::pOK4/542 and SO113::pOK4/1128, were both obtained from the transformation in which *Hin*dII chromosomal fragments were used.

Characterization of insertion 542. SO113::pOK4/542 formed slow-growing translucent colonies on LB plates and began to lyse after four or five generations of growth in LB broth. The *recE4* marker of *B. subtilis* abolishes the SOS-like response to agents which damage DNA (24). This marker was tested as follows for its effect on the expression of alpha-amylase in a strain carrying the 542 insertion. First the insertion was transduced into *B. subtilis* BD393 (a *thyA* · *thyB* auxotroph) by using bacteriophage PBS-1 select-

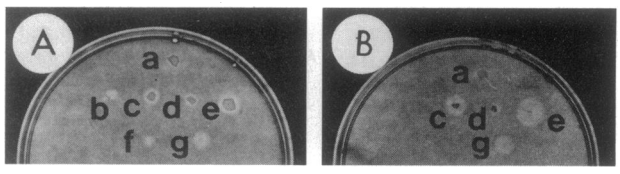

FIG. 5. SOB induction of amylase in different derivatives of insertions 542 and 1128. The strains were plated on LB-starch-chloramphenicol agar without (A) or with (B) mitomycin C (0.05 μg/ml). Strains: a, SO113(pBD64); b, BD393::pOK4/1128 (a *thy*$^+$ *recE4* transductant); c, SO113::pOK411C; d, SO113::pOK411C (a *xin-1* transductant); e, SO113::pOK4/1128; f, BD393::pOK4/542 (a *thy*$^+$ *recE4* transductant); g, BD393::pOK4/542 (a *thy*$^+$ transductant).

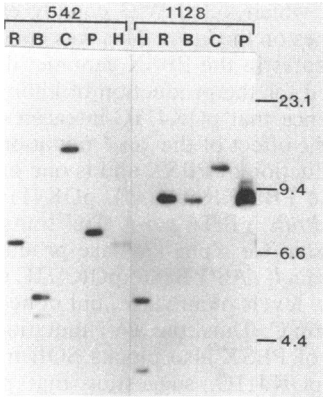

FIG. 6. Hybridization of pOK4 DNA to restriction digests of SO113::pOK4/1128 and SO113::pOK4/542 chromosomal DNA. Chromosomal DNA was digested with *Eco*RI (R), *Hin*dIII (H), *Bcl*I (B), *Cla*I (C), or *Pst*I (P). The autoradiograph is shown after hybridization to nick-translated pOK4. After longer exposure, a 2.3-kb *Cla*I fragment and a 2.2-kb *Hin*dIII fragment were detected in the SO113::pOK4/542 DNA. The *Pst*I digest of SO113::pOK4/1128 DNA was a partial digest, as was shown in further digests.

ing for Cm^r. All Cm^r transductants showed induction of amylase by mitomycin C and formed slow-growing translucent colonies. One such transductant was called BD393::pOK4/542. The *recE4* marker was transduced from SO241 into BD393::pOK4/542 by taking advantage of the linkage of *recE4* and *thyA*. Thy$^+$ transductants were selected on LB-chloramphenicol plates lacking thymidine, and those having *recE4* were detected by testing for sensitivity to mitomycin C. The *recE4* allele reduced but did not eliminate the constitutive alpha-amylase activity as judged by plate tests. It did not eliminate the phenotypes of slow growth and lysis.

Thymidine starvation, which prevents DNA replication, also induces the SOS response in *E. coli* and the SOB response in *B. subtilis* (13, 15, 21). Streaking BD393::pOK4/542 on plates without added thymidine caused the inoculum to synthesize enough amylase for a halo to be visible after 6 to 8 h, even though no growth was visible (data not shown). When streaked on plates with thymidine, there was growth but no halo after 6 to 8 h. Clearly, amylase production was induced by thymidine starvation. Unfortunately, it was not possible to perform any assays on liquid cultures of strains carrying 542 because of the difficulty of getting good growth.

