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Use of the Pre-Pro Part of *Staphylococcus hyicus* Lipase as a Carrier for Secretion of *Escherichia coli* Outer Membrane Protein A (OmpA) Prevents Proteolytic Degradation of OmpA by Cell-Associated Protease(s) in Two Different Gram-Positive Bacteria

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Heterologous protein secretion was studied in the gram-positive bacteria *Bacillus subtilis* and *Staphylococcus carnosus* by using the *Escherichia coli* outer membrane protein OmpA as a model protein. The OmpA protein was found to be translocated across the plasma membrane of both microorganisms. However, the majority of the translocated OmpA was similarly degraded in *B. subtilis* and *S. carnosus* despite the fact that the latter organism does not secrete soluble exoproteases into the culture medium. The finding that purified OmpA, which was added externally to the culture medium of growing *S. carnosus* cells, remained intact indicates that newly synthesized and exported OmpA is degraded by one or more cell-associated proteases rather than by a soluble exoprotease. Fusion of the mature part of OmpA to the pre-pro part of a lipase from *Staphylococcus hyicus* allowed the efficient release of the corresponding propeptide-OmpA hybrid protein into the supernatant and completely prevented the cell-associated proteolytic degradation of the mature OmpA, most likely reflecting an important function of the propeptide during secretion of its natural mature lipase moiety. The relevance of our findings for the biotechnological use of gram-positive bacteria as host organisms for the secretory production of heterologous proteins is discussed.

Many pathogenic bacteria belong to the class of gram-negative bacteria. Components of the cell envelope (e.g., outer membrane proteins [Omps]) of these bacteria are involved in the initial contact between the host and the invading pathogen. In the last few years, great efforts have been made to investigate the potential use of Omps as vaccines against the corresponding microorganisms (9, 26, 46). However, the isolation of sufficient quantities of Omps in pure form from their natural sources is often difficult and time-consuming. For example, the complete removal of endotoxin (lipopolysaccharide [LPS]) from Omps is difficult to achieve, since most Omps have a very high affinity for LPS in the outer membrane (20, 37, 50). Therefore, the use of gram-positive bacteria which, due to the lack of an outer membrane, do not contain LPS as host bacteria might be an attractive alternative for the production of Omps in LPS-free form.

Gram-positive bacteria (e.g., *Bacillus* species) are widely used for the industrial production of technical enzymes which naturally occur in these organisms and which are secreted into the supernatant in very large amounts (4, 6). However, the use of these bacteria as hosts for the secretory production of heterologous proteins has often encountered problems caused by the total lack of translocation or inefficient translocation of the foreign proteins across the plasma membrane (30, 34) or by inefficient release of the exported proteins from the cell surface into the surrounding medium (21, 33). In addition, proteolysis of the secreted heterologous proteins by extracellular proteases was found to be a major problem which can dramatically decrease the yield of the desired product (11, 24, 49). Therefore, a detailed investigation of the mechanism of protein secretion in gram-positive bacteria might lead to strategies permitting the improvement of these bacteria as biological production systems for heterologous proteins by genetic engineering.

With *Escherichia coli* OmpA used as a heterologous model protein, it has previously been shown that, in principle, the authentic OmpA protein can be translocated across the plasma membrane of *Bacillus subtilis* (22). Despite this fact, no soluble, full-length, mature OmpA could be detected in the culture supernatant. In contrast, full-length OmpA was found in the cellular fraction, which most likely was due to aggregation at the membrane surface or trapping in the cell wall. In addition, large amounts of degradation products of lower molecular weights can be detected in the supernatant. In this study, the *E. coli* OmpA protein was expressed in the gram-positive bacterium *Staphylococcus carnosus*, a species which is almost devoid of soluble extracellular proteases (47). Despite this fact, we found that exported OmpA undergoes a degradation in *S. carnosus* similar to that in *B. subtilis*. The results presented in this study strongly suggest that degradation of OmpA in *B. subtilis* and *S. carnosus* is caused by a membrane- or cell wall-associated protease. In addition, we have demonstrated that use of the pre-pro part of a lipase from *Staphylococcus hyicus* as a secretion vector for the mature OmpA protein resulted in the release of a soluble propeptide-OmpA hybrid protein into the supernatant. Furthermore, the presence of the propeptide completely prevented the degradation of the mature OmpA by the above-mentioned protease.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* JM109 (51) was grown at 37°C in L broth (23) containing 100 μg of ampicillin per ml, 0.5% (wt/vol) glucose, or

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1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) as required. *B. subtilis* DB104 (*his nprR2 nprE18* Δ*aprA3*) (14) and *S. carnosus* TM300 (35) were grown at 37°C in L broth supplemented with 15 μg of chloramphenicol per ml, 0.5% (wt/vol) glucose, 0.5% (wt/vol) xylose, or 1 mM IPTG, as required. For the inhibition of extracellular proteases, a complex mixture of protease inhibitors (Complete; Boehringer GmbH, Mannheim, Germany) effective for a broad spectrum of serine, cysteine, and metalloproteases was added to the culture medium according to the instructions of the manufacturer, as required.

