The SecB chaperone is involved in the secretion of the *Serratia marcescens* HasA protein through an ABC transporter

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The secretion pathways of the heme-binding protein HasA from *Serratia marcescens* and of the metalloproteases A, B, C and G from *Erwinia chrysanthemi* have been reconstituted in *Escherichia coli*. They are secreted in a single step from the cytoplasm across both membranes of the Gram-negative envelope, after recognition of their specific C-terminal secretion signal by their cognate ABC transporter. We report strong evidence that both HasA and the metalloproteases bind the SecB chaperone involved in the export of several envelope proteins via the Sec pathway. We also show that the secretion of the HasA protein is strongly dependent upon SecB in the reconstituted system, whereas that of the proteases is not. HasA secretion in the original host is strongly inhibited by a protein known to interfere with *E.coli* SecB function. We propose that the proteins secreted by the ABC pathway may have to be unfolded for efficient secretion.

Keywords: ABC transporter/HasA/maltose-binding protein/metalloproteases B and C/SecB chaperone

Introduction

There are three major pathways for protein secretion in Gram-negative bacteria (Wandersman, 1996): the general secretory pathway (GSP), the ATP-binding cassette (ABC) pathway and the type III pathway. In the GSP pathway, the protein, via its N-terminal signal sequence, first utilizes the Sec machinery to cross the inner membrane and reach the periplasmic compartment, whereupon it folds with the help of periplasmic chaperones (Pugsley, 1993); crossing the outer membrane involves a complex machinery including components localized in all compartments, and it is likely that the protein is translocated in the folded state (Sandkvist and Bagdasarian, 1993). The type III secretion pathway is used by several bacterial pathogens upon contact with the host cell. The protein passes through the envelope in a single step, and the secretion signal is at the N-terminus (Michiels and Cornelis, 1991); the secretion machinery is complex and, in several cases, the secreted protein stably associates with a specific chaperone in the cytoplasm that avoids its premature interaction with other proteins prior to secretion (Woestyn *et al.*, 1996). Protein secretion through the ABC pathway is widespread and concerns several proteins and classes of proteins. The first identified protein secreted by such a pathway was the α -hemolysin (HlyA) secreted by some uropathogenic

Escherichia coli isolates (Goebel and Hedgpeth, 1982; Felmlee *et al.*, 1985; Holland *et al.*, 1990). In all cases, the secretion apparatus is made up of three proteins, the ABC protein, which contains the ABC domain, and the membrane fusion protein (MFP), both in the cytoplasmic membrane, and a specific outer membrane protein (Wandersman, 1992). There appears to be no periplasmic intermediate in this secretion pathway, and functional complexes can be isolated combined with either a secretable substrate or a jammed intermediate (Létoffé *et al.*, 1996). Interactions of the secretion functions have also been detected in the case of the colicin V, which is also secreted by such a pathway (Gilson *et al.*, 1990; Hwang *et al.*, 1997). The ABC protein specifically recognizes the substrate by its C-terminal secretion signal, and this triggers the sequential association of the MFP and outer membrane component into a functional complex (Delepelaire, 1994; Binet and Wandersman, 1995; Létoffé *et al.*, 1996). There are specific interactions between the ABC protein and the secreted substrate, between the outer membrane protein and the MFP component (Binet and Wandersman, 1995) and between the ABC protein and the MFP component (Akatsuka *et al.*, 1997; Hwang *et al.*, 1997). We have described the secretion of the highly homologous metalloproteases PrtA, B, C and G from *Erwinia chrysanthemi* and PrtSM from *Serratia marcescens*, and of the heme-binding protein HasA from *S.marcescens* through their respective ABC transporters reconstituted in *E.coli* (Ghigo *et al.*, 1994; Létoffé *et al.*, 1993, 1994a,b). All these proteins are secreted by the Has secretion apparatus recognizing C-terminal secretion signals despite the lack of primary sequence similarity of their C-terminal secretion signals. The Has secretion apparatus is made up of HasD, the ABC protein, HasE, the MFP component and HasF (Binet and Wandersman, 1996) or TolC, the outer membrane component. In contrast, the protease secretion apparatus, made up of PrtD, the ABC protein, PrtE, the MFP component and PrtF, the outer membrane protein, can secrete only the proteases and not HasA; moreover, overproduction of HasA in a protease-secreting strain leads to an inhibition of protease secretion (Létoffé *et al.*, 1994b). We have used the affinity of HasA and of a chimeric glutathione S-transferase (GST)–PrtC for hemin–agarose and glutathione–agarose respectively to show the association of these substrates with the secretion functions (Létoffé *et al.*, 1996).

