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Analysis of signals for secretion in the staphylococcal protein A gene

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Different constructs of the gene encoding staphylococcal protein A were introduced in *Staphylococcus aureus* and *S. xylosus* as well as *Escherichia coli*. The product of the gene without the cell wall anchoring domain was efficiently secreted in all three hosts. N-terminal sequencing of the affinity-purified mature protein revealed a common processing site after the alanine residue at position 36. In contrast, when an internal IgG-binding fragment of protein A (region B) was inserted after the protein A signal sequence, the product was poorly secreted and N-terminal sequencing revealed no processing at the normal site. This demonstrates that the structure of the polypeptide chain beyond the signal peptide cleavage site can affect cleavage. Another construct, containing the N-terminal IgG-binding part of the mature protein A (region E) followed by region B, gave correct processing and efficient secretion. Unexpectedly, the gene product, EB, was not only secreted and correctly processed, but was also excreted to the culture medium of *E. coli*. Secretion vectors containing the protein A signal sequence were constructed to facilitate secretion of foreign gene products. Insertion of the *E. coli* gene *phoA*, lacking its own promoter and signal sequence, led to efficient secretion of alkaline phosphatase both in *E. coli* and *S. aureus*.

Key words: Staphylococcus/protein A gene/signal peptide/secretion

Introduction

The secretion of proteins into the periplasm of Gram-negative or the medium of Gram-positive bacteria is analogous to the transfer of secretory proteins into the lumen of the rough endoplasmic reticulum in eukaryotes (Randall and Hardy, 1984). The secretion process involves an N-terminal hydrophobic signal sequence, which has been suggested to interact with both soluble and membrane-bound proteins (Walter and Blobel, 1982; Meyer *et al.*, 1982; Oliver, 1985). The signal sequence of Gram-positives is usually longer than that of the Gram-negatives and also contains more charged residues in the N terminus (Watson, 1984). To study the secretion mechanism, several genetic approaches have been used involving both gene fusions (Talmadge *et al.*, 1980; Moreno *et al.*, 1980) and site-directed mutagenesis (Inouye *et al.*, 1982). These studies have primarily been performed using *Escherichia coli* as an *in vivo* model (Talmadge *et al.*, 1980; Moreno *et al.*, 1980; Inouye *et al.*, 1982) but the Gram-positive *Bacillus subtilis* (Palva *et al.*, 1983) and *Staphylococcus aureus* (Nilsson *et al.*, 1985b) have also been used. The fact that bacilli and staphylococci, in contrast to *E. coli*, efficiently excrete the proteins to the culture medium makes these organisms interesting for biosynthesis of foreign gene products by recombinant DNA technology. A better understanding of the translocation mechanism in these organisms is therefore desirable.

In this report we analyze the signals in the staphylococcal protein A gene required for efficient secretion. The promoter and signal sequences of this gene are functional in several prokaryotic host cells, including *E. coli* (Loefdahl *et al.*, 1983), *S. aureus* (Uhlén *et al.*, 1984a), *S. xylosus* (Uhlén *et al.*, 1984a), *S. epidermidis* (Uhlén *et al.*, 1984a), *B. subtilis* (Saunders *et al.*, 1984) and *Streptomyces lividans* (G.Emas, personal communication). This allows a comparison of the secretion and processing efficiencies of the different constructs in several unrelated bacteria. Furthermore protein A can be efficiently purified (95-100% yield) by IgG affinity chromatography (Nilsson *et al.*, 1985a) facilitating N-terminal sequencing of the mature protein. By introducing the constructs into different mutants of *S. aureus* it is relatively easy to study the effect of transcription rates upon secretion. At least a 1000-fold difference in expression from the protein A promoter is obtained in *S. aureus* mutants such as Wood 46 and A676 (Uhlén *et al.*, 1984a). In addition, since the evolution of the protein A gene has involved multiple gene duplications (Uhlén *et al.*, 1984b), a functional comparison of the repeated regions might identify regions necessary for secretion.

The protein processing site of plasmid-encoded protein A was determined by N-terminal sequencing. Based on these results secretion vectors were constructed and used to study the secretion of enzymatically active alkaline phosphatase. Two different regions of the protein A gene encoding IgG-binding domains were also inserted into the secretion vectors to investigate the role of the N-terminal part of the mature protein in the secretion process.