The 542 insert was mapped by using PBS-1 transduction to perform four-point crosses between SO113::pOK4/542 as the donor and TSDNA-A13 as the recipient selecting for Cm^r. A total of 45 Cm^r transductants were screened for other markers. Of these, 23 were *trp*$^+$ *met*$^+$ *ilv*$^+$, 7 were *trp*$^+$ *met*$^-$ *ilv*$^+$, 11 were *trp*$^+$*met*$^-$ *ilv*$^-$, and 4 were *trp*$^-$ *met*$^+$ *ilv*$^+$. These data give 76, 60, and 9% cotransduction of Cm^r with the *ilvA1*, *metB5*, and *trpC2* markers, respectively. The deduced marker order is Cm^r, *ilvA1*, *metB5*, *trpC2*. These data place insertion 542 in the region of the SPβ prophage (53) of *B. subtilis* 168.

There are three reasons which suggest that 542 may be an insertion in SPβ. SPβ is induced as part of the SOB response; 542 maps to the vicinity of the SPβ locus; and the strain carrying the 542 insertion lyses spontaneously. The 542 insertion may have affected some function which is necessary for the stable maintenance of lysogeny of the prophage.

Southern blots of five different restriction digests of DNA from a *thyA*$^+$ *recE4* transductant of BD393::pOK4/542 probed with nick-translated pOK4 gave no evidence for multiple copies of the pOK4 insert (Fig. 6). All the enzymes used cut pOK4 once; therefore, two bands in each digest are expected to hybridize to a pOK4 probe if there is only one copy of the insert in the chromosome. A third band would indicate the presence of tandem copies. Four tracks showed two bands, and two (for the *Eco*RI and *Pst*I digests) showed only one. In the latter two cases, the lengths of homology to the pOK4 probe in the second chromosomal fragment were expected to be short (7 and about 117 base pairs, respectively) and were apparently below the level of detection (Fig. 7). A restriction map of the insert and flanking DNA, deduced from the blotting data, is shown in Fig. 7. There is no evidence from this map that the integration occurred by a Campbell-type event. This is considered further in the Discussion section.

Characterization of insertion 1128. The insertion 1128 in the second *din* operon fusion in SO113::pOK4/1128 was tightly linked to the *metD1*, *glyB133*, and *catA* markers (55, 87, and 92% cotransduction by PBS-1 of Cm^r with these markers, respectively). The mapping data (not shown) place the insertion between *glyB* and *metD*. No genes known or likely to be under SOB control map in this region.

Strain BD393::pOK4/1128, carrying insertion 1128 and the *recE4* marker, was constructed by the same procedure as for insertion 542. The *recE4* marker completely abolished all amylase production, as judged by the plate test (Fig. 5) and the liquid assays. The liquid assay also showed no induction of amylase by mitomycin C in this strain. Amylase was induced by thymidine starvation of BD393::pOK4/1128, as detected on plates and in liquid assays (data not shown). These two pieces of data showed that insertion 1128 was probably fused to an SOB operon.

To characterize this fusion further, it was cloned into *E. coli* by selecting simultaneously for the pBR322 origin of replication and Cm^r. Chromosomal DNA from SO113::pOK/1128 was partially digested with *Hin*dIII (which cuts pOK4 once) and ligated on itself. The ligation mixture was used to transform *E. coli* 5K selecting for Cm^r and screening for Amy$^+$. The Cm^r Amy$^+$ transformants which were obtained contained either plasmid pOK411A or pOK411B. *Bam*HI restriction digests showed that the plasmids probably consisted of pOK4 and approximately 7 and 9 kb, respectively, of additional DNA. pOK411A consisted of five *Hin*dIII fragments of 5.1, 3.8, 2.2, 1.5, and 1.3 kb. pOK411B consisted of five *Hin*dIII fragments of 5.1, 3.8, 3.1, 2.2, and 1.5 kb. These data suggest that the two plasmids consist of pOK4 and overlapping segments of flanking chromosomal DNA. The two largest fragments of pOK411A were shown to hybridize to nick-translated pOK4 in Southern blots (data not shown). When a blot of *Hin*dIII-digested chromosomal

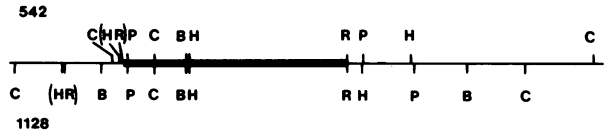

FIG. 7. Restriction maps of the DNA of inserts 542 and 1128 and flanking *B. subtilis* chromosomal DNA. The restriction maps for 542 (above the line) and 1128 (below the line) were based on the Southern blot shown in Fig. 6. ▬, pOK4 (6.8 kb). The abbreviations are as in the legend to Fig. 6. The brackets indicate that the order of the sites is not clear from the data.