The ABC secretion signal is in most cases at the C-terminus of the secreted protein, which implies that the protein is fully synthesized before the recognition step. Thus the secretion apparatus may translocate the fully folded protein and/or unfold the secreted polypeptide. Alternatively, the folding of the polypeptide following synthesis in the cytoplasm may be slow enough with respect to the secretion step for folding to occur outside

Fig. 1. Affinity purification of GST and GST-PrtC fusion protein on glutathione-agarose. Lanes 1–4, stained gel; lanes 5–8, immunodetection with anti-GST antibodies; lanes 9–12, immunodetection with anti-SecB antibodies. Lanes 1, 5 and 9, soluble extracts from *E. coli* C600(pGEX1) synthesizing GST; lanes 2, 6 and 10, soluble extracts from *E. coli* C600(pGST-PrtC) encoding the chimeric GST-PrtC; lanes 3, 7 and 11, proteins eluted from the affinity column with glutathione from C600(pGEX-1); lanes 4, 8 and 12, proteins eluted from the affinity column with glutathione from C600(pGST-PrtC). The asterisk in lane 4 indicates the sequenced protein, SecB.

the cell. The α -hemolysin is secreted from various uropathogenic *E. coli* isolates by an ABC pathway: none of the usual chaperones (SecB, GroEL/ES) are involved in its secretion, leaving both possibilities open (Blight and Holland, 1994); moreover there are late folding steps in the α -hemolysin secretion pathway which occur at the surface of the bacteria (Stanley *et al.*, 1993). In this study, we used the chimeric GST-PrtC protein to detect association with cytoplasmic proteins in the absence of the secretion functions and tested their relevance to the secretion process.

Results

***SecB* is co-precipitated with a chimeric GST-PrtC protein which blocks protease secretion through its ABC transporter**

The chimeric GST-PrtC protein inhibits protease and HasA secretion by making complexes with the secretion functions (Létoffé *et al.*, 1996). We searched for proteins bound to this chimera in the soluble fraction of the cell in the absence of the secretion functions (Figure 1). GST did not bind any protein (Figure 1, lane 3). The preparation of GST-PrtC affinity purified on glutathione-agarose from the soluble fraction gave several protein bands on SDS-PAGE. Besides the full-length GST-PrtC at 70 kDa, the most prominent of these bands was at ~16 kDa (Figure 1,

lane 4); several other bands can be assigned to degradation products of the fusion itself since they were recognized by antibodies specific for GST (Figure 1, lane 8) or PrtC (not shown). The N-terminal sequence of the 16 kDa protein was determined: $_2$ HN-SEQNNTEM-COOH, which is identical to the N-terminus of the SecB protein. The identity of this protein with SecB (17 064 Da) was confirmed by its recognition by anti-SecB antibodies (Figure 1, lane 12). A proportion of the GST-PrtC protein was found in the insoluble fraction after centrifugation of the cell lysate but was nevertheless associated with SecB (as determined by detergent solubilization of that fraction and glutathione affinity chromatography, not shown); this accounts for the lower amount of SecB found in the whole soluble fraction of the C600 (pGST-PrtC) as compared with that of C600 (pGEX1) (Figure 1, lanes 9 and 10).

The precursor of the periplasmic SecB-dependent maltose-binding protein accumulates in the presence of the GST-PrtC chimeric protein

SecB is a cytosolic protein involved in the exportation of various envelope proteins (Collier, 1993). It has two main functions: it retards the folding of precursors by binding to specific sites on the unfolded protein (Collier *et al.*, 1988) and it targets the protein to SecA, the ATPase of the Sec system (Hartl *et al.*, 1990). SecB is not essential, and its absence mainly leads to kinetic defects in precursor processing (Kumamoto and Beckwith, 1985; Kumamoto and Gannon, 1988). The export of the periplasmic maltose-binding protein, product of the *maltE* gene, is very SecB dependent, and some MalE variants interfere with the normal export of wild-type MalE by trapping SecB into stable complexes (Collier *et al.*, 1988). In particular, a variant form of MalE, MalE Δ 323, carrying a deletion from amino acid 7 to 89, from within the signal sequence into the mature part of the protein, leads to a very strong interference phenotype (Collier *et al.*, 1988) which can be used as an *in vivo* test of SecB binding. Interaction between SecB and its natural ligands is by definition transient since SecB can only bind denatured proteins and there is a kinetic partitioning between folding and SecB binding. Stable complexes between SecB and its natural ligands can only be observed under those conditions which stabilize the denatured state or with modified proteins whose folding properties have been strongly altered, like the MalE Δ 323 variant.