Results

Processing of the protein A signal sequence in different species

Protein A is efficiently secreted in *E. coli* (Loefdahl *et al.*, 1983) and in four species of *Staphylococcus* (Uhlén *et al.*, 1984a). To determine if a common processing site is recognized during transport in these species, the plasmid pSPA16 (Uhlén *et al.*, 1984a) was introduced into *E. coli*, *S. aureus* and *S. xylosus*. This plasmid contains a truncated protein A gene, lacking region X coding for the C-terminal end of the protein responsible for cell wall anchorage. *S. aureus* was chosen as the homologous host, *E. coli* as an unrelated bacteria and *S. xylosus* as a non-pathogenic *Staphylococcus* producing little extracellular proteases (F. Götz, personal communication). The latter species may become a host for expression and secretion of foreign gene products. The three bacteria containing plasmid pSPA16 were grown to early stationary phase and protein A from the medium of the Gram-positives or the periplasm of *E. coli* was concentrated and purified by IgG-affinity chromatography (Nilsson *et al.*, 1985b). N-terminal sequencing using Edman degradation revealed a common processing site between the two alanines at position 36 and 37 of the preprotein (Figure 1), in accordance with the consensus

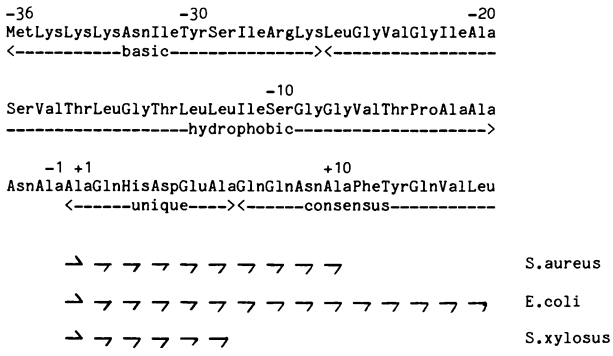

Fig. 1. Structure of the staphylococcal protein A signal sequence. The deduced amino acid sequence (Uhlén *et al.*, 1984b) and relevant characteristics are shown. Unique and consensus indicate the part of region E not homologous and homologous, respectively, to the other IgG-binding regions (Uhlén *et al.*, 1984c). Arrows denote positive identification (⇀) of PTH-amino acids obtained after solid-phase Edman degradation of purified cloned protein A from the different host strains. The coupling method employed does not allow identification of the first amino acid residue (⇀).

model for signal sequence cleavage suggested by von Heine (1984). It is interesting that *E. coli* recognizes this processing site although signal sequences in *E. coli* are usually shorter, consisting of 15–25 amino acid residues (Watson, 1984).

Construction of secretion vectors

The IgG-binding part of protein A is comprised of five homologous domains E, D, A, B and C (Figure 2) each consisting of 56–61 amino acid residues (Uhlén *et al.*, 1984b, 1984c). Region E, the N-terminal domain of the mature protein, differs more from a hypothetical consensus sequence (Uhlén *et al.*, 1984c) than the others. In particular, the first six residues of region E (Ala-Gln-His-Asp-Glu-Ala) differ significantly from the consensus sequence (Asn-Gln-Phe-Asn-Lys-Glu) in the other regions (Uhlén *et al.*, 1984c). To evaluate the role of region E in secretion, two secretion vectors were constructed from a shuttle plasmid pRIT4 (Nilsson *et al.*, 1985b). Using partial *Mst*I digestion followed by ligation to *Eco*RI-linkers of different lengths, six vectors were obtained, i.e., two types of vectors with a unique *Eco*RI site followed by the M13 mp9 multi-linker in all reading frames (Figure 3). The pAS and the pASE series have the multi-linker inserted at the position corresponding to amino acid residues 1 and 59 of the mature protein A, respectively. Thus, a foreign gene can be inserted directly following the signal sequence or region E, simply by choosing the appropriate vector.

