APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1995, p. 3775–3780 Vol. 61, No. 11
0099-2240/95/$04.00+0

Extracellular Enzyme Synthesis in a Sporulation-Deficient Strain of *Bacillus licheniformis*

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Received 2 June 1995/Accepted 12 July 1995

A deletion of the *spoIIAC* gene of *Bacillus licheniformis* was prepared in vitro by using the splicing-by-overlap-extension technique. This gene was introduced into *B. licheniformis* on a temperature-sensitive plasmid, and following integration and excision from the chromosome, a precisely located deletion on the chromosomal gene was prepared. The mutated bacterium was totally asporogenous and formed abortively disporic cells characterized by asymmetric septa at the poles of the cells. Qualitative plate tests revealed that the bacterium synthesized normal levels of DNase, polygalacturonate lyase, protease, RNase, and xylanase, but the hydrolysis zones due to β-1,3-glucanase and carboxymethyl cellulase activity were smaller in the mutant than in the parent strain. The synthesis of alkaline protease was the same in batch cultures of the mutant and the parent during prolonged incubation for 72 h, but the α-amylase yields were reduced by about 30% by the mutation.

Bacillus licheniformis is used extensively for the production of industrial enzymes, such as amylase and protease, and is a popular host for industrial preparation of cloned gene products (7, 8, 29). Hundreds of tons of extracellular enzymes are manufactured annually by using this organism, which results in a considerable mass of spent organisms that must be disposed of; this material is usually disposed of as compost. Inactivation of the organisms used in production before they are distributed into the environment is generally achieved by treating the sludge with lime and/or heat. Although most strains used in production are Spo$^-$, the lesions giving rise to this phenotype have usually been introduced by random mutagenesis. The possibility that low levels of spores are present in waste biomass is therefore an important consideration in the inactivation process. Moreover, the legislation governing strains used in production makes the use of a well-defined sporulation mutant desirable. Such a mutant should preferably be (i) totally defective in sporulation, (ii) completely stable and unable to revert to sporogeny, and (iii) similar or superior to the parent in the ability to synthesize and secrete extracellular enzymes.

Our understanding of the genetics of sporulation in *Bacillus subtilis* is now at an advanced stage (9, 15), and, on the basis of the sporulation genes of *B. licheniformis* which have been well characterized (14, 16, 17, 33), it seems likely that this close relative of *B. subtilis* has a similar, if not identical, developmental pathway. The first morphological change observed during the sporulation process is the synthesis of an asymmetric septum at stage II. The smaller daughter, which ultimately is engulfed by the mother cell, gives rise to a prespore. Gene expression in the mother cell and maturing spore is governed in part by the ordered synthesis and activation of a cascade of sigma factors which direct RNA polymerase to transcribe sporulation-specific promoters in a temporally and spatially oriented fashion (9, 15). The σ^F protein, the product of the *spoIIAC* gene (10, 27), is one sigma factor which is present in predivisional cells, but the activity of this protein is restricted to prespores and becomes evident only after septation (18, 25). This sigma factor is crucial for the development of compartment-specific gene expression (18), and without it, expression of numerous genes in the developing spores is prevented. A major function of σ^F is activation of expression of prespore-specific sigma factor σ^G, which is responsible for gene expression in developing spores (19). Moreover, the processing of pro-σ^E into σ^E, which is responsible for gene expression in mother cells, is also blocked by nonsense mutations in *spoIIAC* (19). Deletion of the *spoIIAC* gene should therefore lead to total disruption of development of spores and blockage of mother cell development which is dependent on σ^E. For these reasons and because the results of previous studies have suggested that *spoIIAC* mutations may have little effect on extracellular enzyme synthesis in *B. subtilis* (4, 30) we chose this gene as a target for deletion in *B. licheniformis.*

In this paper we show that in *B. licheniformis* in vivo recombination can be used to prepare defined gene deletions and that deletion of *spoIIAC* gives rise to a strain which is totally and stably defective in sporulation but exhibits normal synthesis of serine protease but slightly lower levels of amylase synthesis than a wild-type strain.