TABLE 3. SOB inducibility of alpha-amylase in strains carrying insertion 1128

Strain	Amylase activity[a]	
	Uninduced	Induced
SO113	<4	<4
SO113 (pSA33)	147	185
SO113::pOK4/1128	<4	214
SO113::pOK411C	<4	78
SO113::pOK411C C (*xin*-1 transductant)	<4	<4
BD393	<4	<4
BD393::pOK4/1128 (*thy*$^+$ *recE4* transductant)	<4	<4
YB886	<4	<4
YB886::pOK411C	<4	<4

[a] Log-phase cultures were grown for 2 h in the presence or absence of mitomycin C before the supernatants were assayed. Values are units of amylase activity per milliliter per unit of cells at an optical density of 600 nm. The minimum detectable level of activity in the assay was equivalent to 4 units.

DNA from SO113::pOK4/1128 was probed with pOK4, two bands of the same size as these two largest bands hybridized (Fig. 6). The hybridization patterns of five restriction digests of SO113::pOK4/1128 chromosomal DNA gave no evidence (for the same reasons discussed for the case of SO113::pOK4/542) for multiple copies of the integrated plasmid (Fig. 6). The restriction map (Fig. 7) of the DNA of the insertion in SO113::pOK4/1128 did not show evidence for the direct flanking repeats expected from a Campbell-type integration event. This is considered further in the Discussion section.

Since insertion 1128 did not map in the vicinity of any genes known to be under SOB control, the possibility that pOK4 had picked up two chromosomal fragments in the original ligation was investigated. One fragment might mediate the SOB response, and the other might mediate the integration event, resulting in a spurious map position for the SOB promoter. This possibility was checked as follows. A *Hae*III digest of pOK411B was ligated to pOK4 linearized with *Sma*I. The ligation mixture was then digested with *Sma*I to linearize any pOK4 which had recircularized (but not pOK4 which had been ligated to a *Hae*III fragment without a *Sma*I site) and then used to transform *E. coli* 5K. Plasmid DNA was prepared from 14 Cm^r transformants and used to transform *B. subtilis* SO113. Cm^r transformants were selected and tested for SOB induction of alpha-amylase. Four of the 14 plasmids gave transformants which showed SOB induction of amylase; all four had the same restriction digest pattern with *Bam*HI and were apparently identical. One of the four plasmids was designated pOK411C. It consisted of a 600-base-pair fragment in pOK4. The Cm^r markers in four isolates of SO113, each of which had been transformed independently by pOK411C, were mapped by PBS-1 transduction. In each case, Cm^r mapped to the *metC3* region (52% cotransduction) and not in the vicinity of *glyB133* where insertion 1128 had been mapped before.

There is an "operon" in the *metC3* region which is likely to be inducible by SOB. This is the defective prophage PBSX (43). To discover whether pOK411C contained DNA from PBSX, supernatant from a culture of *B. subtilis* SO113::pOK411C induced with mitomycin C was tested for killing activity by spotting 5 μl onto a lawn of the PBSX-sensitive strain *B. subtilis* W23. PBSX adsorbs to this strain and kills it but does not inject its DNA (32). SO113::pOK411C was found to produce no killing activity, unlike its parent strain SO113 or SO113::pOK4/1128, the supernatants of which killed W23 equally effectively, producing clear zones on the lawn. This was good evidence that pOK411C integrates in the PBSX genome, disrupting some function required for the production of killing activity.