Plasmid constructions. The *S. carnosus* expression vector pPS2 was constructed by replacing the *S. hyicus* pre-pro-lipase structural gene, which is located on a *Pst*I DNA fragment in pEF0 (22), with a synthetic multiple cloning site, thereby facilitating the cloning of foreign genes under the control of the lipase promoter.

To obtain a promoterless *ompA* gene, a *Pst*I linker was introduced into the *Eco*RI site located immediately upstream of the *ompA* structural gene in plasmid pRD87 (7). From the resulting plasmid, pRD88, a 1.3-kb *Pst*I fragment containing *ompA* was isolated and ligated into pUC18 (51), resulting in pOA181. From pOA181, a 1.35-kb *Sac*I/*Hin*dIII fragment was isolated and cloned into *Sac*I/*Hin*dIII-digested pPS2, resulting in plasmid pJM100, in which the *ompA* gene is now under the control of the *S. hyicus* lipase promoter.

To replace the ribosome-binding site (RBS) of *ompA* by the RBS of the *S. hyicus* lipase, a *Swa*I site was introduced into pJM1 (22) between the RBS and the ATG start codon of the lipase gene by site-directed mutagenesis, resulting in plasmid pJM1-87. To obtain an *ompA* gene without RBS, a PCR was performed with pOA181 as a template. The primers used were oligonucleotide K24 (5′-G GGATTTAAATGAAAAAGACAGCT-3′), which introduces a *Swa*I site directly upstream of the *ompA* ATG start codon, and the pUC universal sequencing primer. The resulting PCR fragment was digested with *Swa*I and *Hin*dIII and was used to replace the 2-kb *Swa*I/*Hin*dIII fragment containing the lipase structural gene in plasmid pJM1-87. From the resulting plasmid, pOA182, the *ompA* structural gene including the RBS of the lipase was isolated as a 1.2-kb *Sac*I/*Hin*dIII fragment and cloned into *Sac*I/*Hin*dIII-digested pPS2, resulting in plasmid pJM105.

For inducible expression of the *ompA* gene in *S. carnosus*, the *S. hyicus* pre-pro-lipase structural gene in *S. carnosus* expression vector pCX15 (48), which is located on a 2.0-kb *Bam*HI/*Pvu*II fragment, was replaced by a synthetic multiple cloning site, resulting in plasmid pXR1. An additional *Hin*dIII site, located outside the multiple cloning site in pXR1, was removed by filling in of this site with Klenow polymerase and subsequent religation, resulting in plasmid pXR2. The *ompA* structural gene was isolated from pOA181 as a 1.3-kb *Xba*I/*Hin*dIII fragment and ligated into *Xba*I/*Hin*dIII-digested pXR2. The resulting plasmid, pJM110, contained the *ompA* gene under the xylose-inducible control of the *xylA* promoter/operator of *Staphylococcus xylosus* (48).

To allow the construction of precise fusions between the pre-pro part of *S. hyicus* lipase and the mature part of *E. coli* OmpA, a *Sna*BI site was introduced by site-directed mutagenesis into the pre-pro-lipase structural gene in plasmid pJM1 at the position which corresponds to the junction between the propeptide and mature lipase, resulting in plasmid pJM1-23. An *Eco*47-3 site was introduced into the *ompA* gene in plasmid pOA181 by site-directed mutagenesis at the position corresponding to the junction between the signal peptide and mature OmpA. A DNA fragment encoding the mature OmpA protein was isolated from the resulting plasmid, pOA181-K8, by cleavage with *Eco*47-3/*Hin*dIII and ligated into pJM1-23, from which a *Sna*BI/*Hin*dIII fragment encoding mature lipase had been deleted. From the resulting plasmid, pJM3-ompA, a 1.9-kb *Sac*I/*Hin*dIII fragment was isolated and ligated into *Sac*I/*Hin*dIII-digested *S. carnosus* expression vector pPS2, resulting in plasmid pJM30. pJM120, allowing IPTG-inducible expression of the gene encoding the pre-pro(lipase)-OmpA hybrid protein in *B. subtilis*, was obtained by cloning a corresponding 1.9-kb *Pst*I fragment from pJM3-ompA into the *Pst*I-digested expression vector pEF1 (22). The construction of pJM20 has been described elsewhere (22).

All DNA techniques followed standard procedures (31), and the final constructs were verified by DNA sequencing (32). Site-directed mutagenesis and PCRs were performed as described elsewhere (15).

Other methods. Purification of OmpA was done essentially as described by Surrey and Jähnig (45), with the exception that the final anion-exchange chromatography step was omitted. The OmpA concentration in the resulting preparation, which also contained some pre-OmpA protein, was 62 μg/ml.

