Synthesis of OmpA Protein of *Escherichia coli* K12 in *Bacillus subtilis*

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We have inserted a C-terminally truncated gene of the major outer membrane protein OmpA of *Escherichia coli* downstream from the promoter and signal sequence of the secretory α -amylase of *Bacillus amyloliquefaciens* in a secretion vector of *Bacillus subtilis*. *B. subtilis* transformed with the hybrid plasmid synthesized a protein that was immunologically identified as OmpA. All the protein was present in the particulate fraction. The size of the protein compared to the peptide synthesized *in vitro* from the same template indicated that the α -amylase derived signal peptide was not removed; this was verified by N-terminal amino acid sequence determination. The lack of cleavage suggests that there was little or no translocation of OmpA protein across the cytoplasmic membrane. This is an unexpected difference compared with periplasmic proteins, which were both secreted and processed when fused to the same signal peptide. A requirement of a specific component for the export of outer membrane proteins is suggested.

INTRODUCTION

For many purposes it would be highly desirable to obtain outer member (OM) proteins of Gram-negative bacteria free of lipopolysaccharide and other components of the cell envelope. Such pure proteins would be especially useful for evaluating their potential use as vaccines. Their purification is, however, tedious and seldom achievable under mild conditions (for a review, see Lugtenberg & van Alphen, 1983) largely due to their amphipathic or hydrophobic character, which leads to aggregation with other proteins and glycolipids of the cell envelope (Hofstra & Dankert, 1980; Kuusi *et al.*, 1981; Lugtenberg & van Alphen, 1983). Furthermore, at least two classes of major OM proteins – the pore forming porins and the OmpA protein – have a specific affinity to lipopolysaccharide (van Alphen *et al.*, 1979; Lugtenberg & van Alphen, 1983; Osborn & Wu, 1980; Schindler & Rosenbusch, 1981; Schweizer *et al.*, 1978; Yamada & Mizushima, 1980), so that these proteins cannot be separated from lipopolysaccharide without extensive denaturation (Furukawa *et al.*, 1979; Overbeeke *et al.*, 1980; see also Lugtenberg & van Alphen, 1983; Vaara & Nikaido, 1984).

A possible way to obtain lipopolysaccharide-free OM proteins would be to clone the corresponding genes into a Gram-positive bacterium. *Bacillus subtilis* is a host in which a set of cloning vectors is available. The vectors have been constructed from the plasmid pUB110 by insertion of the promoter and signal sequence of the secretory α -amylase of *B. amyloliquefaciens* (Palva *et al.*, 1982, 1983). The signal sequence is immediately followed by a cloning site so that the cloned protein as produced is fused to the signal peptide of the α -amylase (Fig. 2). Foreign secretory proteins, among them the periplasmic β -lactamase of *Escherichia coli* (Palva *et al.*, 1982), can be effectively synthesized and secreted in this system.

We have tested the feasibility of producing OM proteins of Gram-negative bacteria in this system using the OmpA protein of *E. coli* as a model. OM proteins are made as precursors with

Abbreviation: OM, outer membrane.

cleavable signal peptides, and their translocation across the cytoplasmic membrane is believed to take place via the secretory pathway also used by periplasmic proteins (Silhavy *et al.*, 1983). The secretion vector described was therefore expected to provide the *ompA* gene with both a promoter for synthesis in *B. subtilis* and a signal sequence already shown to be effective for translocation of a periplasmic protein in *B. subtilis*.

In this paper we show that the *ompA* gene cloned in the secretion vector indeed directed the synthesis of this protein in *B. subtilis*. However, the signal peptide was not cleaved, suggesting that there was little or no translocation across the cytoplasmic membrane. This surprising finding may indicate a fundamental difference between OM proteins and other exoproteins and suggests that the *Bacillus* secretory mechanism lacks an element required for the processing of OM proteins.