We tested whether the GST-PrtC chimeric protein caused a similar interference phenotype. In the presence of the GST-PrtC protein, the precursor form of the MalE accumulated following maltose induction (Figure 2, lane 8); this was not observed with the GST alone overproduced to a similar level as the GST-PrtC (Figure 2, lane 4 and stained gel, upper part), indicating that overproduction of the cytoplasmic GST does not lead to SecB trapping. As a positive control for interference, we used MalE Δ 323, which shows a very strong interference phenotype (Figure 2, lane 12), and the *secB::Tn5* strain (Figure 2, lane 14). The accumulation of MalE precursor in the presence of GST-PrtC probably reflects slowing down of precursor processing and hence its translocation across the inner membrane due to SecB trapping by GST-PrtC. Thus the observed co-isolation of SecB and GST-PrtC on glutathione-agarose may reflect an interaction *in vivo*.

Fig. 2. Accumulation of maltose-binding protein precursor (pMalE) in various strains. Staining of whole cell lysates (upper part) and immunodetection of MBP (MalE) and of its precursor (pMalE) in those lysates (lower part) of various strains harboring different plasmids, as indicated on the top of the figure; each strain was grown either in the presence or absence of 0.4% maltose to induce synthesis of MalE. Odd lanes, no maltose; even lanes, 0.4% maltose. Cells were grown in rich medium (lanes 1–12) or M9 minimal medium (lanes 13–16). Lanes 1 and 2, C600(pGEX1), no IPTG; lanes 3 and 4, C600(pGEX1), 50 μ M IPTG to induce the synthesis of GST; lanes 5 and 6, C600(pGST-PrtC), no IPTG; lanes 7 and 8, C600(pGST-PrtC), 50 μ M IPTG to induce the synthesis of GST-PrtC; lanes 9 and 10, MC4100; lanes 11 and 12, MC4100(pJF32) synthesizing the SecB-interfering protein MalE Δ 323; lanes 13 and 14, CK1953 (no SecB synthesized); lanes 15 and 16, MC4100. The stained gel was run on a 12.5% polyacrylamide gel, whereas the gel used for immunodetection was 8% polyacrylamide. In the stained gel, 0.2 OD_{600 nm} were loaded on each lane; for immunodetection, 0.025 OD_{600 nm} was loaded in each lane, except for lanes 7 and 8 where 0.050 OD was loaded. GST and GST-PrtC respectively indicate the positions of GST in lanes 3 and 4 and of the chimeric GST-PrtC in lanes 7 and 8. The scale on the right indicates the molecular weight in kDa.

The precursor of MalE accumulates in the presence of overproduced PrtB or HasA

The GST-PrtC chimeric protein, however, is not secreted by an ABC transporter and, on the contrary, blocks secretion. The SecB/GST-PrtC interaction might thus be a property of the fusion itself but not of the secreted protein. We therefore tested whether the full-length PrtB protein, which is 77% identical and 90% homologous to PrtC, and HasA interfere with MalE export (Figure 3). When produced from a high copy number plasmid under the control of the *lac* promoter, PrtB caused the accumulation of the MalE precursor (Figure 3, lane 4); under those

Fig. 3. Immunodetection of maltose-binding protein (MalE) and of its precursor (pMalE) in whole cell lysates from MC4100 cells harboring various plasmids grown at 37°C in rich medium with or without 0.4% maltose to induce MalE synthesis, after polyacrylamide gel electrophoresis. Odd lanes, no maltose; even lanes, 0.4% maltose. Lanes 1 and 2, MC4100(pEMBL18+), control plasmid; lanes 3 and 4, MC4100(pRUW500), encoding the PrtB protease; lanes 5 and 6, MC4100(pUC18), control plasmid; lanes 7 and 8, MC4100(pSYC134), encoding the HasA protein; lanes 9 and 10, MC4100(pBGS18+), control plasmid; lanes 11 and 12, MC4100(pSYC86), encoding HasA Δ 86, a C-terminally truncated form of HasA. The equivalent of 0.025 OD_{600 nm} was loaded in lanes 1, 2, 5, 6, 9 and 10; of 0.075 OD_{600 nm} in lanes 3 and 4; of 0.150 OD_{600 nm} in lanes 7 and 8; and of 0.200 OD_{600 nm} in lanes 11 and 12.

