Mutants of *Bacillus subtilis* Defective in Protein Export

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We have isolated a set of strains with mutations (designated *prs*) that decrease secretion of α -amylase and have a pleiotropic effect on secretion of other exoproteins. The seven mutants were selected in a strain of *Bacillus subtilis* which overproduces α -amylase due to the presence of an α -amylase gene on a multicopy plasmid. The mutations were mapped to four different chromosomal loci. The phenotype of the mutants, especially their pleiotropic effects and the accumulation of α -amylase precursor, indicated that they have defects in the mechanism of protein export. Double mutants with certain pairwise combinations of mutations in different loci had additive effects on secretion, suggesting that these *prs* genes encode different components of the secretion pathway.

INTRODUCTION

The export of proteins requires several cellular components, both membrane-bound and cytoplasmic, that recognize the export signal(s) and direct the protein across the membrane (Walter, 1987). Several components of the export machinery are well characterized in eukaryotic cells. They include signal peptidases (Evans *et al.*, 1986), the signal recognition particle (SRP) (Warren & Dobberstein, 1978; Walter & Blobel, 1981), SRP receptor (Gilmore *et al.*, 1982; Meyer *et al.*, 1982), the signal sequence receptor (Wiedmann *et al.*, 1987) and ribophorins (Kreibich *et al.*, 1978). By contrast, only the signal peptidases are well characterized in *Escherichia coli* (Wolfe *et al.*, 1983; Innis *et al.*, 1984) whereas the involvement of other cellular components is only indicated by mutations affecting protein export. Although some of the genes, e.g. *secA* (Oliver & Beckwith, 1981) and *prfA* (Emr *et al.*, 1981), defined by these mutations are well characterized, their function in secretion is still largely unknown. The nature of the mutations, conditionally lethal or suppressor, has handicapped functional studies (see Silhavy *et al.*, 1983). Furthermore, all these mutations affect early steps of export, probably due to the constraints dictated by the powerful selection needed to find them. It is thus possible that many genes involved in the export of proteins in *E. coli* have remained undetected.

Genetic studies on protein export in *Bacillus* can be expected to demonstrate new types of genes affecting protein export. *B. subtilis* secretes true exoproteins which, in contrast to most major exported proteins of *E. coli*, are not components of the cell envelope. The fact that many *Bacillus* species are devoid of exoproteins indicates that exoproteins are not indispensable in *Bacillus* and suggests that mutations preventing secretion need not be lethal. On the other hand, the large amount of protein secreted by *B. subtilis* (Palva, 1982) suggests easy screening methods for identifying mutants with either complete or partial defects in secretion.

Against this background we set out to isolate mutants of *B. subtilis* defective in protein secretion. Our approach was to start from a strain of *B. subtilis* that carries the structural gene of α -amylase on a multicopy plasmid and thus secretes large amounts of the protein. We screened for decreased secretion of the α -amylase, and analysed the mutants thus obtained by biochemical and genetical methods.

Abbreviation: NNG, *N*-methyl-*N*-nitroso-*N'*-nitroguanidine.

METHODS

Bacteria, phages and plasmids. The *B. subtilis* strains used in mutagenesis experiments and in genetic crosses are listed in Table 1. Mapping experiments were done with phage PBS1; recipient strains were strains KIT1 through KIT9 (Dedonder *et al.*, 1977), obtained from the *Bacillus* Genetic Stock Center (BGSC). Plasmid pKTH10 is a derivative of pUB110 (Palva, 1982) with a 2.3 kb insert from *B. amyloliquefaciens*. The insert contains the gene for secreted α -amylase. pKTH6 is an analogous derivative of pUB110 with an insert at the *EcoRI* site (about 5.4 kb) containing the *penP* (lipopenicillinase) gene of *B. licheniformis*.

Media and growth conditions. Modified L-broth containing 0.5% NaCl (Sibakov *et al.*, 1983) was supplemented with 2% (w/v) soluble starch (Difco) for experiments to determine the secretion kinetics of different exoenzymes and with 5% (w/v) starch and 5% (w/v) agar to make starch plates for screening of mutants defective in α -amylase secretion. In transformation and transduction experiments Spizizen minimal medium (SMS) (Anagnostopoulos & Spizizen, 1961) and agar, antibiotic medium 3 (17.5 g l⁻¹, Difco) and TBAB (tryptose blood agar base) agar (Difco) were used. Sporulation frequency was determined in NSM (nutrient sporulation medium) broth (Vasantha & Freese, 1980). All cultures were incubated at 37 °C; liquid cultures were aerated vigorously.