Expression and secretion of alkaline phosphatase

To test the vectors, the *E. coli* gene *pho*A coding for alkaline phosphatase, without promoter and signal sequence, was ligated into the vectors. The resulting plasmids pASP and pASEP contain *pho*A inserted after the signal sequence and region E, respectively (Figure 2). The two plasmids were transformed into *E. coli* and *S. aureus* and the transformants were screened for alkaline phosphatase activity on XP plates containing 0.9% potassium phosphate to repress endogenous activity. Positive clones were grown in liquid media containing phosphate and the phosphatase activity in the different cellular compartments was measured. The results shown in Tables I and II reveal that alkaline phosphatase is produced from both constructs and secreted to the culture medium of *S. aureus* and into the periplasmic space of *E. coli*. The enzymatic activity is slightly lower in *S. aureus* than in *E. coli* for both gene products (data not shown). This probably reflects proteolytic degradation previously observed with a fusion protein

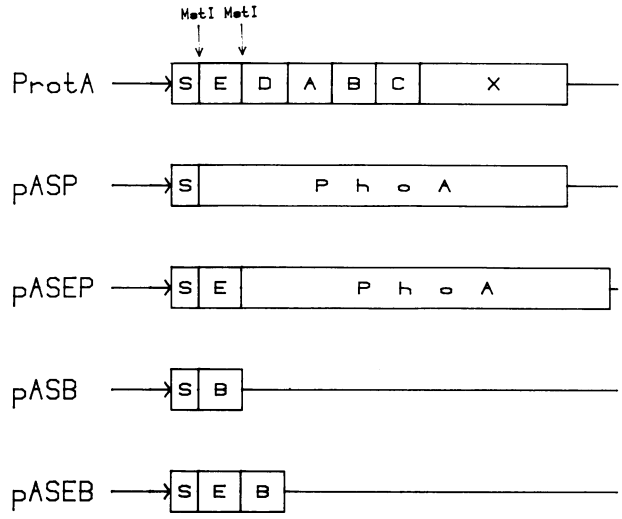

Fig. 2. Schematic drawing of the protein A gene and the different gene fusions. The protein A gene (ProtA) codes for a signal sequence (S), five IgG-binding regions (E, D, A, B and C) and a region responsible for cell wall binding (X). The two *Mst*I restriction sites used for the construction of the secretion vectors are shown. PhoA represents the alkaline phosphatase structural gene lacking its own signal sequence.

containing alkaline phosphatase fused after all the IgG-binding domains of protein A (Nilsson *et al.*, 1985b). Both secretion vectors can obviously be used to secrete a foreign gene product and region E does not seem to be essential for the translocation of alkaline phosphatase in *E. coli* and *S. aureus*.

Expression and secretion of protein A fragments

To elucidate if the divergence of region E has a role in secretion, we decided to fuse region B directly after the protein A signal sequence or after region E. Both domains E and B have been shown to be IgG-binding (T.Moks, unpublished) which simplifies purification and structural analysis of the gene products.

Using the secretion vectors (Figure 3) and three different DNA fragments encoding region B (see Materials and methods) three *in vitro* constructions were assembled: pASB-1, pASB-2 and pASEB (Figure 2). The difference between pASB-1 and pASB-2 is a short stretch of residues at the junction between the signal sequence and region B (Figure 4). Plasmid pASEB encodes a divalent IgG-binding protein (EB) which is identical to native protein A to the end of region E. The three plasmids were transformed into *E. coli* and the amount and localization of the IgG-binding activity was determined using an enzyme-linked immunosorbent assay (ELISA) method. Table III shows that cells harboring plasmid pASEB secreted the protein A fragment but inefficient secretion is observed in cells containing plasmids pASB-1 and pASB-2. Approximately 50% or more of the gene products were not exported, but found in the intracellular fraction (cytoplasm or membrane bound).

The localization studies presented in Table III also reveal an interesting and unexpected phenomena not described before. The expression of the protein A fragments induces changes in the morphology of the *E. coli* host cells, giving filamentous growth. As shown in Table III, the changed morphology is accompanied by a more 'leaky' outer membrane. This results in excretion into the culture medium of normally periplasmic proteins, such as β-lactamase. Although the effect varies depending on the protein A fragment expressed, none of the constructs induces significant cell lysis as assayed by the release of β-galactosidase into the culture medium.