conditions, PrtB accounts for ~1% of the total proteins (not shown). This was also observed with the HasA protein produced from a high copy number plasmid under the control of the *lac* promoter (Figure 3, lane 8); under those conditions, HasA accounts for ~5% of the total proteins (not shown). Accumulation of the precursor form of the MalE protein was lower with overproduction of HasA than PrtB but was higher than with the control plasmid (Figure 3, lane 8). The accumulation of MalE precursor was fully reversed by the overexpression in the same cell of the *secB* gene (not shown). We also tested whether a truncated form of HasA devoid of its C-terminal secretion signal caused interference. HasA Δ 86, deleted from amino acid 132 to 189, led to a very strong interference phenotype, with almost 50% of all MalE produced accumulating as cytoplasmic precursor (Figure 3, lane 12); under those conditions, the HasA Δ 86 accounts for ~5% of the total proteins (not shown). The phenotype was only partially reversed by overexpression of the *secB* gene (not shown). These observations suggest that both HasA and PrtB interact with SecB. We therefore investigated the role of SecB in ABC secretion.

Protease secretion is independent of SecB; HasA secretion is SecB dependent

The secretion of HasA and PrtB was studied in *secB*⁺ and *secB*::Tn5 strains (Figures 4 and 5). The secretion of the protease by the protease transporter was the same in the *secB*::Tn5 and *secB*⁺ backgrounds (Figure 4, lanes 1 and 2); the secretion of C-terminal fragments of the protease was also independent of SecB (not shown). The secretion proteins PrtD, PrtE and PrtF were found in roughly equal amounts in the membrane fractions of both *secB*⁺ and *secB*::Tn5 strains (Figure 4, lanes 3 and 4), indicating that SecB probably does not interfere substantially with the localization of the secretion functions. The amounts and kinetics of PrtB and C secretion in the *secB*⁺ and *secB*::Tn5 backgrounds as assessed by pulse-chase experiments were indistinguishable (not shown).

Fig. 4. Secretion of PrtB protease and detection of protease secretion functions in SecB⁺ and SecB⁻ cells. Analysis of supernatants by SDS-PAGE (lanes 1 and 2, stained gel) and of membrane pellets after immunodetection with anti-PrtD, PrtE and PrtF antibodies (lanes 3 and 4) from MC4100 and MC4100*secB::Tn5* harboring pRUW500-238, encoding the PrtB protease, and pRUW4*inh1*, encoding the PrtD, E and F secretion functions, grown in M9 minimal medium at 30°C. The equivalent of 2 OD_{600 nm} was loaded in lanes 1 and 2 and of 1 OD_{600 nm} in lanes 3 and 4.

In contrast, the steady-state level of HasA secretion in a *secB::Tn5* background was <10% of that in the *secB*⁺ background. Secretion activity was complemented by a *secB*-expressing plasmid (Figure 5A, lanes 1–4). This effect did not depend upon the culture phase or upon the temperature (exponential versus stationary, 30 and 37°C, not shown). Pulse-chase experiments of cells of MC4100(pSYCAC1) and MC4100*secB::Tn5*(pSYCAC1) with [³⁵S]methionine followed either by immunoprecipitation with anti-HasA antibodies in the different compartments or by analysis of the supernatant indicated first that HasA synthesis was unaffected in the *secB::Tn5* background, second that it was secreted rapidly from the MC4100 cell whereupon it is stable in the supernatant, and third that only a small proportion was secreted in the *secB::Tn5* background and the part remaining in the cell is degraded with a half-time of ~30 min under our experimental conditions (Figure 5B and C). In addition, the HasA protein secreted in the *secB::Tn5* background appears to be secreted with slower kinetics than in the wild-type background (Figure 5C). This establishes that the *secB* mutation does not affect the synthesis of the secreted protein but rather affects its secretion.

To test whether SecB could affect the secretion apparatus, we studied the secretion of the C-terminal fragment of HasA via the Has secretion apparatus in both *secB*⁺ and *secB::Tn5* backgrounds (Figure 6): the secretion of

the C-terminal fragment of HasA was not significantly affected by the absence of SecB (Figure 6, lanes 3 and 4). This is very strong evidence that the function of the Has secretion apparatus is unaffected in the *secB::Tn5* background. Moreover, the amount of the C-terminal fragment of HasA secreted in the MC4100 background from the plasmid we used is not very different from that of the whole protein, indicating that this lack of effect on the secretion of the C-terminal fragment of HasA in the *secB::Tn5* background cannot be due to a very different production of the different constructs. Furthermore, the HasA secretion machinery appeared to be unaffected by the absence of SecB: the amounts of both HasD and TolC were similar in the *secB*⁺ and *secB::Tn5* backgrounds (Figure 6, lanes 1 and 2). Although it is not possible to detect the HasE protein, we assume it behaves like PrtE and that its expression will not be affected by the lack of SecB.