METHODS

Bacteria and plasmids. *B. subtilis* IH6140, a prototrophic derivative of *B. subtilis* Marburg 1A289 (Steinmetz *et al.*, 1976) with a reduced level of exoprotease (Palva *et al.*, 1983), was from our collection. *E. coli* K12 strain EH222 (Hrs⁻ Hrm⁻ recA Gal⁻ Pro⁻ Leu⁻ Str⁻ F⁻) was a derivative of *E. coli* HB101 (Boyer & Roulland-Dussoix, 1969). *E. coli* K12 strain UH100 (Thi⁻ pyrD galK Str⁻ Trp⁻ recA *ompA*) (Bremer *et al.*, 1980) was from Ulf Henning, Max-Planck-Institut, Tübingen, FRG.

The plasmids used were pBR322 (Bolivar *et al.*, 1977a, b) and pUB110 (Gryczan *et al.*, 1978). The plasmid pTU100 (Bremer *et al.*, 1980) is a derivative of pSC101 (Cohen & Chang, 1973) containing the *ompA* gene of *E. coli* K12. It was a kind gift from Ulf Henning. pKTH132, pKTH55 and pKTH60 are secretion vectors derived from pUB110 by inserting into the plasmid the promoter and signal sequence of the α -amylase gene of *B. amyloliquefaciens*. The signal sequence is followed by a cloning site in all reading frames (Lundström, 1985; Palva *et al.*, 1982, 1983; Palva, 1983; I. Palva, personal communication). pKTH132 has termination signals for both translation (Pettersson *et al.*, 1983) and transcription (Lundström, 1985) downstream from the cloning site.

Growth conditions. The bacteria were grown in L-broth (Lennox, 1955) either on plates or in liquid cultures on a rotary shaker with vigorous shaking at 37 °C. The antibiotics used were ampicillin (100 µg ml⁻¹), tetracycline (12.5 µg ml⁻¹) and kanamycin (10 µg ml⁻¹). When the expression of *ompA* fragments was studied in *B. subtilis*, the bacteria were grown in twofold concentrated L-broth and supplemented with kanamycin and with potato extract which was prepared as follows. Peeled potatoes were cut, ground and centrifuged at 27000 g, at 4 °C for 30 min. The supernatant was collected and its pH was lowered to 3.0 with 6 M-HCl. The precipitate was removed by centrifugation at 20000 g, at 4 °C for 20 min. The pH of the supernatant was adjusted to 7.0. The extract was heated to 80 °C for 10 min, cooled to room temperature and centrifuged at 20000 g, at 4 °C for 10 min. The supernatant was collected, sterilized by filtration and stored at -20 °C. Potato extract increased the yield of both α -amylase and foreign proteins encoded by genes inserted into the secretion vector. Concentration of the extract in L-broth was optimized by assaying the amount of *E. coli* β -lactamase secreted from *B. subtilis* containing the *bla* gene in a secretion vector (Palva *et al.*, 1982). Usually 30 µl potato extract per ml L-broth was used.

Cell fractionation. *B. subtilis* strains were grown to early stationary phase. Cells were collected by centrifugation. The cell-free supernatant (culture medium) was made 10 mM with EDTA and 1 mM with phenylmethylsulphonyl fluoride. Cells were suspended in 20 mM-potassium phosphate, 15 mM-MgCl₂, pH 7.0, containing 20% (w/v) sucrose and 1 mg lysozyme ml⁻¹ and incubated at 37 °C until the cells turned into protoplasts (usually 30 min). The protoplasts were centrifuged and suspended in 50 mM-Tris/HCl, pH 7.0, and sonicated briefly to break the protoplasts. Membranes (particulate fractions) were collected by centrifugation at 100000 g for 30 min at 4 °C and suspended in 50 mM-Tris/HCl, pH 7.0. The supernatant was used as the fraction of soluble cellular proteins. The cell envelope preparation of *E. coli* was made as described by Nurminen *et al.* (1976). When treated with trypsin, a cell envelope suspension containing 1 mg protein per ml of 50 mM-HEPES, 10 mM-EDTA, 2% (v/v) Triton X-100, pH 8.4, was incubated with 2 µg trypsin ml⁻¹ for 2 h at 37 °C.