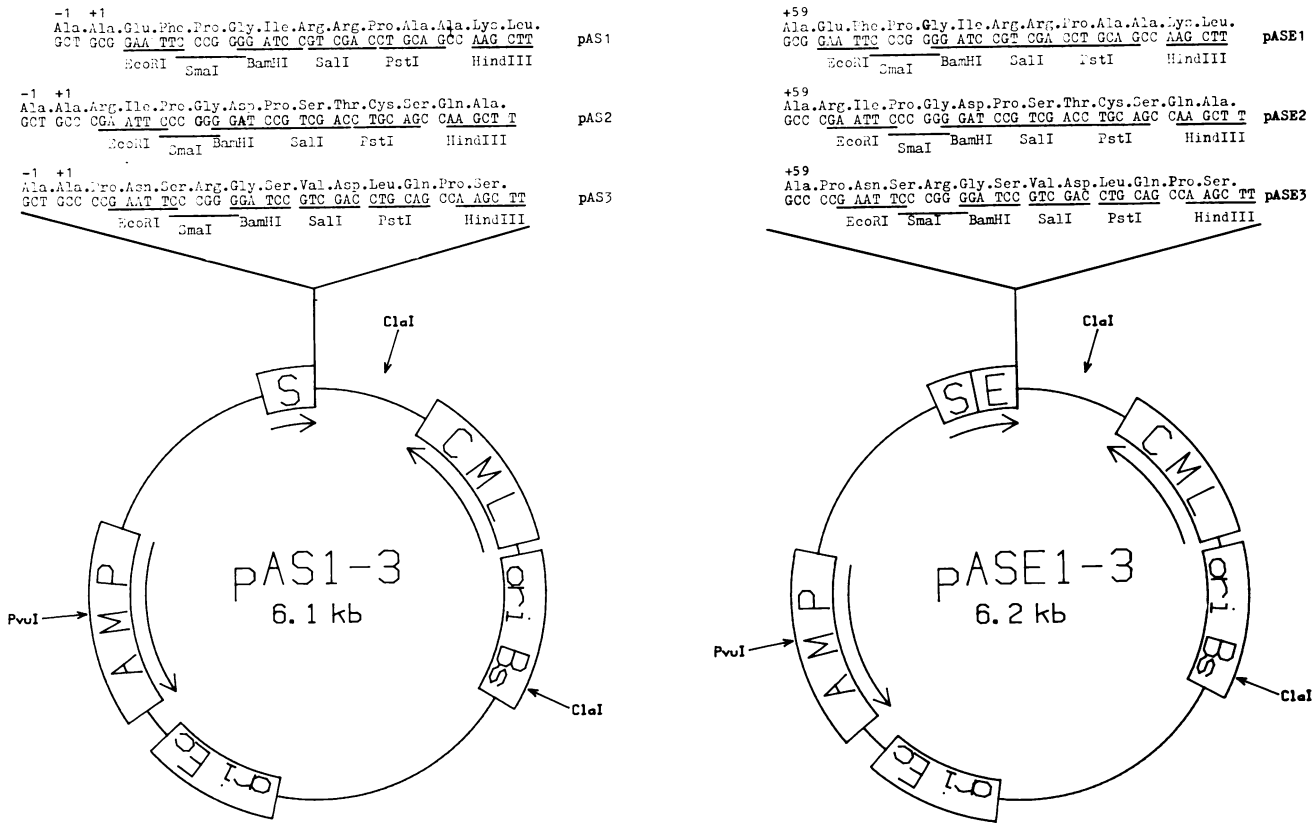

Fig. 3. Schematic drawing of the protein A secretion vectors. Some relevant restriction sites are indicated and the deduced amino acid sequence across the multi-linker region is shown. Boxes represent the position of the genes coding for the signal sequence (S), region E (E), β-lactamase (AMP) and chloramphenicol acetyl transferase (CML). The origins of replication in *E. coli* (OriEc) and in staphylococci/bacilli (OriBs) are also shown.

Table I. Localization of alkaline phosphatase activity in *E. coli* cultures

Plasmid	Periplasm	Intracellular	Medium
pAS1	<0.2	<0.2	<0.2
pASP	89	10	1
pASEP	84	14	2

E. coli HB101 containing the different plasmids were grown to A_{550} of 1.0 and the cells were treated with osmotic shock (periplasm) followed by sonication of the osmotically shocked cells (intracellular). Values for pAS refer to percent activity of pASP-containing cells.