These experiments with the *secB::Tn5* strain were carried out in minimal medium, as this strain does not grow in rich medium (Kumamoto and Beckwith, 1985). HasA secretion was thus also tested in rich medium in the presence and absence of the SecB-interfering protein, MalEΔ323 (Figure 7, lanes 3 and 4); the presence of MalEΔ323 but not of wild-type MalE (not shown) strongly impaired HasA secretion, to a similar extent to that observed in the *secB::Tn5* background. MalEΔ323 did not inhibit the secretion of the protease by its cognate secretion functions (not shown). Thus functional depletion of SecB also leads to inhibition of HasA secretion.

A small proportion of HasA is bound to SecB after short labeling times and co-immunoprecipitation

Direct interactions between SecB and its ligands have first been detected by co-immunoprecipitation with anti-SecB antibodies after short labeling times and inhibition of the translocation by the Sec machinery. There are very few proteins bound to SecB when cells are grown in the presence of glycerol as carbon source; complexes of SecB with the precursor form of MBP have been detected in the presence of maltose as carbon source (Kumamoto, 1989; Kimsey *et al.*, 1995). Studies have also been carried out *in vitro* with purified components, namely the denatured forms of the precursor or the mature maltose- or galactose-binding proteins, and have shown that SecB binds the denatured state of the protein and that stable complexes can only be observed when folding kinetics are slow enough with respect to binding to SecB; furthermore, there is a rapid and dynamic equilibrium of the SecB ligands between the bound and free states (Topping and Randall, 1997). We have tried to show such complexes between HasA and SecB after *in vivo* labeling and co-immunoprecipitation with anti-SecB antibodies. SecB was immunoprecipitated readily, together with other proteins, some of which were specific since they were not observed in the *secB::Tn5* background (Figure 8A, lanes 4–6); overexposure of the autoradiogram (Figure 8B, lanes 4'–6') established that a band co-migrating with HasA was co-immunoprecipitated specifically with SecB under those conditions; this band was identified as HasA since it was not present in immunoprecipitates of MC4100 (Figure 8B, lane 4') and it could be immunoprecipitated with anti-HasA antibodies (not shown). The amount of co-immuno-

Fig. 5. Secretion (A) and synthesis (B and C) of HasA in SecB⁺ and SecB⁻ cells. Analysis of supernatants of MC4100 and MC4100secB::Tn5 harboring either pAC1 (allowing the synthesis and secretion of HasA, lanes 1 and 3) or pAC1 and pAK330 (allowing the overproduction of SecB, lanes 2 and 4) grown at 30°C in M9 minimal medium in stationary growth phase (stained gel); the equivalent of 1.5 OD_{600 nm} was loaded in each lane. The lower panel of (A) shows SecB immunodetection in the different strains (0.2 OD_{600 nm} was loaded) in exponential growth phase. (B) Pulse (lanes 1 and 2) and immunoprecipitation (lanes 3 and 4) with anti-HasA antibodies of whole cultures of MC4100 (lanes 1 and 3) and MC4100secB::Tn5 (lanes 2 and 4) harboring pAC1 and labeled with [³⁵S]methionine for 3 min. (C) Analysis of whole culture and cell pellet by immunoprecipitation with anti-HasA antibodies and of supernatant after TCA precipitation after a pulse-chase experiment of MC4100 and MC4100secB::Tn5 harboring pAC1; cells were pulse-labeled with [³⁵S]methionine for 3 min and chased for the indicated times. Samples in the extracellular medium were 1.5 times more concentrated than in the total or cell fractions.

precipitated HasA was <1% of the synthesized HasA (Figure 8). We also tried to show an interaction between HasA and SecB *in vitro* by using purified proteins; SecB did not alter the behavior of native HasA in non-denaturing polyacrylamide gel electrophoresis; on the contrary, the behavior of HasA which was first denatured and then renatured was clearly affected by the presence of SecB in the renaturation step (data not shown). As in the co-immunoprecipitation experiment, only a very small proportion of HasA was affected.