To test for degradation of purified OmpA by secreted exoproteases of *S. carnosus* TM300, 25 μl of the OmpA stock solution was added to 5 ml of Luria-Bertani medium, freshly inoculated with the corresponding bacteria, and the culture was shaken for 14 h at 37°C. To keep the OmpA protein soluble during this incubation, urea (40 mM final concentration) was included in the growth medium. Protease treatment of *S. carnosus* protoplasts was essentially performed according to the protocol of Puohiniemi et al. (30), except that lysostaphin was used instead of lysozyme for protoplast formation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting) using prolipase- or OmpA-specific antibodies were done as described elsewhere (25). Cellular and supernatant fractions corresponding to an equivalent amount of cells (measured by the optical density at 600 nm) were loaded in each lane of the gels shown below (see Fig. 2 to 5) and electrophoresed.

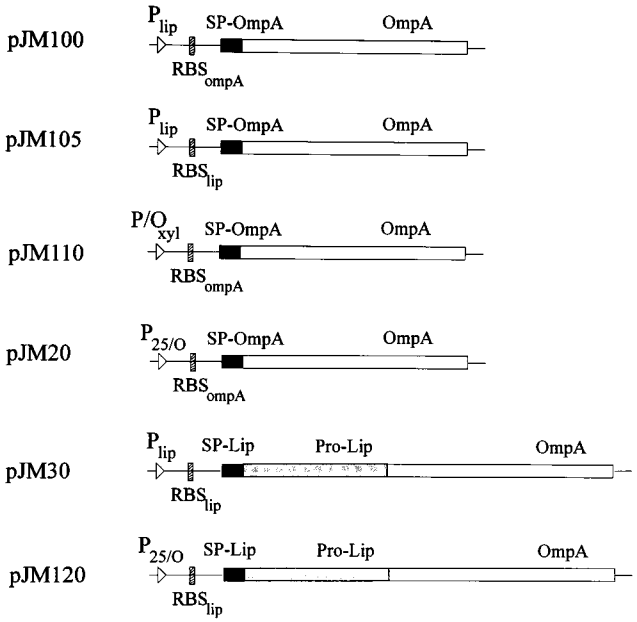


FIG. 1. Schematic structure of the proteins encoded by the indicated plasmids. Black bars, signal peptide derived from pre-OmpA (SP-OmpA) or pre-pro-lipase (SP-Lip); gray bars, propeptide of pre-pro-lipase; open bars, mature region of pre-OmpA; hatched bars, RBS of the *ompA* or lipase gene. The respective promoter and operator regions are indicated (arrowheads): P_{lip}, lipase promoter; $P_{25/O}$, bacteriophage T5 promoter P_{N25}/*lac* operator; and P/O_{xyl}, *xylA* promoter/operator.

RESULTS

Expression of the authentic *E. coli* pre-OmpA protein in *B. subtilis* and *S. carnosus*. The *E. coli* OmpA protein is synthesized as a precursor protein (pre-OmpA) possessing a 21-amino-acid residue signal peptide which is removed by signal peptidase during or shortly after translocation across the plasma membrane (8). It has previously been shown that the pre-OmpA protein is recognized by the *B. subtilis* protein secretion machinery and that the resulting mature protein is translocated across the plasma membrane (22). As described in that report, no intact mature OmpA protein is found in the supernatant of *B. subtilis* DB104 expressing the *ompA* gene from plasmid pJM20 (Fig. 1) (22) when analyzed in Western blots using anti-OmpA antibodies. In contrast, large amounts of degradation products of 16 and 18 kDa can be detected in the supernatant fraction (Fig. 2, lane 11). Although the genes encoding two major soluble extracellular proteases have been deleted in *B. subtilis* DB104 (14), this strain still possesses significant proteolytic activity in the supernatant which is due to at least four additionally secreted exoproteases (49). Therefore, it seems a reasonable possibility that one or several of these exoproteases are responsible for the observed proteolytic breakdown of the mature OmpA protein. In the cellular fraction, two major polypeptides whose sizes correspond to the pre-OmpA protein (38 kDa) and the mature OmpA (36 kDa) can be detected (Fig. 2, lane 10). It has previously been shown that this mature OmpA protein is completely translocated across the plasma membrane and is located at the cell surface, most likely in an aggregated state or trapped in the cell wall (22).

Since, in contrast to *B. subtilis*, *S. carnosus* does not secrete significant amounts of proteases into the supernatant (47), we questioned whether the massive degradation of OmpA which

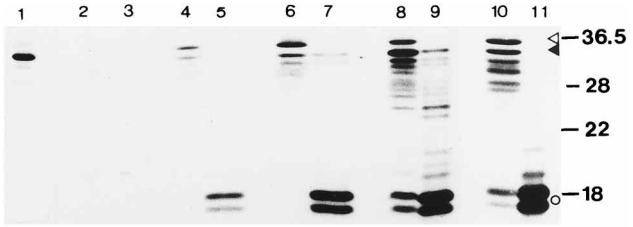
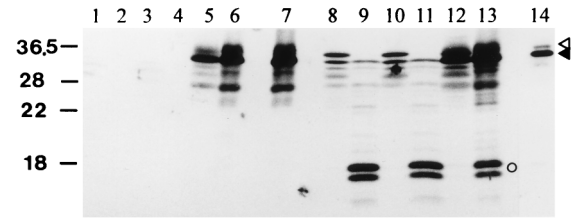