Table II. Localization of alkaline phosphatase activity in *S. aureus* cultures

Plasmid	Medium	Intracellular
pRIT4	<0.2	<0.2
pASP	87	13
pAESP	80	10

S. aureus SA113 containing the different plasmids were grown overnight and the activity in the medium and sonicated cells (intracellular) were determined. The value is given as percent of the total activity of cells. The values for pRIT4 refer to percent activity of pASP-containing cells.

Processing of the protein A fragments

To determine the protein processing sites of the B fragments, N-terminal sequencing was performed after purification of the peptides on IgG columns followed by gel permeation chromatography. The results are presented in Table III and Figure 4. As expected, the secreted protein encoded by pASEB is processed

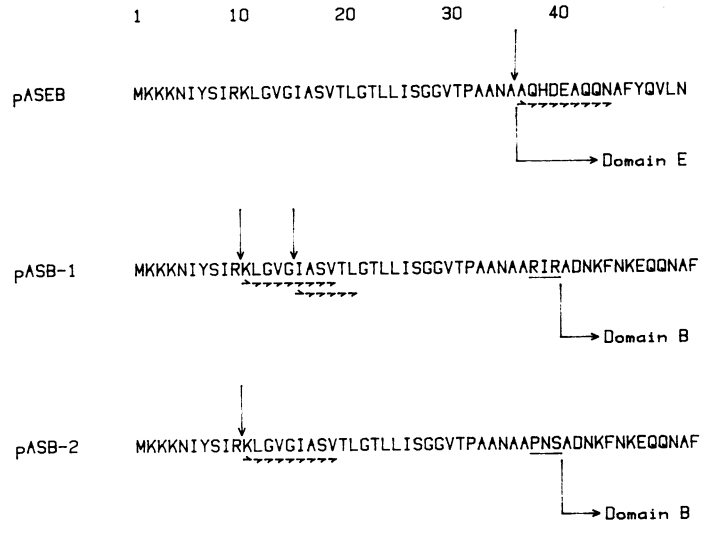

Fig. 4. The structure and cleavage of the different gene fusion products. The deduced amino acid sequence of the N-terminal part of the different gene fusions are shown. The non-homologous parts are underlined and the start of the IgG-binding domains are indicated. The results of the solid-phase Edman degradation are marked as described in Figure 1. The resulting cleavage sites for the different gene products are shown by arrows. Amino acids are given in the one-letter code; i.e., A,Ala, C,Cys; D,Asp; E,Glu; F,Phe. G,Gly; H,His; I,Ile; K,Lys; L,Leu; M,Met; N,Asn; P,Pro; Q,Gln; R,Arg; S,Ser; T,Thr; V,Val; W,Trp; Y,Tyr.

as the native protein A (Figure 4). In contrast, the gene products purified from sonicated cells containing plasmids pASB-1 and pASB-2 were not processed at the plasmid pASB-1 original signal

Table III. Localization of β-galactosidase (lac), β-lactamase (bla) and IgG-binding activity (spa) in *E. coli* cells containing different plasmids

Plasmid (%)	Cleavage after residue	Filamentous growth	Intracellular (%)			Periplasmatic (%)			Medium (%)		
			lac	bla	spa	lac	bla	spa	lac	bla	spa
pBR322	ND	−	99	3	ND	1	94	ND	1	3	ND
pSPA16	36	(−)	99	3	2	<1	83	88	<1	14	10
pASB-1	10,15	+	97	4	89	1	66	6	2	30	5
pASB-2	10	+	92	4	52	1	41	5	7	55	43
pASEB	36	+	96	4	8	1	50	37	3	46	55

E. coli HB101 containing the different plasmids was grown overnight and the cells were treated by an osmotic shock procedure (periplasm) followed by sonication of the osmotically shocked cells (intracellular). Cleavage after residues represents the processing sites as determined by Edman degradation (Figures 1 and 4). Filamentous growth indicates the morphology of the host cells as observed by light microscopy.

peptide cleavage site, but were cleaved after residues 10 or 15 (Figure 4). This shows that, in contrast to the *E. coli* alkaline phosphatase (Tables I and II), an intact protein A signal sequence is not enough to ensure processing and secretion of the internal domain B of protein A.