Isolation of OmpA₂₂₈ protein from *B. subtilis*. For the determination of the N-terminal amino acid sequence, the OmpA₂₂₈ protein was isolated from the membrane fraction of *B. subtilis*. The membranes were solubilized in 5% Triton X-100, 50 mM-EDTA, 50 mM-Tris/HCl, pH 8.0, at 0 °C. OmpA was pelleted by centrifugation (20000 g, 20 min, 4 °C); most other proteins remained in the supernatant. The solubilization and centrifugation were repeated. The pellet was then solubilized in 2% (w/v) Sarkosyl, 1 mM-EDTA, 50 mM-Tris/HCl, pH 8.0, and centrifuged as above. OmpA₂₂₈ remained in the supernatant. To remove the rest of the contaminating proteins the supernatant was electrophoresed in a preparative SDS gel and OmpA₂₂₈ was eluted from the gel as below.

Determination of the N-terminal amino acid sequence. Purified OmpA₂₂₈ was dissolved in 100 µl dimethylallylamine/trifluoroacetic acid buffer, pH 9.5 (in propanol/water, 3:2, v/v) and subjected to manual Edman degradation (Peterson *et al.*, 1972). The released phenylthiohydantoin amino acids were, after conversion in 25% (v/v) trifluoroacetic acid for 30 min at 55 °C, analysed by HPLC on a Spherisorb S5 ODS2 column, using a gradient of acetonitrile in 50 mM-sodium acetate, pH 4.8 (Zimmerman *et al.*, 1977).

Recombinant DNA techniques. Plasmids were isolated on a large scale from *E. coli* as described by Clewell & Helinski (1969) and from *B. subtilis* as described by Palva *et al.* (1981). Some batches were further purified in a neutral sucrose gradient (Palva *et al.*, 1981).

E. coli EH222 and *B. subtilis* IH6140 were transformed as described by Mandel & Higa (1970) and Gryczan *et al.* (1978), respectively.

The methods of Birnboim & Doly (1979), Klein *et al.* (1980) and Barnes (1977) were used to screen for the presence of plasmids and their approximate sizes. The method of Barnes (1977) was modified for *B. subtilis* by treating the bacteria with 2 mg lysozyme ml⁻¹ for 30 min at 37 °C before the SDS/NaOH/EDTA treatment. Exonuclease treatments and DNA linker ligations were done as described by Palva *et al.* (1982).

DNA fragments were sequenced according to Maxam & Gilbert (1980).

In vitro transcription-translation assay. This was done according to Fuchs (1976) and immunoprecipitation according to Sarvas *et al.* (1978). The proteins were labelled with [³⁵S]methionine during the *in vitro* synthesis.

Preparation of antisera against OmpA protein. Most of the antisera that had been raised in rabbits against purified OmpA protein did not detect the N-terminal trypsin fragment (24 kDa) of OmpA, although they detected the complete OmpA protein very well. This means that most of the antibodies were directed against determinants of the C-terminal part. Because this part was not present in the truncated OmpA protein coded by the cloned genes used, we prepared antisera against the N-terminal trypsin fragment of OmpA as follows. Envelope preparation of *E. coli* O111 (Nurminen *et al.*, 1976) (about 5 mg) was treated with trypsin and electrophoresed in 15% preparative SDS-acrylamide gel (Sarvas & Nurminen, 1985). Protein bands were made visible by submerging the gel in 0.1 M-KCl. The 24 kDa OmpA fragment was cut out and eluted with 0.05% SDS at 37 °C overnight. SDS was removed by dialysis and the protein concentrated by vacuum dialysis. Rabbits were immunized by injecting a total of 60 µg protein (as three injections at two week intervals) in Freund's complete adjuvant into the popliteal lymph nodes (Leinonen, 1985). The antisera thus obtained detected, by the immunoblotting method, the complete OmpA protein, the 24 kDa trypsin fragment of OmpA and the OmpA synthesized in *B. subtilis*.

Gel electrophoresis. Plasmids and DNA fragments were analysed in agarose gels (Sharp *et al.*, 1973), using a flat bed apparatus, or in vertical gradient polyacrylamide gels (Jeppesen, 1980).