Growth was determined by measuring culture density in a Klett-Summerson colorimeter with a no. 66 filter.

Mutagenesis and screening of mutants. Bacteria from 10 ml of exponential culture (IH6160 in L-broth) were suspended into 2 ml 0.1 M-potassium phosphate buffer (pH 6.5). NNG (*N*-methyl-*N*-nitroso-*N'*-nitroguanidine) was added to a final concentration of 0.1 mg ml⁻¹, which killed about 99% of the bacteria. After incubation with shaking for 30 min at 37 °C, cells were harvested, washed twice with the above buffer and suspended into 1 ml of the same. Portions (0.1 ml) of this suspension were added to 10 ml L-broth, grown for 4 h and appropriate dilutions plated onto starch plates supplemented with kanamycin (10 µg ml⁻¹). Plates were incubated for 16 h at 37 °C and then transferred to 4 °C for screening mutants defective in α -amylase secretion. A clear halo developed in about 6 h around α -amylase-positive colonies. Colonies with a decreased halo diameter were picked up as putative secretion mutants. The secretion defect was confirmed by assaying α -amylase production in L-broth. Before any further analysis the mutated gene was transformed either to strain IH6157 (*his*⁺ selection) or IH6513 (*thr*⁺ selection) to exclude multiple mutations. The frequency of auxotrophic mutants was about 10% in all of the mutagenesis experiments.

Transformation and transduction. Transformation was done as described by Gryczan *et al.* (1978) and transformants selected on SMS agar. PBS1 lysates were made as follows. A mutant strain in a soft agar lawn on TBAB plates was infected with PBS1 to obtain semiconfluent lysis. The soft agar layer was scraped off into a small volume of antibiotic medium 3, chloramphenicol was added (5 µg ml⁻¹) and the suspension was incubated at 48 °C overnight without aeration. After centrifugation (10000 g, 10 min) the lysate was passed through a Millipore filter (0.45 µm) and stored at 4 °C. This phage preparation was used for transduction as described by Young & Wilson (1974) with slight modifications.

Enzyme assays. α -Amylase was assayed using the Phadebas amylase test (Pharmacia). Samples were incubated for 2 h at 37 °C in 4.2 ml water containing one dispersed Phadebas pellet, after which 0.5 ml NaOH was added to terminate the reaction. After filtration through Whatman no. 1 filter paper, the A_{600} of the filtrate was measured. The results were expressed as µg enzyme ml⁻¹ (commercially available α -amylase of *B. amyloliquefaciens* (Sigma) was used as standard). Protease activity was determined as described by Sibakov *et al.* (1983). One enzyme unit (U) causes an absorbance change of 0.1 A_{595} unit h⁻¹ at 37 °C. Penicillinase was assayed using nitrocefin as described by Simons *et al.* (1978); the results were expressed as µg enzyme ml⁻¹.

Sporulation frequency. This was determined in NSM-broth as described by Sibakov (1986).

Motility. The motilities of different strains were compared on L-broth plates supplemented with 0.4% agar by placing a small drop of exponentially growing culture into the centre of the plate and measuring the spread of bacteria after incubation at 37 °C overnight.

SDS-PAGE and immunoblotting. These were done as described by Sarvas & Nurminen (1985).

Isolation of membranes. Membrane samples were prepared by sonic disruption of lysozyme-treated cells followed by centrifugation at 100000 g for 1 h at 4 °C (Kallio *et al.*, 1986). The pellet was resuspended to a volume one-hundredth that of the culture the bacteria were derived from.

Reagents. NNG was purchased from Koch-Light, Phadebas amylase test and *Staphylococcal* protein A from Pharmacia Diagnostics, α -amylase of *B. amyloliquefaciens* from Sigma, and nitrocefin from Glaxo.