HasA secretion in *S.marcescens* is abolished by the SecB blocker, MalEΔ323

All the experiments described above were in *E.coli* with both secretion functions and secreted proteins expressed from plasmids. This does not represent the situation in the original host. In *S.marcescens*, HasA secretion is under the control of the Fur repressor and is induced only under iron-limiting conditions where it allows growth on heme or hemoglobin as the only iron source (Létoffé *et al.*, 1994a,b). We thus tested whether MalEΔ323 blocked HasA secretion in *S.marcescens* under iron-limiting conditions. A SecB analog has been detected in *S.marcescens* by cross-reaction with anti-SecB antibodies from *E.coli*, the structural gene of which has not been cloned (de Cock and Tommassen, 1991); this analog may be involved in HasA secretion and we therefore tested whether a SecB-interfering species would affect HasA secretion in *S.marcescens*. The MalEΔ323 variant had no effect on the secretion of the metalloprotease from *S.marcescens* SM8000 (Figure 9, lanes 1 and 2), whereas it very strongly inhibited the

Fig. 6. Right panel: analysis of supernatants from overnight cultures of MC4100 and MC4100secB::Tn5 harboring pSYC150 and pSYC138-322 respectively encoding the HasA secretion functions and the C-terminal peptide of HasA containing the secretion signal on tricine gels (lanes 3 and 4) (stained gel); the equivalent of 3 OD_{600 nm} was loaded in each lane. Left panel: immunodetection of TolC and HasD in membrane fractions of MC4100(pSYC150) and MC4100secB::Tn5(pSYC150); the equivalent of 0.5 OD_{600 nm} was loaded in each lane.

secretion of the HasA protein (Figure 9, lanes 3 and 4), which is almost completely abolished. The wild-type MalE had no effect on the secretion of either PrtSM or HasA in this strain (not shown).

Fig. 7. Analysis of supernatants (stained gel) from overnight cultures of MC4100(pAC1) (lane 1) and MC4100secB::Tn5(pAC1) (lane 2) grown in M9 minimal medium at 30°C and MC4100(pAC1) (lanes 3 and 5) and MC4100 (pAC1 + pJF32) (lane 4) grown in rich medium at 37°C (lanes 3 and 4); pAC1 allows the synthesis and secretion of HasA, and pJF32 encodes the SecB-interfering species MalEΔ323. Lane 5 contains one-tenth the amount of protein of lane 3; the equivalent of 0.5 OD_{600 nm} was loaded in lanes 1 and 2 and of 5 OD_{600 nm} in lanes 3 and 4.

Discussion

We report evidence for the involvement of the SecB chaperone in the secretion of a protein (HasA) through an ABC transporter across the Gram-negative *S. marcescens* envelope. SecB was identified in *E. coli* as a non-essential protein contributing to the efficiency of export of various proteins across the cytoplasmic membrane (Kumamoto and Beckwith, 1985; Collier, 1993). We show that a chimeric GST–PrtC protein which inhibits both protease and HasA secretion through their cognate transporters specifically binds SecB; we also show that both HasA and PrtB interfere with the export of the MalE protein, indicative of SecB trapping. However, only HasA secretion was SecB dependent whereas protease secretion was not. Furthermore, the secretion of HasA was completely inhibited in *S. marcescens* when MalEΔ323, which interferes with SecB, was produced. This is strong evidence that HasA secretion from *S. marcescens* might be dependent on the reported SecB analog (de Cock and Tommassen, 1991) or at least that MalEΔ323 might sequester some function involved in HasA secretion. This extends the known functions of SecB to extracellular proteins secreted by a Sec-independent pathway. We have shown that SecB is not involved at the level of synthesis of HasA nor could it be involved at the level of the three secretion proteins.

Direct interaction between SecB and HasA has been difficult to observe, and the co-immunoprecipitation

Fig. 8. Pulse (lanes 1–3) and immunoprecipitation (lanes 4–6) with anti-SecB antibodies of MC4100 (lanes 1 and 4), MC4100 harboring pSYC134-238, which encodes HasA (lanes 2 and 5), and MC4100secB::Tn5(pSYC134-238) (lanes 3 and 6). (A) The cells were pulse-labeled with [³⁵S]methionine for 30 s and immediately processed for immunoprecipitation as described in Materials and methods; 2% of the labeled cells were loaded in lanes 1–3 and 50% of the immunoprecipitates in lanes 4–6. (B) The overexposed HasA–SecB region of the same autoradiogram for lanes 4–6.