Further evidence that pOK411C integrates in PBSX was obtained from the effect of the *xin-1* mutation which specifically blocks induction of PBSX and is one of the mutations which define the PBSX locus (43). pOK411C was used to transform *B. subtilis* YB886 *xin-1*. Cm^r transformants were selected and tested for alpha-amylase production with and without mitomycin C. All YB886::pOK411C Cm^r transformants showed low levels of amylase, but none showed induction by mitomycin C. Thus, the *xin-1* mutation which blocks SOB induction of PBSX also blocks SOB induction of the amylase from pOK411C, suggesting that pOK411C integrates in PBSX. This conclusion was tested further by investigating whether the apparent effect of the *xin-1* mutation blocking the SOB induction of amylase in YB886::pOK411C cotransduces with Cm^r. The Cm^r gene was transduced by PBS-1 from YB886::pOK411C *xin-1* into SO113 *xin*$^+$. Of the transductants, 10% showed induction of amylase by mitomycin C but 90% did not, indicating that the uninducible phenotype and Cm^r are closely linked in YB886::pOK411C and suggesting that the uninducible phenotype is indeed caused by the *xin-1* mutation.

The levels of expression of high-temperature alpha-amylase and the degree of induction by mitomycin C in liquid culture were measured for various strains. The cells were grown for 2 h in the presence or absence of 0.1 μg of mitomycin C per ml, and samples were assayed at 90°C as described above. The results show that the original recombinant strain SO113(pSA33) carrying the amylase gene cloned on the multicopy plasmid pBD64 had a high basal level of activity which was not significantly affected by mitomycin C (Table 3). The fusion strains SO113::pOK4/1128 and SO113::pOK411C had no detectable activity in the liquid assay in the absence of mitomycin C and had high levels, similar to those in SO113(pSA33), when mitomycin C was present. Several strains showed no high-temperature alpha-amylase activity in the presence or absence of mitomycin C, including SO113, BD393, and YB866, which are Amy$^-$. The effect of *recE4*, which blocks SOB-inducible gene expression, is seen in the result for the *thyA*$^+$ *recE4* transductant of BD393-pOK4/1128 which has no detectable activity in the presence or absence of mitomycin C. The *xin-1* mutation has a similar effect, there being no measurable activity with or without mitomycin C for YB886::pOK411C and the *xin-1* transductant of SO113::pOK411C. These data taken together are convincing evidence that pOK411C inserts into a tightly regulated SOB-inducible operon in the PBSX genome.

DISCUSSION

We described a method using the plasmid pOK4 for generating gene fusions in *B. subtilis* in vivo by homologous recombination. The system is versatile. As with previously described integrating plasmids used in *B. subtilis* (12, 16, 31, 33), pOK4 can be used for insertion mutagenesis, gene mapping, and gene cloning. In addition, it can be used to isolate and analyze transcriptional fusions and to study gene regulation, making use of the promoterless alpha-amylase gene as an indicator. The only system of comparable versatility presently available in *B. subtilis* is based on the transposon Tn*917*. There are a number of differences between the pOK4 and Tn*917* systems.

First, it should be possible to fuse the promoterless alpha-amylase gene to any site in chromosomal DNA where a double-stranded break can be made. Blunt-ended fragments, which can be produced by many different methods essentially at any site, can be inserted in the *SmaI* site of pOK4; by using a variety of restriction enzymes or other methods for breaking DNA, it should be possible to generate insertions into any *B. subtilis* operon. Because the fusions are generated by homologous recombination, the probability of inserting into a particular operon should depend primarily on the size of that operon. This expectation contrasts with the apparently more extreme site specificity of insertion mediated by Tn*917*. In one study, 90% of the auxotrophs recovered were glutamate requirers (50).