Proteins were analysed in SDS-PAGE gels (Laemmli, 1970; Sarvas & Nurminen, 1985) and then either stained with Coomassie brilliant blue or immunoblotted (Sarvas & Nurminen, 1985; Towbin *et al.*, 1979) and visualized by autoradiography using ¹²⁵I-labelled protein A from *Staphylococcus aureus*.

Enzymes and chemicals. Restriction enzymes were from BRL, New England Biolabs, Amersham or Boehringer-Mannheim; they were used according to the manufacturers' instructions. T4 DNA ligase and T4 polynucleotide kinase were from BRL, S1 nuclease and *E. coli* exonuclease III were purchased from Calbiochem and New England Biolabs, respectively. Reverse transcriptase was a kind gift from J. Beard, Life Sciences Inc., USA. DNA linkers were obtained from BRL.

RESULTS

Modification of the *ompA* gene

Synthesis of a large amount of OmpA protein is lethal in *E. coli* (Bremer *et al.*, 1980). For this reason the *ompA* gene had initially not been cloned in a high copy number plasmid, in which the synthesis of OmpA from the efficient *ompA* promoter would be lethal, but instead in pTU100 (Fig. 1), a derivative of the low copy number vector pSC101 (Bremer *et al.*, 1980). Since *B. subtilis* does not usually start transcription from *E. coli* promoters, the *ompA* gene was not expected to be expressed or to be lethal even in a high copy number plasmid in this host. To facilitate the isolation of plasmid DNA needed for the modifications described below we subcloned an *ompA* gene fragment into the high copy number plasmid pUB110 of *B. subtilis*.

In the plasmid pTU100 the *ompA* gene is located in two *Bam*HI fragments (Fig. 1) (Bremer *et al.*, 1980, 1982). The 1.78 kb fragment contains the promoter, the signal sequence and the first 228 codons, whereas the 1.83 kb fragment codes for the last 98 residues of the OmpA protein. This C-terminal part of OmpA is believed to be entirely located on the periplasmic side of the OM. Since the 1.78 kb fragment was known to be sufficient to direct the synthesis and incorporation of the truncated OmpA protein in the OM in *E. coli*, we decided to use it as the basis for further constructions. That fragment was subcloned into pUB110. The resulting hybrid plasmid was designated pKTH42 (Fig. 1).

To construct an *ompA* derivative lacking the promoter region, the plasmid pKTH42 was cleaved at the unique *Ava*I site (Fig. 1) located at about 1.2 kb upstream from the *ompA* promoter. After partial digestion with *E. coli* exonuclease III and S1 nuclease, fill-in with reverse

Fig. 1. Modification of *ompA* gene. The *Bam*HI fragment of pTU100 coding for the amino terminus (up to residue 228) of OmpA protein was inserted into the *Bam*HI site of pUB110. The promoter and signal sequence of *ompA* were removed by an Exo III/S1 nuclease treatment starting from the *Ava*I site of pKTH42. The truncated *ompA* gene was inserted into pBR322 by the aid of *Hind*III linkers. For details see the text. B, *Bam*HI site; $\curvearrowright$, signal peptide of OmpA.

transcriptase and addition of an *Eco*RI linker the plasmid was cut with *Bam*HI; the *Eco*RI–*Bam*HI fragments were cloned in pBR322. This cloning excluded any *ompA* fragment that would retain a functional promoter after the exonuclease treatment because that would have led to lethal expression of *ompA* from the high copy number plasmid pBR322.

The extent of nuclease digestion in the plasmids isolated from the surviving transformants was analysed by gradient polyacrylamide gel electrophoresis after cutting the plasmid with restriction enzymes *Hpa*I, which has a unique site in the *ompA* gene fragment (Beck & Bremer, 1980), and *Eco*RI. Several plasmids of the desired size were picked up and their 5' end sequences determined. The promoter, signal sequence and seven N-terminal codons of *ompA* were deleted in one of the plasmids (Fig. 2). From this plasmid the *ompA* fragment was cut out with *Eco*RI and *Bam*HI. *Hind*III linkers were ligated and the fragment was inserted into the *Hind*III site of pBR322. This gave us an *ompA* construction (pKTH102; Fig. 1) coding for amino acid residues 8 to 228 of mature OmpA protein.