MATERIALS AND METHODS

Strains and growth conditions. Strain DN286 is a *B. licheniformis* wild-type strain obtained from the collection of Novo Nordisk. *B. licheniformis* NCIMB 6346 and *B. subtilis* 168 were obtained from our laboratory collection. *Escherichia coli* JM 83 was used for all plasmid constructions. Luria broth and Luria broth agar (11) were used routinely and were supplemented with the following antibiotics for selection: ampicillin (100 μg/ml) and erythromycin (1 μg/ml). Spizizen's minimal salts medium containing 2% maltose as a carbon source (11), Schaefer's sporulation medium (11), and brain heart infusion (BHI) (Oxoid) were also used. Cultures were incubated at 37°C unless indicated otherwise.

Plasmids. Plasmid pUC19 was used for cloning in *E. coli* JM 83. The complete *spoIIA* operon from *B. licheniformis* cloned in pUC13 (33) was provided by M. Yudkin (University of Oxford, Oxford, United Kingdom), and pE194ts, a mutant of pE194 which is temperature sensitive for replication, was a gift from P. Youngman (University of Georgia, Athens).

DNA manipulations. Most of the methods used for DNA manipulations were methods described by Sambrook et al. (24) or Aquino de Muro et al. (1). DNA was excised from agarose gels and was purified prior to ligation by using a Gene-Clean II kit (Bio 101, Inc.). The DNA sequences of PCR products were determined by the automated DNA sequencing service provided by the Department of Molecular Medicine, Kings College, University of London, London, United Kingdom (an ABI model 373A DNA sequencer is used to determine sequences). The sequencing primer used, 5′-CGATCATGGAAAATTTCATG

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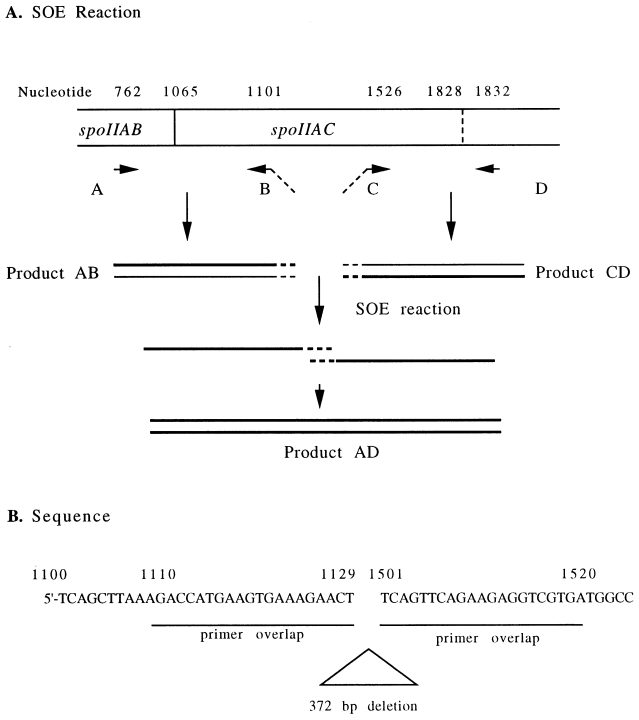

FIG. 1. SOE reaction used to create the deletion in *spoIIA*. (A) Details of the primers used. The numbers are the nucleotide positions of the 3′ ends of primers A, B, C, and D. (B) Sequence of the *spoIIAC* gene obtained by sequencing the PCR product generated with primers A and D from the deleted strain, *B. licheniformis* HWL 10.

GATG-3′, was complementary to a region approximately 100 bases upstream of the proposed recombination junction, corresponding to bases 943 to 966 in the previously published sequence (33).