RESULTS

Isolation of mutants with decreased secretion of α -amylase

Strain IH6160, a hyperproducer of α -amylase due to multiple copies of plasmid pKTH10 (see Table 1), was mutagenized with NNG and plated onto starch plates. About 40000 colonies were screened for decreased secretion of α -amylase, and several were found that had smaller haloes around the colony. In order to determine whether the mutations were located on the plasmid or

Table 1. *Bacillus subtilis* strains

Strain	Relevant genotype/ plasmid carried*	Parent/source/reference
IH6064	<i>metB5 sacA321 amyR1</i>	Sibakov <i>et al.</i> (1983)
IH6090	<i>hisA1 metB5 sacA321 amyR1</i>	IH6064
IH6131	IH6090(pKTH6)	IH6090
IH6157	IH6090(pKTH10)	IH6090
IH6160	IH6064(pKTH10)	IH6064
IH6509	QB934(pKTH10)	QB934
IH6513	QB917(pKTH10)	QB917
IH6531	<i>glyB1 hisA1 trpC2</i> (pKTH10)	IH6513†
IH6532	QB943(pKTH10)	QB943
QB917	<i>hisA1 thr-5 trpC2</i>	1A10 in BGSC‡
QB934	<i>tre-12 metC3 glyB133 trpC2</i>	1A5 in BGSC
QB943	<i>pyrD1 ilvA1 thyA1 thyB1 trpC2</i>	1A6 in BGSC§

* pKTH6 is a derivative of pUB110 with an insert of the *penP* gene from *B. licheniformis*. pKTH10 is a derivative of pUB110 with an insert of the α -amylase gene from *B. amyloliquefaciens*.

† Transformant with DNA from QB934.

‡ BGSC, Bacillus Genetic Stock Center, The Ohio State University, Department of Biochemistry, 484 West 12th Avenue, Columbus, Ohio 43210, USA.

§ Received from F. Kunst, Institut Pasteur, 28, Rue du Dr Roux, 75724 Paris Cedex 15, France.

on the chromosome, the plasmid was transferred from all these mutants to a plasmidless strain, IH6064. In only one mutant was there complete loss of α -amylase, and this coincided with loss of the plasmid, concordant with the described stability of pKTH10 (Vehmaanperä & Korhola, 1986). In about 30 mutants the decreased secretion of α -amylase was co-transferred with the plasmid; these mutated plasmids were presumed to have mutations in the structural gene or control region of α -amylase and were not studied further. In seven cases the transformants appeared to secrete as much α -amylase as IH6160, as judged by the size of the halo, implying that the mutations in the original strains were chromosomal. These *protein secretion* (*prs*) mutations were transformed into new host strains IH6157 or IH6513 (both carrying pKTH10), to exclude as far as possible the effects of multiple mutations.

Growth and secretion of exoproteins in the prs mutants

Decreased α -amylase secretion in the *prs* mutants was not caused by a decreased growth rate. All mutants and their parents had identical growth rates and the same maximal growth in L-broth supplemented with 2% soluble starch, with variation between several experiments of less than 10%.

The exoamylase production of mutant *prs-3* is shown and compared to the parent strain, IH6157, in Fig. 1(a). In this mutant the concentration of exoamylase did not exceed the detection threshold before the early stationary phase (approx. 8 h). At this point the amount of exoamylase was about 3% of that in the parent strain, and in the later stationary phase it was only about 1% of the parental level.

To compare different mutants we therefore used the amount of the α -amylase in the culture supernatant in the early stationary phase (Table 2). In the seven mutants this amount varied between 4 and 28 $\mu\text{g ml}^{-1}$ or 2–14% of that in the parent. In the late stationary phase the relative amount of exoamylase further decreased to below 3% ($<45 \mu\text{g ml}^{-1}$) except in *prs-33*, in which it was about 15%.

Because a mutation affecting the export machinery is likely to cause defects in the export of several exoenzymes, we also determined the exoprotease activity of the *prs* mutants. Fig. 1(b) shows the kinetics of exoprotease production in *prs-3* and Table 2 shows the level of exoprotease in the early stationary phase in other *prs* mutants. The production of exoprotease was decreased in all *prs* mutants compared to the parent, but the decrease was comparatively less (to 18–62% of the parent) than the decrease of α -amylase (to 2–14%).