FIG. 2. Identification of *ompA* gene products in *S. carnosus* and *B. subtilis*. Cultures of *S. carnosus* TM300 containing plasmid pPS2 (lanes 2 and 3), pJM100 (lanes 4 and 5), or pJM105 (lanes 6 and 7) were grown in L broth for 16 h at 37°C. *S. carnosus* harboring pJM110 (lanes 8 and 9) was pregrown in L broth containing 0.5% glucose for 16 h. The cells were washed twice, resuspended in fresh medium containing 0.5% xylose, and incubated for 3 h at 37°C. *B. subtilis* DB104 containing plasmid pJM20 (lanes 10 and 11) was treated in the same way except that 1 mM IPTG was used for induction of *ompA* gene expression. Cellular (lanes 2, 4, 6, 8, and 10) and supernatant (lanes 3, 5, 7, 9, and 11) fractions were subjected to SDS-PAGE and immunoblotting using anti-OmpA antibodies. Mature OmpA produced by *E. coli* JM109 (lane 1) is also shown. Open arrowhead, pre-OmpA; solid arrowhead, mature OmpA; open circle, 16- and 18-kDa OmpA degradation products. The positions of molecular mass markers (in kilodaltons) are indicated on the right.



	C	S	C	S	C	S	Control	C	S	C	S	C	S
Urea	-		+		-		-	-		+		-	
OmpA in Urea	-		-		+		+	-		-		+	

FIG. 3. Stability of purified OmpA added to the growth medium of *S. carnosus*. Cells of *S. carnosus* TM300 containing plasmid pPS2 (lanes 1 to 6) or pJM105 (lanes 8 to 13) were grown for 14 h at 37°C in L broth to which purified urea-denatured OmpA protein (final concentrations, 0.3 μg of OmpA per ml and 40 mM urea) or urea alone (final concentration, 40 mM) had been added as indicated (+). As a control, purified OmpA was incubated for 14 h in L broth without cells (lane 7). Cellular (C lanes) and supernatant (S lanes) fractions were subjected to SDS-PAGE and immunoblotting using anti-OmpA antibodies. Lane 14, OmpA polypeptides produced by *E. coli* JM109 containing plasmid pRD87 (7). Open arrowhead, pre-OmpA; solid arrowhead, mature OmpA protein; open circle, 16- and 18-kDa OmpA degradation products. The positions of molecular mass markers (in kilodaltons) are indicated on the left.

is observed in *B. subtilis* could be avoided by using this species as a host. The promoterless *ompA* gene including its authentic RBS was cloned into *S. carnosus* expression vector pPS2 (this study) under the control of the promoter of a lipase from *S. hyicus* (18). The resulting plasmid, pJM100 (Fig. 1), allowed constitutive expression of the *ompA* gene in *S. carnosus*. To achieve different levels of *ompA* expression, two additional plasmids were constructed. In pJM105 (Fig. 1), the authentic *ompA* RBS was replaced by the RBS of the *S. hyicus* lipase, which is somewhat more efficient in directing protein synthesis in *S. carnosus* (21a). Plasmid pJM110 (Fig. 1) contains the promoterless *ompA* gene cloned in expression vector pCX15 (48) under the regulatory control of the *xylA* promoter/operator of *S. xylosus*, allowing xylose-inducible high-level expression of *ompA* in *S. carnosus*. The level of *ompA* expression is lowest from plasmid pJM100 and highest from fully induced pJM110, whereas an intermediate level is obtained with plasmid pJM105 (Fig. 2).

pJM100, pJM105, and pJM110 were used to transform *S. carnosus* TM300, and the cellular and supernatant fractions of the corresponding transformants were analyzed by SDS-PAGE and Western blotting using anti-OmpA antibodies. Similar to the situation in *B. subtilis*, two polypeptides (38 and 36 kDa), which correspond in size to the pre-OmpA and mature OmpA proteins, respectively, are detected in the cellular fractions of all *S. carnosus* strains expressing the *ompA* gene (Fig. 2, lanes 4, 6, and 8). Also in parallel to *B. subtilis*, digestion of protoplasts of the corresponding *S. carnosus* cells with trypsin revealed that the mature OmpA polypeptide is completely degraded by the added protease, demonstrating that the OmpA is indeed translocated across the plasma membrane (data not shown). In the *S. carnosus* supernatant fractions (Fig. 2, lanes 5, 7, and 9), two major polypeptides of 16 and 18 kDa, identical in size to the OmpA degradation products which are detected upon *ompA* expression in *B. subtilis*, were found (Fig. 2, lane 11). In addition, small amounts of soluble, full-length, mature OmpA protein which are not observed in *B. subtilis*(pJM20) can be detected in the supernatant fractions of *S. carnosus*. The amount of mature OmpA in the supernatant fraction increases with increasing amounts of total synthesized OmpA proteins, but in all *S. carnosus* strains, this still represents only a minor fraction of the soluble OmpA species. From these results, we conclude that the use of *S. carnosus* as a host for the secretory production of OmpA does not prevent the massive proteolytic degradation of exported mature OmpA protein, despite the fact that this species is known to be exoprotease deficient.