Preparation of a deletion of *spoIIAC*. The splicing-by-overlap-extension (SOE) reaction was used to prepare a deletion of *spoIIAC* (13). The promoter-proximal region of the *spoIIAC* gene was amplified in a reaction with primers A (5′-GCGGCGAATTCAGCTTGACCCGACGATGGATGAACTG) and B (5′-CACGACCTCTTCTGAACTGAAGTTCTTTCACTTCATGGTCTTTAAGCTG-3′), and the promoter-distal region of the *spoIIAC* gene was amplified in a separate reaction with primers C (5′-GACCATGAAGTGAAAGAACTTCAGTTCAGAAGAGGTCGTGATGGGC-3′) and D (5′-GCGGCGGATCCTGCCTGCAACATGAGCAGCCTCAGC-3′) (Fig. 1) (the underlined sequences in primers A and D are *Eco*RI and *Bam*HI sites, respectively). Each reaction mixture contained 200 ng of template DNA (cloned *spoIIA* operon of *B. licheniformis*), 100 pmol of each primer, each deoxynucleoside triphosphate at a final concentration of 125 mM, 6 μl of $MgCl_2$ (25 mM), 10 μl of 10× *Taq* buffer, and enough sterile Millipure water to bring the volume to 100 μl. The amplification program used began with denaturation at 95°C for 10 min, after which 1 U of *Taq* polymerase (Promega) was added and the reaction mixture was covered with 100 μl of light mineral oil. This was followed by 35 cycles consisting of denaturation at 95°C for 2 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min. An additional cycle consisting of denaturation at 95°C for 2 min, annealing at 55°C for 2 min, and extension at 72°C for 6 min completed the program.

The 464- and 382-bp PCR products were concentrated by ethanol precipitation, purified by using a Gene-Clean kit, and used as templates (300 ng each) with primers A and D in an SOE reaction under the conditions described above to generate the 806-bp *spoIIAC* deletion that made up fragment AD (Fig. 1).

Introduction of the deletion into *B. licheniformis* and *B. subtilis*. Fragment AD was cloned into pUC19 through the *Bam*HI and *Eco*RI restriction sites in primers A and D, respectively, and was transformed into *E. coli* JM 83, and colonies containing plasmids with insertions were screened on Luria broth plates containing ampicillin and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per ml. A recombinant plasmid from a clone was then ligated to pE194ts through the unique *Pst*I sites in each replicon after the recombinant plasmid was treated with calf intestinal alkaline phosphatase. Ligation mixtures were transformed into *E. coli* JM 83, and recombinants were detected by performing agarose gel electrophoresis with minipreparations. The plasmid prepared from *E. coli* was then used to transform protoplasts of *B. licheniformis* DN286 (28) and competent cells of *B. subtilis* 168 (6).

B. licheniformis and *B. subtilis* containing the pE194-based deleted *spoIIAC* allele were grown overnight in 50 ml of Luria broth containing erythromycin at 28°C. Samples were diluted, mixed with 3 ml of soft agar containing erthromycin, and overlaid onto Luria broth agar plates containing erythromycin, and the plates were incubated at 28°C (permissive for replication) and 40°C (not permissive for replication). The frequency of integration was the ratio of the number of colonies growing at the permissive temperature to the number of colonies growing at the nonpermissive temperature. Integrants isolated at 40°C were subsequently grown routinely at 37°C.

The integrated plasmid was excised by growing cells at 30°C in Luria broth with no antibiotic selection. Diluted samples were plated onto Luria broth agar and incubated overnight at 37°C, and colonies were replica plated onto Luria broth agar with and without erythromycin. The frequency of excision was determined by calculating the ratio of total number of colonies to number of erythromycin-resistant (Em^r) colonies.

Enzyme assays. Qualitative estimates of enzyme secretion were determined by using plate detection methods described previously (22). Alkaline protease activity was assayed in culture supernatants by using azocasein as the substrate. Potassium phosphate buffer (50 mM, pH 9.0) (1 ml) and culture supernatant (1.0 ml) were equilibrated at 37°C for 3 min, and the reaction was started by adding 0.5 ml of 0.8% azocasein solution in phosphate buffer. At appropriate times, 0.5-ml portions of the reaction mixture were precipitated with 0.5-ml portions of ice-cold 20% trichloroacetic acid, the mixture was centrifuged, and the A_{405} of the supernatant was determined. Activity was expressed as the change in absorbance per minute per milliliter of supernatant.