Second, in contrast to insertion by transposition, which is usually mutagenic, random insertion of integrating plasmids is less likely to cause mutation. Insertion of a transposon is mutagenic unless it inserts near the 3′ end of an operon or outside an operon. On the other hand, if the integrating plasmid carries a chromosomal fragment which contains either end of an operon, integration by a single crossover event will not be mutagenic (Fig. 3) (54). It may even be possible to tune the system to favor either mutagenic or nonmutagenic insertion by using chromosomal fragments of different sizes in the ligation reactions; larger fragments are more likely to contain one end of an operon. Preliminary data, using the frequency of auxotrophs as a measure of mutagenesis from experiments which used fragments of different sizes in the ligations and which might have been expected to show this effect of size on mutagenesis (Table 2), did not show statistically significant differences in the proportion of auxotrophs produced. Apart from the small numbers involved, there are a number of other possible factors to take into account in explaining these data. (i) The size distribution of chromosomal fragments on plasmids which successfully transform is not expected simply to reflect the size distribution of chromosomal fragments in the digest used in the ligation. Integrating plasmids with longer lengths of homology to DNA in the recipient cell are known to transform more efficiently, presumably because the probability of recombination is greater (26). The factors behind this observation (in the study cited, the homologous sequences were on a resident plasmid) will be a source of bias against insertions mediated by small fragments. (ii) There may also be a bias against larger fragments similar to that encountered in cloning random fragments in *B. subtilis* (27). (iii) If auxotrophic operons are on average very long in *B. subtilis*, and the *trp* operon is estimated to be 6 kb in length (17), the use of longer fragments in the range which successfully transforms may not greatly decrease the probability that any fragment will contain an internal fragment of such an operon. (iv) A proportion of the auxotrophic transformants may have arisen by a double crossover involving the chromosome and a circular or linear ligation product containing at least one plasmid molecule flanked by two chromosomal fragments (31); the frequency and the dependence on fragment size of such events are difficult to predict. In general, however, because integration of pOK4 plasmids need not be mutagenic (Fig. 3B), we expect that it should be possible to use pOK4 to isolate fusions to essential genes without undue difficulty, provided there is an effective method for screening.

Third, the method of generating Tn*917-lac* fusions can lead to the recovery of multiple colonies which arise from a single transposition event (50). This is not expected to occur with the integrating plasmid method.

Fourth, the pOK4 integrating plasmid carries the alpha-amylase gene as an indicator instead of the β-galactosidase of the transposons derived from Tn*917*. In our hands, in *B. subtilis* the starch-iodine plate test for alpha-amylase seems to be more sensitive than either the X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) or methylumbelliferyl-β-D-galactoside plate tests for β-galactosidase. This might partly reflect the difference in half-lives for the two enzymes; high-temperature alpha-amylase has a half-life of approximately 48 h in culture supernatants of *B. subtilis* (Ortlepp, Ph.D. thesis), whereas β-galactosidase has a very short half-life (J. Errington, personal communication). However, although alpha-amylase is a sensitive measure of accumulated enzyme levels, it is less suited than β-galactosidase for measurements of rates of synthesis over short time periods.

Some of the strains examined gave no detectable amylase in the liquid assay (Table 3) but gave small haloes in the plate assay (Fig. 4 and 5). This is presumably owing to the effect of accumulated synthesis and activity of the enzyme during overnight growth on plates compared with 2 h of growth in dilute liquid culture followed by a 30-min incubation for the liquid assay.

A possible disadvantage of using alpha-amylase as an indicator gene is that many strains of *B. subtilis* in general use are Amy^+, including any of the Dedonder mapping-kit strains (9) which we have examined. During this work, it was discovered unexpectedly that strains BD393 and YB886 are amy^-, which was convenient. In practice, it is easy to transfer the amy^- marker into other *B. subtilis* strains carrying the amy^+ gene by congression (cotransformation); about 3% of transformants selected for transformation by a marker not linked to *amy* are found also to have been transformed to Amy^- (34). Although it is necessary to do initial screens on plates in an Amy^- host, liquid assays at high temperature do not detect the low-temperature *B. subtilis* alpha-amylase, which facilitates some experiments in Amy^+ hosts.

The main reason for caution in using the pOK4 system is the possibility that insertions in the vector may be mapped to spurious positions on the chromosome, owing either to the ligation of more than one chromosomal fragment to the same plasmid molecule or to the occurrence of homologous sequences at a number of chromosomal locations. For insertion 1128 in which the initial map position was misleading, such homologous sequences might include the sequences of related cryptic or defective prophages. This problem of spurious mapping is, however, easily checked once the DNA of the insertion has been cloned into *E. coli* and subclones are used to redirect integration into the *B. subtilis* chromosome.