A cell-associated protease is responsible for the degradation of the translocated OmpA protein in *S. carnosus*. As shown above, the majority of the translocated OmpA protein was also found to be degraded in the exoprotease-deficient bacterium *S. carnosus*. Therefore, we considered the possibilities that degradation of OmpA might be caused by a cell-associated protease, located at the outer surface of the plasma membrane or in the cell wall area, or, alternatively, by a minor proteolytic activity in the culture supernatant of *S. carnosus*. To distinguish between these possibilities, purified OmpA protein was added to the freshly inoculated growth medium of *S. carnosus* cells harboring control vector pPS2 or plasmid pJM105 (containing the *ompA* gene), and the cultures were grown to stationary phase for 14 h at 37°C (Fig. 3). To keep the OmpA protein soluble during this incubation, 40 mM urea (final concentration) was included in the growth medium, the addition of which had no adverse effects on the growth of *S. carnosus* cells or *ompA* expression (Fig. 3, compare lanes 8 and 9 with lanes 10 and 11). As a further control, purified OmpA was incubated in L broth without cells (Fig. 3, lane 7). Addition of small amounts of proteinase K (1 μg/ml) to the purified OmpA protein in L broth resulted in rapid digestion of the OmpA (data not shown). Also, addition of the purified OmpA to the culture supernatant of *B. subtilis* DB104 grown to the stationary phase, in which this strain is known to secrete different proteases (14, 49), resulted in complete degradation of the protein (i.e., without any detectable leftover degradation products) within 30 min, demonstrating that the purified OmpA is a substrate sensitive to proteases per se (data not shown). Figure 3 clearly shows that the purified OmpA protein which was added to the supernatants of *S. carnosus* containing pPS2 or pJM105 was completely stable throughout the growth of the cultures (compare lane 7 with lanes 6 and 13). Although the majority of the OmpA protein remained soluble, some of the externally added OmpA was found in the cellular fraction (Fig. 3, lanes 5 and 12), which might be due to aggregation at the cell surface or formation of aggregates in the supernatant which would cofractionate with the cellular fraction upon centrifuga-

tion. In pJM105-containing cells, OmpA degradation products of 16 and 18 kDa were found in the supernatant fraction, the amounts of which were the same irrespective of whether purified OmpA protein had been added to the culture supernatant (Fig. 3, compare lanes 9 and 11 with lane 13). This result clearly demonstrates that the 16- and 18-kDa OmpA degradation products are derived solely from proteolysis of newly synthesized and exported OmpA and not from degradation of the added purified OmpA. From these data, we conclude that degradation of in vivo-synthesized and translocated OmpA protein in *S. carnosus* is caused not by a soluble extracellular protease but rather by a protease located at the outer surface of the plasma membrane or in the cell wall. Furthermore, our data confirm previous findings which indicated that *S. carnosus* does not secrete significant amounts of soluble exoproteases (27, 36, 47).

Use of the pre-pro part of an *S. hyicus* lipase as a secretion carrier prevents the degradation of newly translocated *E. coli* OmpA in *S. carnosus* and *B. subtilis* by cell-associated proteases. In addition to the signal peptide (or prepeptide), which is required for the initiation of protein export (28, 41), many precursors of exoenzymes of gram-positive bacteria contain a so-called propeptide which, in some cases, is thought to play an important role in the folding and release of the respective mature protein from the plasma membrane after translocation (38, 41). To test whether the presence of such a propeptide would improve the secretion of an Omp by *S. carnosus*, the mature part of OmpA was precisely fused to the pre-pro part (38 and 207 amino acid residues, respectively) of a lipase from *S. hyicus* (10). When pre-pro-lipase is expressed in *S. carnosus*, only the prepeptide is processed since the extracellular metalloprotease, which is responsible for the removal of the propeptide in *S. hyicus*, is lacking in *S. carnosus* (1, 47). Previously, it has been shown that the propeptide of the lipase, although not absolutely required for the formation of an active enzyme, is necessary for efficient secretion and proteolytic stability of the lipase in *S. carnosus* (5).