Amylase activity was assayed in culture supernatants by using the Phadebas amylase substrate (Pharmacia) at 37°C. The values were converted to amounts of reducing sugar (glucose) released from soluble starch by using a standard curve constructed with dilutions of amylase assayed by the Phadebas and Nelson-Somogyi reducing sugar assay. The units were micromoles of maltose equivalents released per minute per milliliter of supernatant. All of the batch cultures were grown at least twice; the values presented below represent reproducible patterns of results.

Electron microscopy. Cells grown in minimal medium containing 0.5% maltose as a carbon source were collected by centrifugation and washed in 10 mM Tris buffer (pH 8) containing 50 mM NaCl. The cells were stained with 4% aqueous uranyl acetate for 2 h and counterstained with lead citrate as described by Robards and Wilson (23). Thin sections were viewed with a JEOL model 100S transmission electron microscope.

RESULTS

Preparation of a deletion in *spoIIAC*. A deletion in the *spoIIAC* gene of *B. licheniformis* was prepared by using the SOE technique. Two independent PCR amplification preparations were obtained from the cloned *spoIIA* operon by using the primers described in Materials and Methods. One product (product AB) comprised the distal end of the downstream gene (*spoIIAB*) and the proximal region of the *spoIIAC* gene. The second product (product CD) comprised the distal region of *spoIIAC* and the proximal region of the upstream noncoding region. The extensive regions of homology (overlap) in primers B and C were used to initiate a third PCR amplification in which we used products AB and CD and primers A and D. This resulted in a fragment that contained the ends of the *spoIIAC* gene encompassing a 372-bp deletion (fragment AD) (Fig. 1).

Fragment AD was cloned into pUC19 and ligated to pE194ts through the unique *Pst*I sites in each replicon to produce shuttle plasmid pHWM2 (Fig. 2). This plasmid was transformed into *E. coli*. A plasmid prepared from *E. coli* was then used to transform protoplasts of *B. licheniformis* DN286 and competent cells of *B. subtilis* 168. We verified that clones from each transformation preparation contained pHWM2 by performing a restriction enzyme analysis, and these clones were used for integration and excision studies.

Integration and excision of the deleted *spoIIAC* allele. *B. licheniformis* and *B. subtilis* containing pHWM2 were grown overnight at 28°C and then plated onto selective (erythromycin-containing) and nonselective media and incubated at 45°C. The integration frequency was determined by estimating the proportion of cells which could grow on erythromycin-containing plates at 45°C. The integration frequency for *B. licheniformis* was about 10^{-4}, and the integration frequency for *B. sub-*

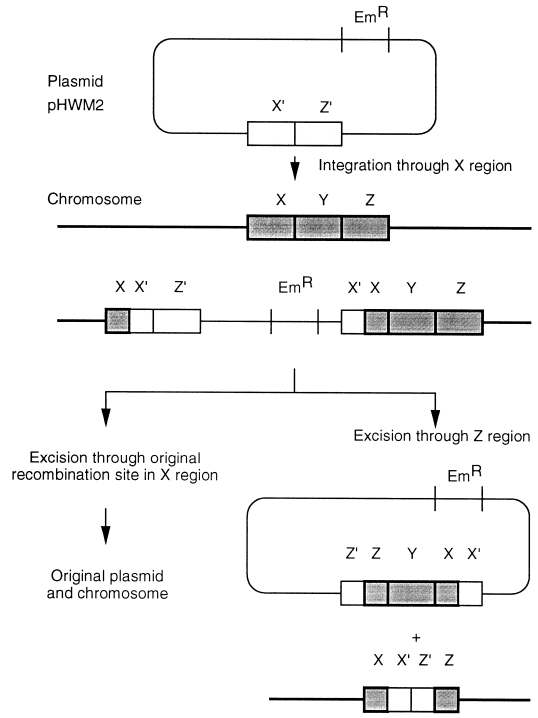

FIG. 2. Integration and excision reactions resulting in the production of a deletion in *spoIIAC*.