experiment indicated that a very small proportion of HasA co-precipitated with SecB; however, the stability of those complexes might be strongly dependent upon the physico-chemical conditions and the range of stability of the denatured protein, substrate of SecB. In the case of the MalE protein, between 10 and 40% of the synthesized MalE precursor is bound to SecB in similar experiments and it should be borne in mind that the signal sequence contributes to the maintenance of the unfolded state (Kumamoto, 1989; Kimsey *et al.*, 1995); the absence of SecB leads to a reduction of 40% of the exported MalE, with slower kinetics. In the case of HasA, the absence of SecB leads to a much greater defect in secretion since <10% of HasA is secreted in the secB::Tn5 background and almost no secretion occurs in *S. marcescens* in the presence of the SecB-interfering species MalEΔ323. It is thus possible that HasA folding is much faster than that of MalE precursor and that SecB–HasA complexes are much less stable than SecB–pMalE complexes; this would account for both the very strong effect of SecB depletion on HasA secretion and the very low amount of HasA bound to SecB in the co-immunoprecipitation experiment. Studies of folding kinetics of HasA *in vitro* after denaturation might help to answer this question. Our experiment strongly suggests that HasA is unfolded at some stage of the secretion process and that SecB helps maintain this state rather than acting indirectly. In this respect, the

Fig. 9. Analysis of *S.marcescens* supernatants by Coomassie Blue staining (lanes 1–4) and immunoblotting with anti-PrtSM (lanes 5–8) or anti-HasA (lanes 9–12) antibodies. *Serratia marcescens* SM8000 was grown in rich medium either without (lanes 1, 5 and 9) or with 0.4 mM dipyrityl (lanes 2, 6 and 10); SM8000 (pJF32) was grown in rich medium either without (lanes 3, 7 and 11) or with 0.4 mM dipyrityl (lanes 4, 8 and 12); pJF32 encodes the SecB-interfering species MalEΔ323. The equivalent of 1 OD_{600 nm} was loaded in each lane.

absence of structure of the C-terminal fragment of HasA in aqueous environments (Wolff *et al.*, 1997) is consistent with the lack of effect of SecB on its secretion, which in turn argues against an effect of SecB on the secretion apparatus. It is, however, still possible to make the ad hoc hypothesis that SecB could also act on an as yet unidentified exported protein specifically involved in the secretion of full-length HasA. The dynamic nature of the interaction of SecB with its ligands is clearly a distinctive feature as compared with the binding of the chaperones involved in type III secretion where very stable complexes can be isolated, and those chaperones are more likely to be anti-association factors rather than anti-folding factors (Woestyn *et al.*, 1996).

The residual secretion of HasA observed in the *secB::Tn5* background which occurs with slower kinetics than in the wild-type might be accounted for either by intrinsic secretion in the absence of SecB or by the action of other chaperones complementing SecB function, since the *secB::Tn5* mutation leads to elevated levels of other chaperones (Wild *et al.*, 1993).

The interacting domains of other SecB substrates have been mapped and appear to cover a central region of ~150–200 residues (Topping and Randall, 1994; Khisty *et al.*, 1995). In the case of HasA, which is 189 residues long, further studies should be carried out in order to identify those regions involved in SecB binding.

The protease does not need SecB for its secretion although it can cause interference, suggesting an interaction between the protease and SecB; possibly, as has

been observed with the R-BPTI (Randall, 1992), the protease is at least partially unfolded in the cytoplasm, exposing potential SecB-binding sites with no physiological relevance. Alternatively the lack of a SecB requirement for protease secretion may reflect a competition between folding and secretion strongly in favor of secretion, and sequences within the protease itself may allow only slow folding, thereby bypassing the requirement for SecB. MalE variants have been isolated which do not require SecB for translocation across the inner membrane but still bind SecB very tightly. They have been shown to be very slow folding forms of MalE (Weiss and Bassford, 1990). The case of PrtB protease may be analogous, and SecB could be required to secrete some protease variants. Two structural determinants of the protease may be involved in this process: the propeptide, which is autocatalytically cleaved after secretion, and the glycine-rich repeat region, which is not part of the secretion signal but improves the secretion of some passenger polypeptides (Létoffé and Wandersman, 1992). A similar glycine-rich repeat region is also found in α -hemolysin. Finally, it is conceivable that several chaperones might fulfill the same function due to their redundancy and that their effect can only be observed if several of them are altered.

α -hemolysin secretion and protease secretion are SecB independent, and hemolysin secretion is also independent of GroEL/ES (Blight and Holland, 1994). Other unidentified chaperones may be involved in their secretion, or their secretion may be fast with respect to the folding process, in which case they would not need chaperones at all. These observations are consistent with proteins secreted by ABC transporters being in at least partially unfolded conformations when interacting with the secretion machinery. Possibly this conformation is required for the secretion signal to interact with the ABC protein.