The gene encoding the hybrid protein consisting of the mature OmpA protein fused to the pre-pro part of the *S. hyicus* lipase was cloned into *S. carnosus* expression vector pPS2 under the control of the endogenous lipase promoter, and the resulting plasmid (pJM30; Fig. 1) was used to transform *S. carnosus* TM300. When the cellular and supernatant fractions of the corresponding strain were analyzed by Western blotting, a polypeptide of 78 kDa which reacted with lipase-specific as well as OmpA-specific antibodies (Fig. 4A) and most likely represents the processed form of the pre-pro precursor protein was found in the culture supernatant. Typically, concentrations between 20 and 25 mg/liter were determined for the secreted propeptide-OmpA hybrid protein in the culture supernatant of *S. carnosus*(pJM30) grown to stationary phase (data not shown). It should be noted that it has been previously observed that the presence of the lipase propeptide results in an aberrant (i.e., lower) mobility in SDS-PAGE of the corresponding proprotein compared with that expected from its calculated molecular weight (18). In contrast to *S. carnosus* expressing the authentic *ompA* gene (Fig. 2), no degradation products were found in the supernatant of pJM30-containing cells. In the cellular fraction, two polypeptides of 78 and 82 kDa, which most likely correspond to the unprocessed pre-pro-protein and the processed proprotein can be detected (Fig. 4A, lanes 2 and 4). To determine the localization of these cell-bound polypeptides, protoplasts of *S. carnosus* synthesizing the hybrid protein were treated with trypsin (Fig. 4B). Already during the preparation of the protoplasts, a considerable amount of the 78-kDa polypeptide was lost from the cellular fraction (Fig. 4B, compare lanes 1 and 2), indicating that this polypeptide represents part of the translocated proprotein which is loosely associated with the cells. The remaining 78-kDa polypeptide was completely digested by trypsin (Fig. 4B, lane 3), showing that it had indeed crossed the cytoplasmic membrane. As expected, the 82-kDa polypeptide was totally resistant to trypsin treatment in protoplasts (Fig. 4B, lane 3), indicating that this protein represents the unprocessed precursor of the hybrid protein which has accumulated in the cytosol. Upon disruption of the protoplasts by sonication, the 82-kDa polypeptide was completely digested by trypsin (Fig. 4B, lane 4).

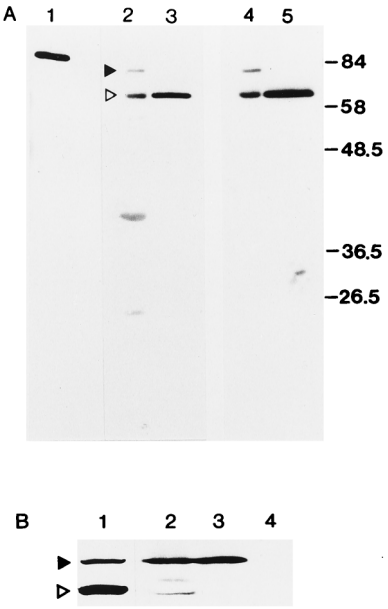


FIG. 4. (A) Identification of pre-pro(lipase)-*ompA* gene products in *S. carnosus*. *S. carnosus* TM300 containing plasmid pJM30 was grown for 5 h in L broth and the cellular (lanes 2 and 4) and supernatant (lanes 3 and 5) fractions were analyzed by SDS-PAGE and immunoblotting using anti-lipase (lanes 1 to 3) or anti-OmpA (lanes 4 and 5) antibodies. Lane 1, prolipase secreted by *S. carnosus* containing plasmid pLipPS1 (18). The positions of molecular mass markers (in kilodaltons) are indicated on the right. (B) Trypsin treatment of *S. carnosus* protoplasts. *S. carnosus* containing pJM30 was grown in L broth for 12 h. Lane 1, cellular fraction of *S. carnosus*(pJM30) before protoplast formation; lanes 2 to 4, protoplasts of *S. carnosus*(pJM30) after no treatment, trypsin treatment of intact protoplasts, and trypsin treatment after sonication, respectively. Solid arrowheads, pre-pro(lipase)-OmpA protein; open arrowheads, pro(lipase)-OmpA protein.

Next, we compared secretion of the authentic pre-OmpA protein with secretion of the pre-pro(lipase)-OmpA hybrid protein in *B. subtilis* DB104, which, in contrast to *S. carnosus*, secretes significant amounts of extracellular proteases, in the absence or presence of a mix of protease inhibitors (Complete; Boehringer). For these studies, plasmids pJM20 (harboring the authentic *ompA* gene; Fig. 1) (22) and pJM120 [harboring the gene encoding the pre-pro(lipase)-OmpA hybrid protein; Fig. 1] were used, both allowing IPTG-inducible expression in *B. subtilis* of the corresponding genes. As shown in Fig. 5B, in the absence of Complete, a 78-kDa polypeptide, which corresponds to the processed and translocated propeptide-OmpA hybrid protein, was detected in the supernatant of *B. subtilis*-(pJM120) 1 h after induction of gene expression (lane 2), and this polypeptide was completely degraded upon prolonged incubation (Fig. 5B, lanes 2, 4, 6, 8, 10, and 12), which is very likely caused by the increased secretion of soluble exoproteases by *B. subtilis* upon transition from exponential growth to sta-