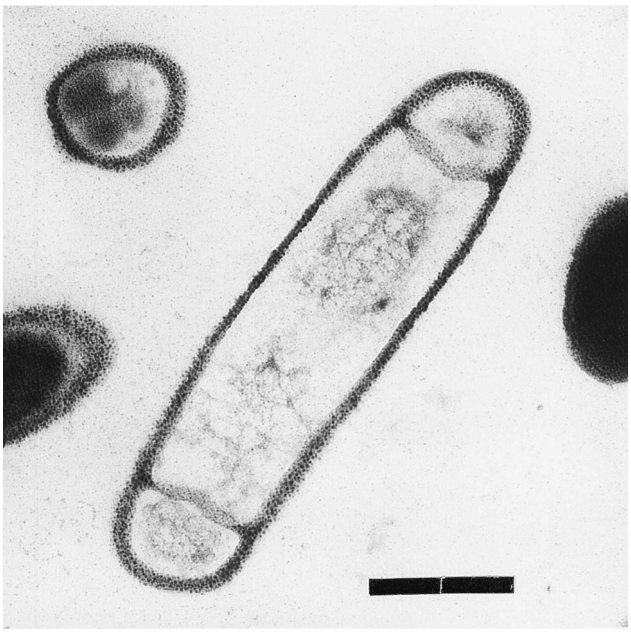

FIG. 3. Thin section of a typical cell from a culture of *B. licheniformis* HWL 10 containing the deletion *spoIIACΔ3*, showing the abortive disporic phenotype. Bar = 0.5 μm.

tilis (containing the *B. licheniformis* allele) was 100-fold lower. Several integrants were purified from the erythromycin-containing plates. These integrants were designated *B. licheniformis* HWL 1 through HWL 5 and *B. subtilis* HWS 1 through HWS 3 (Table 1) and were tested for excision of the plasmid following activation of rolling circle replication in the integrated plasmid by culturing at 28°C. The excision frequencies, which were determined by estimating the proportions of erythromycin-sensitive (Em^s) cells in the cultures, were between 10^{-2} and 10^{-3} for the *B. licheniformis* strains. In two clones (strains HWL 1 and HWL 2) excision was invariably through the integration site, and wild-type Spo^+ progeny were recovered. However, in the other three clones excision was also through a second-site recombinational event (Fig. 2), which gave rise to deletions of the *spoIIAC* gene in the Em^s derivatives. Interestingly, the insertion was very unstable in *B. subtilis*, but invariably excision occurred at the insertion point and no Spo^- progeny were recovered even after a long outgrowth period (48 h) (Table 1).

TABLE 1. Excision frequency of pE194ts carrying the deleted *spollAC* allele of *B. licheniformis*

Organism	Integrant	Outgrowth period (h)	No. of colonies tested	Excision frequency	% Spo^- colonies
B. licheniformis	HWL 1	24	150	9×10^{-2}	0
	HWL 2	24	100	2×10^{-2}	0
	HWL 3	24	150	6×10^{-2}	44
	HWL 4	24	150	7×10^{-2}	20
	HWL 5	24	150	5×10^{-2}	25
B. subtilis	HWS 1	24	560	1×10^{-1}	0
		48	114	2×10^{-1}	0
	HWS 2	24	358	1×10^{-1}	0
		48	152	2×10^{-1}	0
	HWS 3	24	442	2×10^{-1}	0
		48	184	3×10^{-1}	0