Insertion of the ompA fragment into the secretion vector

As described above, the plasmid pKTH102 carried a DNA fragment coding for the amino acid residues 8 to 228 (OmpA₂₂₈) of OmpA protein flanked by *Hind*III linkers (Fig. 1). The fragment was cut out with *Hind*III endonuclease and inserted into the *Hind*III site of the secretion vector (pKTH132) (Fig. 2). The insert was thus placed between the signal sequence of α -amylase and a stretch of DNA containing a synthetic oligonucleotide with translational stop codon TGA in all reading frames (Pettersson *et al.*, 1983) and the transcription termination signal of the α -amylase gene (Lundström, 1985) (see Fig. 2). Hybrid plasmids containing an insert of the right size were screened by the methods of Birnboim & Doly (1979) and Barnes (1977). The orientation of the insert was analysed by restriction endonuclease digestion (*Cla*I, *Pvu*II; see Fig. 2) followed by electrophoresis of the fragments in agarose gel. The orientation and the reading frame were confirmed by sequencing the region joining the insert and the vector (Fig. 2). The primary translation product is expected to start with the complete signal peptide (31 residues) of α -amylase, followed by six linker-derived amino acid residues, then the residues (8–228) of the mature OmpA protein and finally five residues derived from the linker and the synthetic oligonucleotide containing the translational stop codon (Fig. 2).

Synthesis of OmpA protein in B. subtilis

B. subtilis IH6140 was transformed with the plasmid pKTH158. The presence of OmpA protein was detected by SDS-PAGE of various cellular fractions followed by immunoblotting with anti-OmpA serum. The membrane (particulate) fraction of such a transformant contained

Fig. 2. Truncated *ompA* gene inserted in the *HindIII* site of the secretion vector and the nucleotide and amino acid sequences around the junctions. The nucleotides in the solid outline boxes derive from the linkers and those in the dotted outline box derive from the oligonucleotide with the stop codons. -1 is the last amino acid residue of the signal peptide of α -amylase; +8 and +228 are the respective amino acid residues of mature OmpA protein. *ZZZZ*, DNA of α -amylase gene of *B. amyloliquefaciens*; *P_{amy}*, promoter of α -amylase gene; *SS_{amy}*, signal sequence of α -amylase gene; *ZZZZ*, an oligonucleotide with the stop codon TGA in all reading frames; *TTTTT*, transcription termination region of α -amylase gene.

a protein labelled with anti-OmpA serum (Fig. 3, lane D). This serum detects specifically the OmpA protein of *E. coli* or its N-terminal trypsin fragment (24 kDa) (lanes B and C). No proteins were labelled with this serum in the membrane preparation of a strain carrying the secretion vector pKTH132 (lane E).

The membranes for Fig. 3 were prepared from cells at the early stationary phase of growth. At that time point the level of OmpA protein was near its maximum (to be shown in detail elsewhere) and the amount of exoproteases, which appear during the stationary phase, was still very small (Millet, 1970; M. Sibakov, personal communication). No OmpA was detected among the soluble cellular proteins or in the culture medium of the pKTH158 transformant (data not shown).

The amount of the OmpA protein synthesized in the *B. subtilis* transformant was estimated from the immunoblottings by comparing the intensity of the OmpA band with those in envelope preparations of *E. coli* containing known amounts of OmpA protein. Although not exact this evaluation showed that the maximal amount of OmpA produced in the *B. subtilis* cultures was about 1–2 mg per litre of culture.

Fig. 3

Fig. 4

Fig. 3. Synthesis of OmpA protein in *B. subtilis*. Immunoblots of: lane A, molecular mass standards (kDa); lane B, envelope of *E. coli* K12, undigested; lane C, as B, but digested with trypsin; lane D, particulate fraction from *B. subtilis* transformed with pKTH158; lane E, as D, but the plasmid used in transformation was pKTH132, which contained no *ompA* insert.

Fig. 4. Synthesis of OmpA protein in an *in vitro* transcription-translation assay. Fluorography of SDS-PAGE. The DNA templates used were pKTH158 (lane B) and pKTH132 (lane C). Lane A is an immunoprecipitate of lane B with anti-OmpA serum. The positions of the molecular mass standards (kDa) are indicated on the right-hand side.