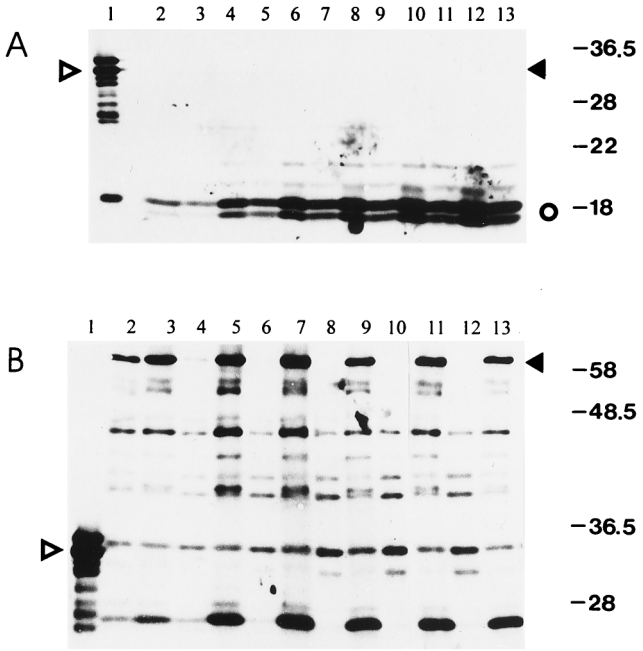


FIG. 5. Secretion of authentic OmpA (A) and pro(lipase)-OmpA (B) polypeptides in *B. subtilis* in the presence or absence of protease inhibitors. Cells of *B. subtilis* DB104 containing plasmid pJM20 (A) or pJM120 (B) were pregrown in L broth containing 0.5% glucose for 16 h at 37°C. The cells were washed, resuspended in 2 volumes of fresh medium containing 1 mM IPTG, and grown at 37°C in the absence (lanes 2, 4, 6, 8, 10, and 12) or presence (lanes 3, 5, 7, 9, 11, and 13) of a mixture of protease inhibitors (Complete; Boehringer). Supernatant fractions were analyzed by SDS-PAGE and immunoblotting using anti-OmpA antibodies after 1 (lanes 2 and 3), 2 (lanes 4 and 5), 3 (lanes 6 and 7), 4 (lanes 8 and 9), 5 (lanes 10 and 11), and 6 (lanes 12 and 13) h of cultivation. Lanes 1, OmpA polypeptides of *E. coli* JM109 containing plasmid pRD87 (7). Open arrowheads, mature OmpA protein; solid arrowhead, expected position of secreted authentic OmpA (A) or pro(lipase)-OmpA (B) polypeptides; open circle, 16- and 18-kDa OmpA degradation products. The positions of molecular mass markers (in kilodaltons) are indicated on the right.

tionary growth phase (43). In the presence of Complete, this degradation was significantly suppressed (Fig. 5B, lanes 3, 5, 7, 9, 11, and 13). In contrast, despite the presence of Complete, no full-length mature OmpA protein could be detected in the supernatant of *B. subtilis*(pJM20), expressing the authentic *ompA* gene. Instead, only degradation products, 16 and 18 kDa in size, were found in large amounts in the supernatant fraction, irrespective of whether Complete had been added to the culture medium (Fig. 5A, lanes 3, 5, 7, 9, 11, and 13) or not (Fig. 5A, lanes 2, 4, 6, 8, 10, and 12).

From these results, we conclude that degradation of newly translocated OmpA protein, also in *B. subtilis*, is caused mainly by one or more cell-associated proteases which cannot be inhibited by the protease inhibitors included in Complete. In addition, fusion of the mature OmpA protein to the pre-pro part of the *S. hyicus* lipase significantly inhibits this cell-associated proteolytic breakdown and allows efficient release of the corresponding pro(lipase)-OmpA hybrid protein into the supernatant, probably reflecting one of the functions of the propeptide during secretion of its natural mature (lipase) moiety.

DISCUSSION

Expression of the authentic pre-OmpA precursor protein in *B. subtilis* and *S. carnosus* resulted in the translocation of mature OmpA across the plasma membrane. In both organisms, part of the translocated OmpA was found associated with the cellular fraction. Whereas in *B. subtilis* no full-length mature OmpA could be detected in the culture medium, small amounts of intact mature OmpA were found in the supernatant when *S. carnosus* was used as a host. However, large amounts of degradation products, 16 and 18 kDa in size, were detected in the supernatants of both organisms. The latter finding was somewhat surprising, since *S. carnosus* is known to secrete very little, if any, soluble exoprotease into the surrounding medium (47). The finding that purified OmpA protein, although intrinsically very sensitive to proteolysis, remained completely undigested when added externally to growing cells of *S. carnosus* strongly suggests that newly synthesized and exported OmpA is degraded by one or more cell-associated proteases during or shortly after membrane translocation rather than by a secreted soluble exoprotease. In *E. coli*, Omps are sorted into the outer membrane by a still-unknown mechanism. Recent results indicate that periplasmic proteins such as Skp (3) or SurA (17) might guide Omps along their way through the periplasm to the outer membrane. Since these Omp-specific periplasmic proteins and an outer membrane are lacking in gram-positive bacteria, it is very likely that Omps which have been translocated across the plasma membrane of these microorganisms exist in an unfolded or misfolded state. Such a state is very prone to aggregation and/or proteolytic degradation, and, in fact, both of these phenomena have been observed for the exported OmpA protein in *B. subtilis* and *S. carnosus.*