Characterization of *B. licheniformis spoIIACΔ3*. Deletion strain *B. licheniformis* HWL 10, which was obtained as an Em^s clone from strain HWL 3 (Table 1), was used for further study. Hybridization of a labeled *spoIIA* operon from *B. licheniformis* to Southern-blotted chromosomal DNAs from the wild-type and deleted strains which had been cut with *Eco*RI revealed that there was a small 3.8-kb hybridizing fragment in the mutant (compared with the 4.2-kb fragment in the parent) (data not shown). PCR amplification from chromosomal DNAs of the mutant and parent strains by using primers A and D revealed that the mutant had a deletion of about 370 bp. When the PCR product from the mutant was sequenced, a deletion located precisely at the junction between primers B and C was revealed (Fig. 1B). Morphological confirmation of a SpoIIA mutation was provided by electron microscopy. The two asymmetric septa in an abortively disporic cell typical of a *B. subtilis spoIIAC* mutant (5) were clearly visible (Fig. 3).

The deletion gave rise to completely asporogenous cultures in which spores could not be detected. After growth in BHI, minimal medium (Fig. 4), and Schaeffer's sporulation medium (data not shown) for 72 h, the sporulation frequency of the parent was about 2% in each case. The naturally low sporulation rate of strain DN286 was confirmed by comparing this strain with *B. licheniformis* NCIMB 6346, which exhibited a sporulation rate of 90 to 100% under the same conditions. Spores were never detected in the *spoIIACΔ3* derivative in any of the media. Despite the absence of spores, there was no evidence of loss of viability or cell lysis in the mutant strain when it was grown in BHI, and the cell population remained steady at about 4×10^9 CFU/ml throughout the incubation period. In minimal medium, however, there was some loss of viability when the mutant was incubated for more than 36 h, and the final cell population contained approximately 3×10^8 CFU/ml after the population peaked 10-fold higher. Loss of