Lack of cleavage of the signal peptide

The expected molecular mass of the primary translation product from the *ompA*₂₂₈ gene construction in pKTH158 is about 29 000 Da. The removal of the signal peptide would decrease it to about 26 000 Da. The apparent molecular mass of the OmpA in *B. subtilis* carrying pKTH158 was about 31 000 Da as estimated by SDS-PAGE (Fig. 3). This is very close to the above estimate of the size of the primary translation product, and much larger than the expected cleavage product.

To obtain the primary translation product in the absence of membranes we synthesized OmpA₂₂₈ *in vitro* using pKTH158 DNA as template. In such systems the signal peptide of precursors of secretory proteins is usually not cleaved (Walter & Blobel, 1981; Ohno-Iwashita *et al.*, 1984). The pKTH158 template directed the *in vitro* synthesis of a protein of 31 000 Da (Fig. 4, lane B). This protein was precipitated by anti-OmpA serum (lane A). No such protein was present among the proteins synthesized from pKTH132, which lacks the *ompA*₂₂₈ insert (lane C). The primary translation product of *ompA*₂₂₈ of pKTH158 had thus indeed the same apparent mass as the OmpA₂₂₈ in the particulate fraction of *B. subtilis* harbouring pKTH158.

Fig. 5. *In vivo* and *in vitro* synthesis of OmpA protein from plasmids pKTH174 and pKTH175. (a) Immunoblots of membrane fractions of *B. subtilis* carrying either the vector pKTH132 (lane B), pKTH174 (lane C) or pKTH175 (lane D). Molecular mass standards (kDa) are shown in lane A. (b) *In vitro* synthesized proteins from pKTH174 (lane D) and pKTH175 (lane B). Lanes A and C are immunoprecipitates with anti-OmpA serum of B and D, respectively. The size of the precipitated protein and that of OmpA in the membrane fraction is 32 kDa.

These findings strongly suggest that the signal peptide of α -amylase was not cleaved off the OmpA protein in *B. subtilis*.

To confirm that the signal peptide of OmpA₂₂₈ was indeed not cleaved in *B. subtilis*, the N-terminal amino acid sequence of the protein was determined. When the purified OmpA₂₂₈ was subjected to four cycles of Edman degradation, a single N-terminal amino acid sequence Met-Ile-Gln-Lys was obtained, confirming the purity of the analysed protein. The quantity of the released phenylthiohydantoin amino acids also corresponded to the amount of the degraded protein. The sequence obtained was identical to the N-terminal sequence of the α -amylase signal peptide deduced from the DNA sequence (Palva *et al.*, 1981). The result shows that the signal peptide was not cleaved when OmpA₂₂₈ was synthesized in *B. subtilis* from the plasmid pKTH158.

To make sure that the lack of cleavage of the signal peptide was not due to an unfavourable sequence at the cleavage site in this particular construction (pKTH158), we inserted the *ompA*₂₂₈ fragment into two other expression vectors, pKTH55 and pKTH60. They are similar to

pKTH132, the vector from which pKTH158 was derived, but they contain before the cloning site four and 17 codons, respectively, of the mature α -amylase in addition to the signal sequence. The two plasmids thus obtained, pKTH174 and pKTH175, coded for a protein in which the amino acid sequence around the cleavage site of α -amylase signal peptide was natural.

The OmpA proteins produced from both of these vectors in *B. subtilis* were found in the membrane fraction and had an apparent molecular mass of 32 kDa (Fig. 5). The same size was obtained for these proteins when produced *in vitro* from DNA templates (Fig. 5). Thus these constructions behaved in an exactly analogous manner to pKTH158, without cleavage of the signal peptide, in spite of the shorter or longer natural amino acid sequence around the cleavage site.