The Southern blots (Fig. 6) and the restriction maps (Fig. 7) of the chromosomal DNA around the site of integration of 1128 and 542 do not show whether integration occurred by Campbell-type events (Fig. 3). For both insertions, the flanking direct repeats characteristic of such integrations may have been either too short or too long to have been detected. For 1128, there is the added complication that some of the restriction sites mapped may have been derived from a different chromosomal location than the site of integration, thus obscuring the pattern of restriction sites generated by direct repeats.

The fact that 70% of the insertions produced detectable levels of alpha-amylase after overnight growth on plates is surprising and interesting. It suggests that much of the *B. subtilis* chromosome is transcribed on both strands to some extent at some stage of the growth cycle during growth on

LB plates. Many of the haloes are small, but they are quite clear. It is interesting to speculate that this evidence of symmetrical transcription may be related to the phenomenon of regulation by anti-sense RNA, for which there is now good evidence in other systems (7, 30, 38, 45). However, at this stage we must be cautious. It is possible that the alpha-amylase gene is so sensitive an indicator that it detects low-level transcriptional noise which has no specific biological significance. In an earlier study, a promoter probe was used on a very well defined transcriptional system, coliphage T7, and low-level transcription was detected from weak promoters for which there is no known function (47).

In this paper, the use of the integrating plasmid system in analyzing gene regulation was demonstrated for the SOB (or *din*) genes, which are expressed after DNA is damaged. It has previously been shown that gene fusions can be useful in the study of *din* operons by using a Mu d *lac* phage in *E. coli* (20) and the transposon Tn*917* in *B. subtilis* (23). Using pOK4, two strains, SO113::pOK4/542 and SO113::pOK4/1128, carrying insertions were isolated in which alpha-amylase was strongly induced by mitomycin C. The evidence shows that both had insertions in prophages, in SPβ and PBSX, respectively. The plasmids pOK411A, B, and C may be valuable in analyzing the PBSX prophage. This is a defective prophage whose DNA has not yet been characterized. The fact that both 524 and 1128 insertions were in prophages probably reflects two things. First, the SPβ genome is about 120 kb, so that prophages together represent at least 3% of the *B. subtilis* genome (52). Since all genes of a prophage may be induced in the SOB response (directly by cleavage of a phage repressor protein or indirectly through the expression of late genes), insertions of the alpha-amylase gene at many points in prophage genomes will produce a strain with SOB-inducible alpha-amylase. The prophages are, therefore, large targets compared with other SOB-inducible genes. Second, the SOB respnse of an alpha-amylase gene in a prophage may be large because it probably results not only from induction of gene expression from phage promoters but also from gene amplification during DNA replication of the phage. The two strains were indeed chosen for study because the response was so high, more than 50-fold in the case of 1128 and its derivatives (Fig. 4 and 5; Table 3). We have not determined whether the SOB induction of amylase in these two strains is due mainly to activation of transcription or to increased copy number through phage DNA replication. The fact that induction in strain SO113::pOK4/1128 occurs when the insertion is not in the PBSX locus suggests that in this case there is a strong effect on transcription.

The operon fusion system based on pOK4 should be directly applicable in any species into which the plasmid DNA can be introduced efficiently, which is proficient in homologous recombination, and in which the alpha-amylase gene can be expressed and the enzyme can be secreted. As an additional caveat, nonhomologous recombination should not occur at a significant rate relative to homologous recombination. For the shotgun approach described here to be used effectively, it is probably necessary to be able to obtain about 10^3 independent transformants (which we obtained in these experiments) to detect fusions to members of a class of coordinately regulated genes and at least 10^4 independent transformants to detect a fusion to a particular gene. We achieved 10^4 transformants in transformations with a ligation mixture containing an integrating plasmid and an *Eco*RI digest of *B. subtilis* chromosomal DNA (unpublished results). The alpha-amylase gene of *B. licheniformis* used in pOK4 is expressed in *B. subtilis* and *E. coli* and detected in both species in the plate test. Indeed, it is normally found that gram-positive genes are expressed effectively in many gram-positive and gram-negative species. It is possible that pOK4 may be useful for generating gene fusions in other species without much or any adaptation.

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