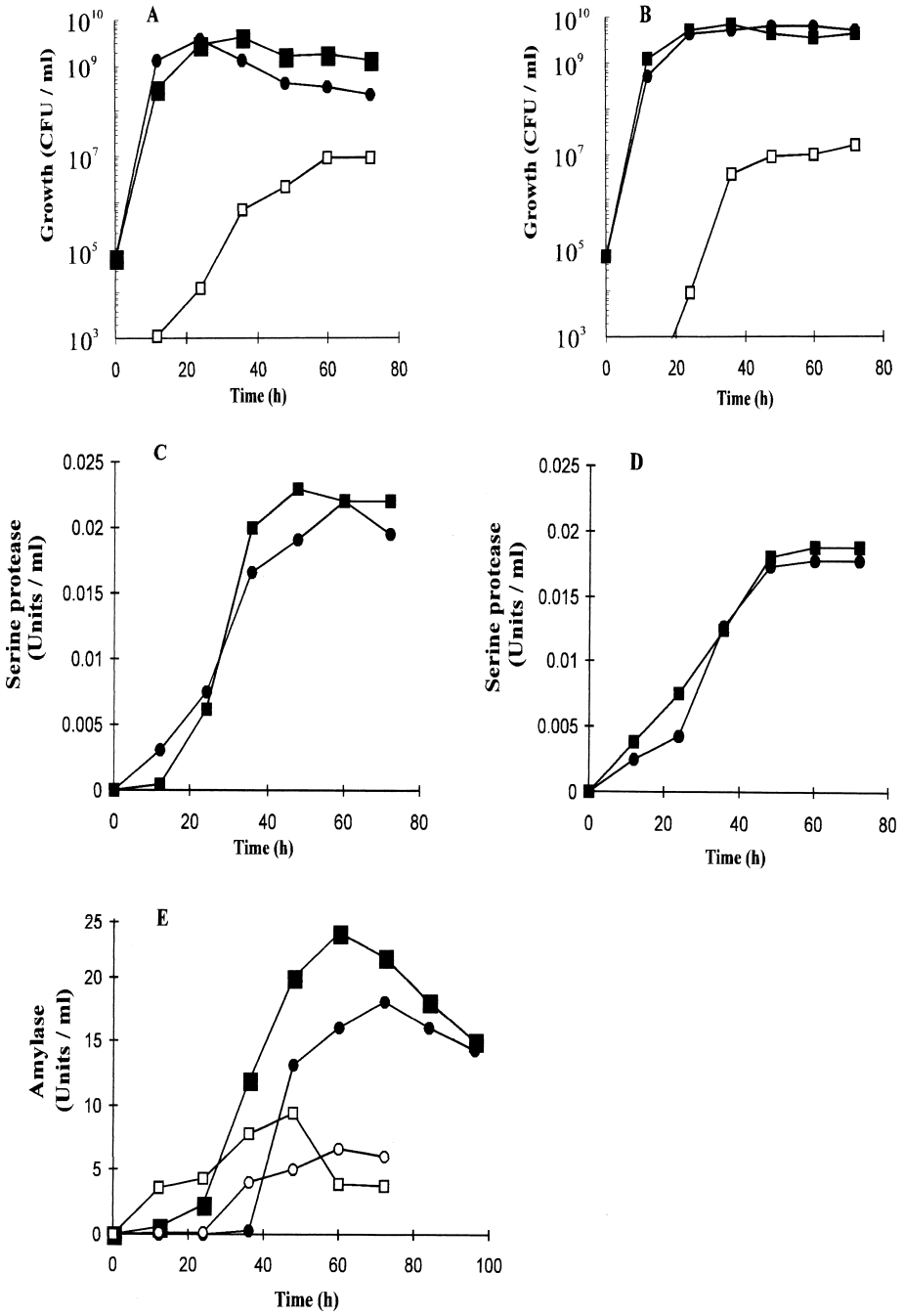

FIG. 4. Growth, sporulation, and extracellular enzyme synthesis in *B. licheniformis* DN286 and HWL 10. (A and B) Growth (■) and sporulation (□) of strain DN286 and growth of strain HWL 10 (●) in minimal medium and BHI, respectively. (C and D) Serine protease synthesis by strain DN286 (■) and strain HWL 10 (●) in minimal medium and BHI, respectively. (E) Amylase synthesis in strain DN286 (squares) and strain HWL 10 (circles) in minimal medium (open symbols) and BHI (solid symbols).

viability was less pronounced in the parent strain growing in minimal medium.

Extracellular enzyme synthesis in *B. licheniformis spoIIAC*Δ*3.* The results of the initial plate tests suggested that the mutant strain differed slightly from the parent strain in the amounts of extracellular enzymes synthesized. In particular, the hydrolysis zones due to β-1,3-glucanase and carboxymethyl cellulase were smaller in the mutant than in the parent strain, but synthesis of α-amylase, DNase, polygalacturonate lyase, protease, RNase, and xylanase was largely unaffected.

The mutant and its parent were grown in rich (BHI) and minimal media for 72 h, and sporulation and the synthesis of serine protease were monitored (Fig. 4). Protease production was similar in the parent and the mutant. In minimal medium, the enzyme yield peaked after incubation for about 40 h in both strains and then remained steady. In BHI, the enzyme

yield was slightly repressed and peaked after incubation for about 48 h in both strains. The subsequent decline in enzyme yield was less pronounced in BHI than in the salts medium. In both media, the yield of serine protease was not affected by the *spoIIAC* deletion.

During batch culture α-amylase synthesis consistently began later in the mutant than in the parent, and consequently these cultures were incubated longer than the protease preparations (84 h). In both media, the yield of α-amylase obtained from the mutant was about 70% of the yield obtained from the parent strain. However, if the enzyme yields were expressed as specific activities per unit of biomass, the mutant performed almost as well as the parent, particularly later in the growth cycle, when the amount of biomass in the minimal medium declined.