Discussion

All signal sequences seem to have the same general structure, a positively charged N-terminal region, a central hydrophobic core and a more polar C-terminal region that defines the cleavage site during processing from preprotein to mature protein (von Heine, 1984). These regions are present in all signal sequences both of prokaryotic and eukaryotic origin, although the variations in length and composition can be large. Comparative studies to determine the structural limits of the signal sequence have revealed that the protein A signal sequence is 'extreme' in its structure (von Heine, 1985). First, because it is the longest out of some 300 entries, second because its N terminus is unusually charged. However, in this comparison only three examples of signal peptides from Gram-positive bacteria were included and therefore we show additional examples from this class of prokaryotes in Figure 5. The collection reveals that most Gram-positive signal sequences are unusual both in length and charge. The average positive charge in the N terminus is $>+3$ as opposed to $+2$ for all prokaryotes and $+1$ for eukaryotes (von Heine, 1984). Hence, the protein A signal sequence, although unique compared with a standard signal sequence, is not unusual when compared with other signal sequences of proteins from Gram-positive bacteria.

There are conflicting data about the role of the N-terminal part of the mature protein in the secretion process. Russel and Model (1981) showed that a mutation downstream of the signal peptide affects cleavage but not membrane insertion of an *E. coli* phage coat protein. Furthermore, Haguenauer-Tsapis and Hinnen (1984) showed that a deletion in the beginning of the mature protein partially impairs the *in vitro* processing of yeast acid phosphatase. In contrast, Takahara *et al.* (1985) concluded that the structural determinants necessary for correct processing in *E. coli* are located at the N-terminal side of the signal peptidase cleavage site in the *ompA* protein. Here we investigate these questions in *E. coli* and *S. aureus* using different vectors based on the staphylococcal protein A signal sequence.

Two sets of secretion vectors have been constructed, with and without region E (Figure 3) which is the region proximal to the signal sequence of the protein A gene. Alkaline phosphatase, lacking its own signal sequence, was found to be efficiently secreted using both vectors in *E. coli* (Table I) and *S. aureus* (Table II). The large difference in size between the signal sequence provided by the protein A vectors (36 residues) and the native alkaline phosphatase (21 residues) does not significantly influence the transport efficiency. Interestingly, most promoters, ribosome-binding sites and signal sequences from Gram-positive bacteria seem to be functional in *E. coli*, but not the reverse because of blocks at the level of transcription, translation and secretion (Goebel *et al.*, 1979). The obvious conclusion is therefore that broad host range expression vectors should be based on genes from Gram-positives.

Alkaline phosphatase was secreted using these secretion vectors, but an internal IgG-binding fragment of protein A (region B) was poorly excreted and accumulated in the intracellular fraction (cytoplasm or membrane bound), most likely due to lack of signal peptide processing. This was observed in two different constructs with different residues at the fusion point (pASB-1