DISCUSSION

The protein secretion machinery of both eukaryotic (Talmadge *et al.*, 1980a; Gray *et al.*, 1984; Müller *et al.*, 1982) and prokaryotic cells is usually capable of exporting heterologous as well as homologous natural secretory proteins. Many types of foreign secretory proteins have been shown to be exported into the periplasmic space of *E. coli* (see Talmadge *et al.*, 1980a; Pugsley & Schwartz, 1985). Furthermore, the signal peptides targeting proteins for export are largely exchangeable between proteins from different cells or from different extracytoplasmic locations (Tommassen *et al.*, 1983; Yu *et al.*, 1984; Talmadge *et al.*, 1980b; Chan *et al.*, 1981; Talmadge & Gilbert, 1982). Previous observations on the secretion of foreign proteins in *B. subtilis* conform with this general principle. Thus both human and mouse interferons (Palva *et al.*, 1983; Shiroza *et al.*, 1985) and periplasmic β -lactamase of *E. coli* (Palva *et al.*, 1982; Ohmura *et al.*, 1984) were secreted and processed correctly in *B. subtilis* when fused to the signal peptide of the secretory bacilliary α -amylase.

The behaviour of the OmpA₂₂₈ protein in *B. subtilis* is different. Although fused to the same signal peptide of α -amylase and synthesized in relatively large amounts, all of the OmpA₂₂₈ protein synthesized in *B. subtilis* was found in the particulate fraction and was in its precursor form with the signal peptide uncleaved. A possible reason for the lack of cleavage could be an unfavourable amino acid sequence at the expected cleavage site, which in these constructions contained six amino acid residues coded by the *Hind*III linker. Although much variation is allowed at the cleavage site of signal peptides, there are examples showing that the signal peptidase is not entirely non-specific (Ohmura *et al.*, 1984; Kadonaga *et al.*, 1984; Koshland *et al.*, 1982). To test for this possibility we inserted the *ompA*₂₂₈ into two secretion vectors so that OmpA₂₂₈ became fused to the signal peptide followed by either four (pKTH174) or 17 (pKTH175) residues of the mature α -amylase. Thus both these constructions had an intact cleavage site of α -amylase signal peptide. A comparison of the OmpA proteins produced from these plasmids *in vivo* and *in vitro* showed that the signal peptide was not cleaved (Fig. 5), and thus the lack of cleavage must be due to something other than the sequence at the cleavage site.

The observed lack of cleavage strongly suggests that the protein was not even partially translocated across the cytoplasmic membrane [see Pugsley & Schwartz (1985) for discussion on processing of the signal peptide and translocation]. This striking difference, compared with the efficient secretion and processing of the *E. coli* β -lactamase fused to the same signal peptide, indicates that, rather unexpectedly, the export of OM proteins may require specific components not present in the cytoplasmic membrane of *B. subtilis*. We are currently pursuing this interesting possibility by studying in more detail the location and conformation of the OmpA protein made in *B. subtilis*.

No OmpA protein was found in the growth medium of *B. subtilis* harbouring the plasmid pKTH158. The possibility that some OmpA protein could have been secreted into the growth medium and rapidly destroyed by exoproteases, a problem encountered to various degrees with all foreign proteins secreted from *B. subtilis* (Lundström, 1984; Lundström *et al.*, 1985; Palva *et al.*, 1983; Ulmanen *et al.*, 1985), cannot be formally excluded. However, no OmpA protein was detected in the culture fluid even in the presence of protease inhibitors (phenylmethylsulphonyl fluoride and EDTA or *o*-phenanthroline) or during exponential phase growth, when there is no detectable protease activity in the growth medium of the host strain used (data not shown).

In *E. coli*, the OmpA protein like other OM proteins, must be transported from cytoplasm to its final location in the OM. The mechanism of this transfer is unknown (for a review, see Lugtenberg & van Alphen, 1983; Pugsley & Schwartz, 1985). Some hypothetical models (Henning *et al.*, 1983; Osborn & Wu, 1980) suggest that OM proteins may travel across the periplasmic space in soluble form not bound to membrane structures. Such a form would seemingly be analogous to periplasmic proteins and expected to be secreted – like the β -lactamase – also across the cytoplasmic membrane of *B. subtilis* when fused to the signal peptide of α -amylase. Our failure to find any secreted OmpA protein in the *B. subtilis* cultures does not support this model in its simplest form.

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