Fig. 1. Secretion of α -amylase and protease by IH6157 and its *prs-3* derivative IH6480. (a) α -Amylase: ●, wild-type; ○, *prs-3*. (b) Growth rate: ●, wild-type; ○, *prs-3*. Protease: ■, wild-type; □, *prs-3*.

Table 2. Exoenzymes in the culture supernatant of *prs* mutants

Control strain . . .	α -Amylase*		Protease*		Penicillinase†	
	Plasmid-coded	Chromosomally encoded	With pKTH10	Without pKTH10	Cell-bound	Secreted
	IH6157 or IH6513	IH6064 or QB917	IH6157 or IH6513	IH6064 or QB917	IH6131	IH6131
Enzyme activity . . .	210 $\mu\text{g ml}^{-1}$	0.2 $\mu\text{g ml}^{-1}$	2 U ml ⁻¹	8 U ml ⁻¹	2.2 $\mu\text{g ml}^{-1}$	0.3 $\mu\text{g ml}^{-1}$
Mutant	Percentage of the enzyme activity of the control:					
<i>prs-3</i>	2	51	18	83	100	100
<i>prs-29</i>	3	72	23	67	100	100
<i>prs-40</i>	6	70	33	81	100	100
<i>prs-26</i>	7	84	31	93	67	22
<i>prs-13</i>	9	88	62	100	75	28
<i>prs-33</i>	10	51	30	32	58	130
<i>prs-11</i>	14	100	53	62	5	1200

* Determined in the early stationary phase (6 h after the culture density reached 100 Klett units).

† Determined in late-exponential-phase cultures (200 Klett units). Cell-bound penicillinase was from intact whole cells.

All the mutant strains carried the plasmid pKTH10 and were therefore hyperproducers of α -amylase in spite of the decreased level of the enzyme. Since it is possible that the protein export machinery is saturated by high levels of α -amylase secretion (Sibakov *et al.*, 1983), we determined the production of exoproteases and exoamylase coded by the chromosomal *amyE* gene by the mutant strains in the absence of pKTH10. Table 2 shows that the effect of the *prs* mutations under these conditions was much less.

Effect of the prs mutations on the export of lipopenicillinase

The lipopenicillinases form a unique class of *Bacillus* exoproteins which, unlike other exoproteins, involve a membrane-bound form from which the exopenicillinase is proteolytically released. Furthermore, at least one component of the secretory machinery, the lipoprotein signal

Fig. 2. Secreted (in culture supernatant) and cell-bound (in intact whole cells) penicillinase of *prs* mutant IH6505 (*prs-11*) (■, secreted; □ cell-bound penicillinase) and its parent IH6131 (●, secreted; ○, cell-bound penicillinase).

peptidase, is specific for lipoproteins. It was therefore of interest to study the effect of the *prs* mutations on this secretory pathway.

The plasmid pKTH6, a derivative of pUB110 carrying the lipopenicillinase gene *penP* of *B. licheniformis*, was transformed into the set of *prs* mutants. Table 2 shows the amount of membrane-bound penicillinase and exopenicillinase in these strains in the late-exponential phase of growth. The ratio of the membrane-bound and exopenicillinase remained about 7 : 1 in the non-mutated parent strain throughout the exponential phase as shown previously (Imanaka *et al.*, 1985). The *prs* mutants exhibited two types of changes to this pattern. In *prs-26* and *prs-13* the level of exopenicillinase was decreased to about 25% of that in the parent, with a lesser decrease of the membrane-bound form. In *prs-11* there was a change in the opposite direction, so that essentially all the penicillinase was in the form of exopenicillinase; the total amount of penicillinase synthesized was also slightly higher than in the parent (Fig. 2). The mutation *prs-33* had very little effect on lipopenicillinase secretion despite its effect on the other exoenzymes. The remaining three *prs* mutations had no effect on penicillinase (Table 2).

Effects of the prs mutations on sporulation and other phenotypic properties

Decreased production of proteases is frequently found in sporulation mutants (Piggot, 1985). We therefore determined the sporulation frequency of the *prs* mutants (Table 3). In one mutant (*prs-33*) there were no spores. Some other mutants showed a slightly decreased frequency of sporulation, but not to the extent seen in most *spo* mutants (Piggot, 1985).