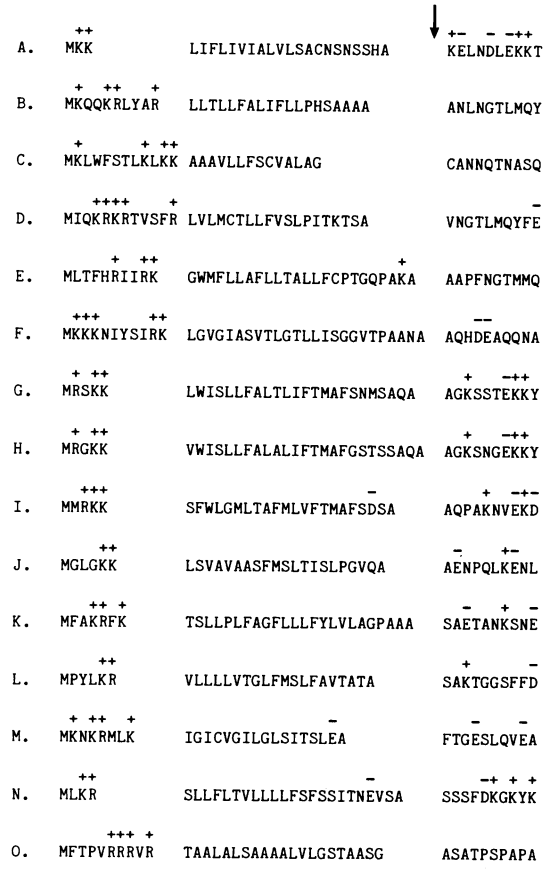

Fig. 5. A collection of signal sequences from Gram-positive bacteria. The amino acid sequence as deduced from the nucleotide sequence is shown. Charged residues are indicated by + or − and the proposed cleavage site for the signal peptidase by the arrow. The processing site for the first six sequences (A−F) has been confirmed by amino acid sequencing. **A,** *S. aureus* β-lactamase (McLaughlin *et al.*, 1981; **B,** *B. licheniformis* α-amylase (Stephens *et al.*, 1984); **C,** *B. licheniformis* β-lactamase (Chang *et al.*, 1982); **D,** *B. amyloliquefaciens* α-amylase (Palva *et al.*, 1981); **E,** *B. stearothermophilus* α-amylase (Nakajima *et al.*, 1985); **F,** *S. aureus* protein A (this paper); **G,** *B. subtilis* subtilisin (Wong *et al.*, 1984); **H,** *B. amyloliquefaciens* subtilisin (Wells *et al.*, 1983); **I,** *B. licheniformis* subtilisin (M.Eliasson, personal communication); **J,** *B. amyloliquefaciens* neutral protease (Vasantha *et al.*, 1984); **K,** *B. subtilis* α-amylase (Ohmura *et al.*, 1984); **L,** *B. subtilis* β-glucanase (Murphy *et al.*, 1984); **M,** *B. cereus* type I β-lactamase (Sloma and Gross, 1983); **N,** *S. aureus* staphylokinase (Sako and Tsuchiba, 1983); **O,** *Streptomyces plicatus* endoH (Robbin *et al.*, 1984).

and pASB-2). When the gene fusion also contained region E (pAEB), correct processing and efficient secretion was observed. The signal sequence is identical in all constructs and the sequences start to differ at residue 2 of the mature protein (Figure 4). Most differences are accounted for by the structural difference between regions E and B, but three residues result from the linker. It is not at present possible to rule out that these linker residues are responsible for the lack of signal peptide processing. However, the fact that two constructs (pASB-1,2) with different linker regions, both failed to give signal peptide processing suggests that region B, in contrast to region E, interferes with signal peptide processing and thus with secretion. This implies that the divergence between region E and the other four IgG-binding domains is an evolutionary adaptation for efficient secretion.

The alternative cleavage site for fragments B-1 and B-2 occurs between the two basic residues Arg-Lys at positions 10 – 11 and for fragment B-2 also between the Gly-Ile residues at positions 15 – 16 (Figure 4). The question arises whether this abnormal processing reflects enzymatic activities which are normally involved in signal peptide degradation. In this respect, it is interesting that the cleavage after the glycine is rather unusual, but the cleavage after the arginine could be made by a trypsin-like protease.

An interesting and unexpected observation is the fact that the fragment EB encoded by pASEB is not only secreted and correctly processed but it is also excreted into the culture medium of *E. coli* (Table III). The expression of this fragment, but also fragments B-1 and B-2, seems to interfere with the formation of the cell wall of the *E. coli* host cells, as judged by the filamentous shape of the bacteria. It is likely that the outer membrane is also affected, thereby allowing passage of proteins which are usually found only in the periplasm.

Although the protein A promoter is constitutively expressed, the changed morphology does not seem to affect seriously the viability of the cells. Little cell lysis can be detected using an intracellular marker (β-galactosidase) and the generation time during growth is only slightly longer than for the host cell containing plasmid pBR322 (data not shown). Filamentous growth has also been observed when highly expressed promoters are induced, producing foreign gene products that form crystals ('inclusion bodies') in the cell (Nilsson *et al.*, 1985b). Although, in contrast to our system, this results in non-viable cells, it is likely that the two phenomena have similar causes.