DISCUSSION

Gene integration and excision is a common procedure for producing specific mutations in *B. subtilis* and other gram-positive bacteria (3, 28, 32). In this paper we show that this procedure is also an efficient way to introduce defined mutations into the *B. licheniformis* chromosome. Given the close relationship between *B. subtilis* and *B. licheniformis* (reviewed in reference 21), we wanted to examine the possibility of using heterologous alleles to introduce genes into the chromosome. In this way, the large number of *B. subtilis* genes that have been sequenced might be used for genome manipulation in *B. licheniformis*. Indeed, the level of homology was great enough that the *spoIIAC* allele of *B. licheniformis* could be integrated into the *B. subtilis* chromosome, albeit at a lower frequency than the homologous reaction, and integration presumably occurred at the correct site since the integrants were asporogenous (data not shown). However, excision in the *B. subtilis* host invariably occurred during a second-site recombination event at the integration crossover point, and no deletions were recovered. Presumably, a high level of homology in this region led to a recombinational hot spot, and consequently all progeny (at least 2,000 progeny from several integrants were examined) retained an intact *spoIIAC* gene. It seems likely that the reverse situation occurs in *B. licheniformis* and that *B. subtilis* genes forced into the *B. licheniformis* chromosome should excise perfectly. Therefore, it seems that *B. subtilis* genes may be used to generate specific insertions in the *B. licheniformis* chromosome, but are less useful for the recovery of deletions.

Assuming that *B. subtilis* and *B. licheniformis* follow similar developmental pathways, the effect of the deletion in *spoIIAC* in *B. licheniformis* can be examined in light of the *B. subtilis* model. Indeed, the differentiation of *B. licheniformis* DN286 containing *spoIIACΔ3* into abortively disporic cells, exactly like those found in *spoIIAC* mutants of *B. subtilis* (9), supports this assumption. Extracellular enzymes are generally synthesized in the late stages of exponential growth and during the early stationary phase prior to stage II of sporulation (reviewed in reference 20). Therefore, we predict that mutations of *spoIIAC* should have little or no effect on the expression of extracellular enzyme genes, and indeed, with the exception of the β-glucanases, plate tests revealed no gross changes in extracellular enzyme yields in the mutant compared with the parent strain.

Genes expressed before septation is complete are expressed in SpoIIA cells. This includes all stage 0 genes and some stage II genes, as well as regulatory genes such as *abrB*, *hpr*, *sen*, and *sin* (26). Most or all of the controls that affect *aprE* expression in *B. subtilis* (there are at least nine independent genes or systems [26]) are early-stationary-phase phenomena and probably function effectively in the *spoIIAC* mutant. It follows that the pattern of serine protease synthesis and the enzyme yield in the mutant would match the pattern and yield of the parent (Fig. 4). Indeed, similar results for serine protease synthesis in *B. subtilis* have been reported by Arbidge et al. (2), who used an undefined asporogenous mutant.

The expression of *amyE* is subject to far fewer regulatory genes in *B. subtilis* than is the expression of *aprE*. Catabolite repression and the DegSU systems are the major control systems for this gene (26), although *sen* (31) and *pai* (12) are also involved. It is not readily apparent why the synthesis of amylase was affected by the *spoIIAC* mutation. It is possible that the early sporulation sigma factor, σ^H, accumulates in the *spoIIAC* mutant because the blockage prevents σ^F and σ^E synthesis, the former because of the deletion and the latter because active σ^E production from its precursor is dependent on σ^F (19). Indeed, there is evidence that enhanced accumulation of σ^H occurs in a *spoIIA* mutant of *B. subtilis* (9a). Thus, the lower α-amylase yield may be due to the partial replacement of E-σ^A by E-σ^H.

The major aim of this work was to see if an asporogenous mutant of *B. licheniformis* could be prepared by molecular techniques. We constructed such a mutant, and our mutant is totally asporogenous under laboratory conditions. No spores were detected among 10^{10} cells when we used conditions under which the level of sporulation of a wild-type strain (*B. licheniformis* NCIMB 6346) was 90 to 100%. The likelihood that a deletion spanning almost 400 bp will revert is minimal, and we have no evidence which suggests that suppression may be a problem. Therefore, we concluded that such a bacterium can be prepared by introducing a deletion in the *spoIIAC* gene and that the mutant obtained has retained its important industrial properties.

ACKNOWLEDGMENTS

A.B.F. is grateful to the BBSRC and Novo Nordisk for financial assistance through the CASE scheme.

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