Mutants *prs-29* and *prs-33* showed changes of colony morphology (Table 3). *prs-29* caused a clearly altered colony morphology (very small raised colonies) when the mutant carried pKTH10 and therefore produced large amounts of α -amylase, but the colony size was normal if pKTH10 was replaced by pKTH6, which produces penicillinase at a much lower level than pKTH10 produces α -amylase. Three mutants (*prs-13*, *prs-26* and *prs-33*) were nontransformable.

Genetic mapping

The *prs* mutations were mapped by transduction with phage PBS1 and transformation (Table 4). Five of the seven *prs* mutations mapped very close to *glyB* (cotransfer frequencies by transduction about 90%) with no (< 5%) linkage with *metC* or *trpC*. Interestingly, *prs-13* and *prs-26* had a tighter linkage (about 100%) to *glyB* than did the other *glyB*-linked *prs* mutations,

Table 3. *Some phenotypic properties of prs mutants*

The properties (except transformability) were determined in strains carrying pKTH10.

Strain	Mutation	Sporulation frequency*	Other properties
IH6513	Wild-type	2.0×10^8	
IH6525	<i>prs-3</i>	0.5×10^8	
IH6527	<i>prs-29</i>	1.5×10^7	Decreased colony size†
IH6537	<i>prs-40</i>	1.3×10^8	
IH6521	<i>prs-26</i>	2.0×10^5	Not transformable‡
IH6522	<i>prs-13</i>	2.0×10^6	Not transformable‡
IH6529	<i>prs-33</i>	0	Not transformable‡; decreased motility; 'rough' colony
IH6520	<i>prs-11</i>	0.5×10^5	

* No. of spores determined as described in Methods.

† Colonies were of nearly normal size if there was no pKTH10 in the mutant strain.

‡ The mutant was transformed with pKTH10. The frequency of transformation was less than 10^{-4} of that of the parent.

Table 4. *Mapping of prs mutations*

prs mutations were mapped with both transduction and transformation. Contrtransfer of *prs* with a linked genetic marker is shown as percentage of transformants. In all crosses more than 50 colonies were screened for *prs*.

Donor		Recipient*	Selected marker and linkage (%)		
Strain	Mutation		Marker	Transduction	Transformation†
IH6480	<i>prs-3</i>	IH6509	<i>glyB</i>	92	38
IH6482	<i>prs-29</i>	IH6509	<i>glyB</i>	95	63
IH6484	<i>prs-40</i>	IH6509	<i>glyB</i>	88	56
IH6491	<i>prs-26</i>	IH6509	<i>glyB</i>	> 95	72
IH6497	<i>prs-13</i>	IH6509	<i>glyB</i>	> 95	81
IH6483	<i>prs-33</i> ‡	IH6532	<i>pyrD</i>	29	ND
IH6489	<i>prs-11</i>	IH6513	<i>hisA</i>	93	89

ND, Not done.

* For the genotypes see Table 1.

† In transformation crosses the recipient was IH6531, which was a Gly⁻ derivative of IH6513.

‡ *prs-33* also showed slight linkage with *thyA* (not presented).

suggesting the presence of two separate *prs* loci in this area. By analogous reasoning *prs-33* was localized in the *pyrD-thyA* region, and *prs-11* near *hisA* (about 90% linkage). Transformation confirmed all the linkages with *glyB* and *hisA* loci. The transformation data also confirmed the tighter linkage with *glyB* of *prs-13* and *prs-26*. Thus the seven mutants indicate the presence of at least four genes (loci) involved with protein secretion.

Recombinants with two prs mutations

To investigate the possible synergistic effect of *prs* mutations at the four separate loci, the *his*-linked *prs-11* and the *pyrD-thyA*-linked *prs-33* were recombined with the rest of the mutations as shown in Fig. 3. The amount of α -amylase secreted by each of the constructs was determined in the early stationary phase of growth (Table 5). Both *prs-11* and *prs-33* alone caused a similar moderate decrease of α -amylase (to about $10 \mu\text{g ml}^{-1}$ and $6 \mu\text{g ml}^{-1}$ respectively). However, their combination with the other *prs* mutations had very different effects. *prs-11* combined with most of the other *prs* mutants had hardly any additional effect. The only exception was *prs-33*: the level of α -amylase in the double mutant *prs-11 prs-33* was only $1.4 \mu\text{g ml}^{-1}$. On the other hand,

Fig. 3. Construction of recombinants with two *prs* mutations. The *his*-linked *prs-11* and the *pyrD-thyA*-linked *prs-33* were recombined with the other *prs* mutations as indicated. '*prs-n*' denotes the set of *prs* mutants as in Table 5. Tf, transformation; Td, transduction; the marker used for selection is indicated.