The nature of the cell-associated protease that is responsible for the degradation of exported OmpA is unknown so far. The mixture of protease inhibitors included in Complete, according to the specifications of the manufacturer, Boehringer, is effective in inhibiting the activities of a broad spectrum of serine, cysteine, and metalloproteases. Since Complete does not prevent the cell-associated degradation of OmpA in *S. carnosus* (14a) or in *B. subtilis* DB104 (but significantly inhibits the soluble exoproteases), our results might suggest that the protease(s) in question belongs to none of these classes of proteases. In *E. coli*, the periplasmic space contains the DegP protease, which is involved in the degradation of unfolded or misfolded proteins (19, 42). It is tempting to speculate that gram-positive bacteria might contain a cell-associated protease which performs a function similar to that of DegP, i.e., the selective degradation of unfolded or misfolded proteins trapped in the cell wall. Recent results obtained with a SubC-PhoA fusion protein in a *B. subtilis prsA* mutant strain (13) might support this hypothesis. In *B. subtilis*, the extracellular lipoprotein PrsA is involved in the proper folding and release of secretory proteins from the plasma membrane into the supernatant (16). Synthesis of a SubC-PhoA hybrid protein in a *prsA* mutant strain resulted in misfolding of the translocated protein and in its rapid degradation. Also in this case, the investigators speculated that proteolysis of incorrectly folded SupC-PhoA hybrid protein might be caused by an extracellular cell-associated proteolytic activity (13).

When the mature OmpA protein was precisely fused to the pre-pro part of the lipase from *S. hyicus*, a major part of the translocated propeptide-OmpA hybrid protein was released into the supernatants of *B. subtilis* and *S. carnosus*. In *B. subtilis* DB104, the propeptide-OmpA hybrid protein was rapidly degraded by soluble exoproteases unless these proteases were inhibited by a mixture of protease inhibitors. In contrast, the secreted hybrid protein was found to be very stable in *S. carnosus*, even in the absence of protease inhibitors. Furthermore, the complete absence of the 16- and 18-kDa OmpA degradation products demonstrates that the presence of the propep-

tide effectively prevented the cell-associated proteolysis which was observed when OmpA was secreted without the lipase propeptide. Previously, it has been shown that the propeptide of the *S. hyicus* lipase is required for efficient secretion of active prolipase by *S. carnosus*. In contrast to the situation in *S. hyicus*, where the propeptide is cleaved off from the mature lipase by an extracellular metalloprotease (1), the secreted prolipase is not further processed in *S. carnosus* due to the absence of the respective proteolytic enzyme (47). Deletions of parts of the propeptide or removal of the entire propeptide in pre-pro-lipase were found to affect lipase secretion, stability, and activity (5). However, in contrast to the propeptides of subtilisin E from *B. subtilis* (12), α-lytic protease from *Lysobacter enzymogenes* (40), or elastase from *Pseudomonas aeruginosa* (2), the lipase propeptide does not seem to be absolutely necessary for the correct folding of the mature enzyme in vivo, since deletion of the entire propeptide still allowed formation of biologically active lipase in *S. carnosus*, albeit in small amounts (5). In addition, with either the authentic pre-pro-lipase or pre-propeptide (lipase)–β-lactamase hybrid proteins it has been shown that deletion of major parts of the propeptide resulted in reduced secretion and proteolytic instability of the corresponding proteins in *S. carnosus* (5, 18). It has been speculated that degradation of the respective proteins might occur intracellularly or at the cell surface after their membrane translocation. The results described in this paper clearly favor the latter possibility. We can only speculate on the mechanism by which the propeptide prevents the cell-associated degradation of the attached homologous and heterologous mature proteins. Since the propeptide not only prevents the mature lipase from being degraded but also is effective in protecting various heterologous proteins from proteolysis, i.e., OmpA (this study), β-lactamase (18), or different antibody fragments (27, 36), we think it very unlikely that a direct folding interaction between the propeptide and the corresponding passenger protein, analogous to the one described for the subtilisin propeptide and mature subtilisin (39), is responsible for the observed protective effect. One possibility would be that the propeptide might directly inhibit the cell-associated protease. If this were the case, the expression of the propeptide in *trans* or the addition of purified propeptide to the culture supernatant should result in the protection of heterologous proteins secreted without propeptide. On the other hand, it might also be possible that the lipase propeptide is directly involved in the efficient secretion (i.e., release from the plasma membrane and/or passage through the cell wall) of the attached passenger protein and thus shortens the time span during which the passenger would be in the range of the cell-bound protease. Recently, such a secretion-enhancing function has been reported for the 19-amino-acid residue propeptide of *Staphylococcus aureus* nuclease in *E. coli* (44).

Since the pre-pro part of the *S. hyicus* lipase is able to function as a carrier for different proteins (see above), this expression system might be potentially very useful for the biotechnological secretory production of a wide range of heterologous proteins using *S. carnosus* as a host. For example, with respect to their potential applications as parts of a subunit vaccine or as antigens in diagnostic test systems, the secretion of Omps from gram-negative bacteria into the culture supernatant represents an attractive alternative to the direct isolation of Omps from their native membrane or to their intracellular production (29). Furthermore, the identification of the gene encoding the cell-associated protease and its subsequent inactivation might be a promising strategy for the construction of a gram-positive bacterial host strain which alleviates the need for an N-terminally attached propeptide and which might then allow authentic heterologous proteins to be secreted without their concomitant degradation at the cell surface.

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