It is obvious that this expression/secretion system is potentially useful for secretion and purification of foreign gene products. Recently, we have used this sytem, to express in *E. coli* human insulin-like growth factor-I (IGF-I). The gene product was efficiently secreted into the growth medium of the *E. coli* host cells and could be purified simply by passing the culture medium through an IgG affinity column (T.Moks, unpublished).

Materials and methods

Bacterial strains and plasmids

E. coli strain HB101 (Boyer and Roulland-Dussoix, 1969), *S. aureus* strain SA113 (Iordanescu, 1975) and *S. xylosus* strain KL117 (Schleifer and Kloos, 1975) were used as bacterial hosts. The plasmid vectors were pRIT4 (Nilsson *et al.*, 1985b) and pCH40 (C.Hoffmann, personal communication) which is pBR322 (Bolivar *et al.*, 1977) containing the *E. coli* gene *pho*A coding for alkaline phosphatase.

DNA preparations

Plasmid DNA was prepared by a modified alkaline extraction method (Kieser, 1984). Transformation of *E. coli* was made according to Morrison (1979) and transformation of *Staphylococcus* by a protoplast transformation procedure, as described elsewhere (Götz *et al.*, 1981; Lindberg, 1981). Restriction endonucleases and T4 DNA ligase were obtained from Boehringer Mannheim or New England Biolabs and were used according to the suppliers' recommendations.

DNA construction

The details for the subcloning of the gene fragment coding for region B will be presented elsewhere (T.Moks, preliminary manuscript). Essentially the fragment was obtained by exonuclease *Bal*31 treatment followed by addition of *Eco*RI-linkers (5′ end) and *Bam*HI-linkers (3′ end). A linker containing a translation stop codon was subsequently inserted in the *Bam*HI site. In this way a fragment of 180 bp was obtained coding for region B except for the last lysine residue, which has been substituted with a glycine (pASB-1) or an alanine (pASB-2).

Protein preparations

Cell extracts were made by sonication (4 × 30 s in a MSE-sonicator) in a buffer containing the serine protease inhibitor phenylmethylsulphonyl fluoride (PMSF, Merck No. 7349). An osmotic shock procedure was used to release protein from the periplasmic space (Nossal and Heppel, 1965).

IgG-Sepharose 4B (Pharmacia, Uppsala, Sweden) affinity chromatography was used to purify and concentrate protein A and protein A fragments from crude preparations. Protein to be NH_2-terminally sequenced were further purified using gel-permeation chromatography on AcA202 (LKB, Stockholm, Sweden).

Enzymatic assays

The *pho*A gene products were assayed on plates containing 5-bromo-4-chloro-3′-indolyl phosphate (XP, No. B-8503 Sigma) and 0.9% phosphate to suppress endogenous activity. Detection and quantification of alkaline phosphatase was done by a colorimetric procedure using *p*-nitrophenylphosphate (Sigma product No. S-6750 in accordance with the supplier's recommendation).

β-Lactamase activity was determined spectrophotometrically as described by O'Callaghan *et al.* (1968) and β-galactosidase using a colorimetric procedure exactly as described by Uhlén *et al.* (1983). Detection and quantification of protein A was performed by ELISA. Human IgG (Kabi-Vitrum, Stockholm, Sweden) was used to coat the microtiter wells. Cell extracts were added to allow binding of protein A fragments to IgG. The Fab fragment of rabbit anti-protein A antibodies conjugated to β-galactosidase (kindly supplied by Mats Inganäs, Pharmacia) were allowed to bind to the protein A fragments. After washing, the β-galactosidase activity was measured in a colorimetric procedure using ONPG (*o*-nitro-phenyl-β-D-galactoside, Sigma No. N-1127) as substrate.

Protein sequence analysis

The sample was attached to amino-propyl glass via lysyl residues by the diisothiocyanate method (Laursen *et al.*, 1972) and subjected to automated solid-phase Edman degradation (LKB solid phase peptide sequence 4020). After manual conversion the phenylthiohydantoin amino acids were analyzed by reversed phase h.p.l.c. (Laboratory DNA Control) on a Nucleosil C18 5 μ column (× 300 mm) by a gradient of acetonitrile in 6 mM NaAc pH 4.9. The column was operated at 37°C, the flow-rate was 0.9 ml/min and the eluate was monitored at 269 nm.

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