Table 5. Secretion of α -amylase by double *prs* mutants

The set of *prs* mutations were recombined with *prs-11* and *prs-33* to construct double mutants. All double mutants were made in strain IH6513 or its derivative (IH6531) as described in Fig. 3. α -Amylase is expressed as μg enzyme per ml of culture determined in the early stationary phase of growth.

First mutation	Second mutation . . .	None*	<i>prs-33</i> †	None‡	<i>prs-11</i> §
<i>prs-3</i>		0.1	0.03	0.3	0.3
<i>prs-29</i>		0.7	0.05	0.5	0.3
<i>prs-40</i>		1.1	0.2	1.1	0.9
<i>prs-26</i>		1.0	1.5	3.1	1.2
<i>prs-13</i>		0.9	1.5	2.4	1.1
<i>prs-33</i>		—	—	5.7	1.4
<i>prs-11</i>		9.8	1.4	—	—
None		—	6.0	—	10.0

* Derivatives of IH6531 (set B, Fig. 3).

† Derivatives of IH6531 (set A, Fig. 3).

‡ Derivatives of IH6513 (set C, Fig. 3).

§ Derivatives of IH6513 (set D, Fig. 3).

|| The host of this double mutant is IH6531.

prs-33 together with *prs-3*, *prs-29* or *prs-40* decreased the very low level of α -amylase of these mutants ($0.1\text{--}0.7\ \mu\text{g ml}^{-1}$) even further, down to 7–30% of that in the single mutant. However, it had no effect on *prs-13* and *prs-26*, the mutations with very tight linkage to *glyB*.

Because the mapping data indicated at least two separate groups of mutations linked to *glyB* (Table 4), we also combined mutations of both groups as described in Table 6. Determination of α -amylase of the double mutants (Table 6) showed two very different phenotypes. *prs-40 prs-26* produced slightly less α -amylase than each of the single mutants. No double mutants combining

Fig. 4. Growth rates of a set of *prs* mutants linked to *glyB* and two recombinants of them (see Table 6). ●, IH6521 (*prs-13*); ○, IH6522 (*prs-26*); ■, IH6527 (*prs-29*); □, IH6550 (*prs-29 prs-26*); ▲, IH6551 (*prs-29 prs-13*).

Table 6. Secretion of α -amylase by recombinants with two *glyB*-linked *prs* mutations

Construction of recombinants with two *glyB*-linked *prs* mutations was based on the presence of two groups of *prs* mutations with different degrees of cotransformational linkage to *glyB* (see Table 4). In the first step, strain IH6531 (a Gly⁻ derivative of strain IH6513) was transformed with DNA from *prs* mutants weakly linked to *glyB*. His⁺ transformants were selected and among them *prs-gly* recombinants were screened. These single mutants were then transformed with DNA from mutants tightly linked to *glyB* by selecting Gly⁺ transformants. These recombinants were screened on starch plates by looking for decreased α -amylase secretion. Secretion of α -amylase by double mutants, and the corresponding single mutants, was determined in liquid culture in the exponential phase (culture density 100 Klett units) and in the early stationary phase (6 h later).

Mutation	α -Amylase ($\mu\text{g ml}^{-1}$) in the culture supernatant	
	Exponential phase	Early stationary phase
<i>prs-13</i>	0.2	11.0
<i>prs-26</i>	0.1	11.0-29.0
<i>prs-29</i>	0.04-0.08	2.0-3.0
<i>prs-40</i>	ND	14.0
<i>prs-29 prs-13</i>	<0.01	<0.01
<i>prs-29 prs-26</i>	<0.01	<0.01
<i>prs-40 prs-26</i>	ND	5.0

ND, Not done.

prs-3 with *prs-26* were identified, suggesting that the secretion level of this combination did not differ from that of the respective single mutants. By contrast, the combination of *prs-29* with either *prs-26* or *prs-13* had dramatic effects on the phenotype. Firstly, the growth pattern differed from that of the wild-type in a manner not seen in any single *prs* mutants or any other combinations of two mutations (Fig. 4). In the exponential phase of growth the double mutants were identical to the single mutants, but they then abruptly stopped growing (Fig. 4); 6 h later, they resumed growth, so that the overnight densities were identical to that of the wild-type (not shown). Secondly, in the early stationary phase there was an approximately 1000-fold decrease of α -amylase secretion in these double mutants compared with each single mutant. This decrease was already clear by mid-exponential phase (100 Klett units) when the growth of the double mutants was still indistinguishable from that of the single mutants (Fig. 4).

Fig. 5. SDS-PAGE and immunoblotting with rabbit antiserum against α -amylase of *B. amyloliquefaciens*. Culture supernatant (S) and cell membrane fraction (M) of different *prs* mutants in early stationary phase (6 h after 100 Klett units) were analysed. (a) The amount of membrane fraction was from bacteria in 1 ml of culture; the amount of culture supernatant was 10 μ l in lanes A, F, H, K and N, and 5 μ l in lanes B, D, J and M. Staining was with 125 I-labelled protein A from *Staphylococcus aureus*. The arrow indicates exoamylase. (b) The amount of membrane fraction was from bacteria in 0.9 ml of culture in lanes F and I; in 0.3 ml of culture in lanes A, E and H; in 0.1 ml of culture in lanes B, D and G; in 0.03 ml of culture in lane C. The amount of culture supernatant was 5 μ l in lanes J, K and L. Other designations as in (a).

Immunoblots of the *prs* mutants

A block in the secretion pathway might cause cellular accumulation of the secretory proteins or their precursors. We therefore studied the possible accumulation of α -amylase in the *prs* mutants by SDS-PAGE followed by immunoblotting with anti- α -amylase serum. Fig. 5 shows that the anti- α -amylase serum recognized one protein of 55 kDa in the culture supernatant and two major proteins of 58 and 55 kDa in the cell membrane of an α -amylase-producing parent strain. None of these proteins was seen in preparations of IH6064, which does not produce α -amylase (not shown). The larger of the membrane-bound proteins is of the size of the *in vitro*

Fig. 6. Comparison of α -amylase in the membrane fractions of single and double *prs* mutants. Cultures were sampled in the early stationary phase (6 h after 100 Klett units) unless otherwise indicated. Membrane preparations (3 μ l, concentrated 100-fold) were electrophoresed in SDS-PAGE and immunoblotted with anti- α -amylase serum. Lane A, α -amylase of IH6527 (*prs-29*); lanes B and C, double mutant IH6550 (*prs-29 prs-26*), at 100 Klett units and 6 h later, respectively; lane D, IH6521 (*prs-26*); lane E, IH6522 (*prs-13*); lanes F and G, double mutant IH6551 (*prs-29 prs-13*) at 100 Klett units and 6 h later, respectively; lane H, IH6513 (wild-type); lane I, IH6525 (*prs-3*); lane J, IH6529 (*prs-33*); lanes K and L, double mutants IH6533 (*prs-33 prs-3*) and IH6534 (*prs-33 prs-29*). The arrow indicates the exoamylase-like protein.

precursor (Palva, 1982) of α -amylase, and is most probably the precursor form from which the signal peptide has not been cleaved. The 55 kDa membrane-bound protein has the same molecular mass as the purified exoamylase, and may represent exoamylase trapped in the cell membrane preparation or a degradation product of the 58 kDa precursor.

Four of the *prs* mutants (*prs-3*, *prs-29*, *prs-40* and *prs-11*) resembled the parent strain in containing α -amylase proteins of 58 and 55 kDa in the membrane preparations (pattern 1) (Figs 5 and 6; data for *prs-11* not shown). Membrane preparations of mutants *prs-33*, *prs-13* and *prs-26* contained only the 58 kDa precursor of α -amylase (pattern 2, Fig. 5).

In each case, the amount of α -amylase in the supernatant (visually estimated on the immunoblots) was much less than that in the supernatant of the parent, confirming the data of the enzyme assays (Table 2). The size of the α -amylase in the supernatant was 55 kDa in each case, indicating that processing had taken place.

We also tried to make quantitative estimates of the 58 kDa precursor from the immunoblots. Titration of membrane preparations derived from 0.3–0.03 ml of the culture of the wild-type showed that the detection limit of the 58 kDa band in the blot was between 0.3 and 0.1 ml of culture (Fig. 5b). In all mutants the 58 kDa protein was clearly visible in blots made from 0.3 ml of culture. When membrane preparations of mutants *prs-13* and *prs-26*, both of pattern 2, were titrated, the 58 kDa protein was still detectable in preparations from 0.1 ml of culture, suggesting a relative increase of the precursor in these mutants compared to the parent. In the double mutants combining mutations producing pattern 1 and mutations producing pattern 2, both the banding pattern and the amount of precursor were similar to pattern 2 (Fig. 6).

DISCUSSION

Screening for mutations with decreased secretion of α -amylase produced mutations in four chromosomal loci, designated *prs*. All these mutations were pleiotropic and decreased the secretion of several exoproteins. Thus they either affect the protein export machinery or the transcriptional regulation system of exoprotein genes (Shimotsu & Henner, 1986; Aymerich *et al.*, 1986; Yang *et al.*, 1987). We consider the first alternative to be more likely for the following reasons. Firstly, the decrease in the amount of exoamylase, to 2–15% of the parental level, was not paralleled by a decrease of the membrane-bound precursor. Secondly, in some of the

mutants, decreased exoprotein levels were seen only in the presence of the plasmid pKTH10 (Table 2), when the *amyE* gene is very highly expressed (Palva, 1982). Thus the pleiotropic effect of these *prs* mutations seems to require a relatively high overall level of protein secretion.

Impaired secretion of exoproteins might also affect the structure of the cell wall, which is itself synthesized by exported proteins. Indeed, some *prs* mutants had properties suggestive of such effects (Table 3). The *prs-29* mutant formed small colonies, but only in the presence of pKTH10 and hence a high level of α -amylase secretion. The *prs-13*, *prs-26* and *prs-33* mutations conferred non-transformability. The somewhat decreased ability of many *prs* mutants to sporulate may also be due to alterations of the cell wall as a consequence of the secretion defect. The *prs* mutations did not map in known *spo* loci. Only two of the secretion defective mutations (*prs-13* and *prs-26*, linked to *glyB*) mapped close to a sporulation locus, *scoC*, and the phenotype of *scoC* mutants (Milhaud *et al.*, 1978) is very different from that of the *prs* mutants.

The *prs* mutations mapped in four distinct chromosomal loci and *prs* mutations in each of the four loci caused a characteristic secretion phenotype, including two patterns of accumulation of α -amylase precursor (Fig. 5) and distinctive effects on the release of lipopenicillinase (Table 2). Some of the mutations also had clearly additive effects. The combination of *prs-29* with *prs-13* or *prs-26* decreased the amount of secreted α -amylase about 1000-fold compared to each mutation alone. *prs-33* combined with *prs-3*, *prs-29* or *prs-40*, all linked to *glyB*, also decreased the level of secretion below that of the single *prs* mutants. These findings suggest that the four types of *prs* mutations affect different components of the secretion machinery. Furthermore, the *prs-11* mutation was hypostatic to both groups of *prs* mutations near *glyB* and its effect on the release of penicillinase was unique and very unexpected. This might indicate that some components of the secretion mechanism are specific for certain types of proteins.

Mutant genes (*sacU*, *sacQ*, *hpr*, *pri*) that have pleiotropic effects on the expression of exoproteins have recently been described in *B. subtilis*. They all increase initiation of transcription of several exoproteins at the promoter level (Shimotsu & Henner, 1986; Aymerich *et al.*, 1986; Henner *et al.*, 1988; Yang *et al.*, 1987). The *prs-11* and *prs-33* mutations mapped at loci far removed from these genes. Only the *prs* genes linked to *glyB* map close to the *hpr* gene (Higerd *et al.*, 1972). Our mapping data do not allow conclusions of possible identity of *hpr* with the *glyB*-linked *prs* genes. However, the phenotype of the *prs* mutants, especially the accumulation of α -amylase precursor, the pleiotropy only in the presence of high α -amylase production, and the presence of two *prs* loci near *glyB*, are not compatible with previously described *hpr* mutations.

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