Materials and methods

Strains and media

Escherichia coli C600(F⁻ *thr leu fluA lacY thi supE*) and MC4100(*a-rad139 Δlac169 rpsL relA thi*) were from our laboratory collection; CK1953 (MC4100 *secB::Tn5*) was kindly provided by C.Kumamoto (Kumamoto and Beckwith, 1985); and *S.marcescens* SM8000 was kindly provided by K.Omori. They were grown either in rich medium (C600, MC4100 and SM8000) or in M9 minimal medium with 0.4% glycerol as a carbon source and thiamine (MC4100 and CK1953) with the appropriate antibiotics (Miller, 1972).

Plasmids

The following plasmids were used (see Table I). pGEX-1 encodes the wild-type GST (Smith and Johnson, 1988). pGST-PrtC encodes a chimeric GST–protease C fusion protein (Létoffé *et al.*, 1996). pJF2 and pJF32 encode the wild-type MalE and MalEΔ323 respectively (Fikes and Bassford, 1987; Collier *et al.*, 1988) and were kindly provided by T.Topping and L.Randall. pRUW500 is a high-copy number plasmid and encodes the protease B (Delepelaire and Wandersman, 1990). pSYC134 is a high copy number plasmid and encodes HasA (Létoffé *et al.*, 1994b). pSYC86 was generated during *hasA* sequencing; it encodes the 132 N-terminal amino acids of HasA and was kindly provided by S.Létoffé; these plasmids are derivatives of pEMBL8+ (Dente *et al.*, 1983), pUC18 (Yanisch-Perron *et al.*, 1985) and pBGS18+ (Spratt *et al.*, 1986) respectively. pRUW500-238 contains the same insert as that of pRUW500 in the low copy number plasmid pAM238 and was kindly provided by S.Létoffé. pRUW4*inh1* (Wandersman *et al.*, 1987) encodes the protease secretion proteins PrtD, PrtE and PrtF. pAK330 (Kumamoto and Nault, 1989) is a derivative of pBR322; it encodes SecB and was a gift from C.Kumamoto. pSYCAC1 encodes the HasA, HasD and HasE proteins and leads to HasA secretion in a *tolC*⁺ strain

Table I. Plasmids used in this study

Name	Encoded proteins	Promoter	Derivative of
pUC18			pUC
pEMBL18+			pUC
pBGS18+			pBR322
pGEX-1	GST	plac	pBR322
pGST-PrtC	GST-PrtC	plac	pEMBL
pRUW500	PrtB	plac	pAM238
pRUW500-238	PrtB	plac	pACYC184
pRUW4inh1	PrtDEF	ptet	pACYC184
pSYCAC1	HasADE	ptet	pUC
pSYC134	HasA	plac	pAM238
pSYC134-238	HasA	plac	pBGS
pSYC86	HasAΔ86	plac	pBR322
pSYC138-322	CterHasA	plac	pACYC184
pSYC150	HasDE	ptet	pBR322
pAK330	SecB	ptet	pBR322
pJF32	MalEΔ323	ptet	pBR322
pJF2	MalE	ptet	pBR322

GST-PrtC, chimeric GST-protease C fusion protein; PrtB, secreted metalloprotease B; PrtDEF, metalloprotease secretion functions; HasA, secreted heme-binding protein; HasDE, HasA secretion functions (the chromosomal *tolC* gene encodes the outer membrane component); HasAΔ86, N-terminal fragment of HasA (amino acids 1–132); CterHasA, secreted C-terminal peptide of 56 residues of HasA fused to the first 12 residues of β -galactosidase; SecB, SecB chaperone; MalE, maltose-binding protein; MalEΔ323, variant MalE protein with an internal deletion (amino acids 7–89), strongly interfering with MalE export.

(Ghigo *et al.*, 1997). pSYC150 encodes the HasD and HasE secretion functions (Létoffé *et al.*, 1994b). pSYC134-238 contains the same insert as pSYC134 in the low copy number plasmid pAM238 (Létoffé *et al.*, 1994b). pSYC138-322 contains a 0.52 kb *PvuII*–*SalI* fragment from pSYC138 between the *EcoRV* and *SalI* sites of pBR322 and encodes the C-terminal fragment of HasA containing the secretion signal; it was kindly provided by N. Wolff.

Purification of GST and GST-PrtC

C600 cells harboring the recombinant plasmid pGEX1 encoding GST under the control of the *lac* promoter, or plasmid pGST-PrtC encoding a chimeric GST-PrtC fusion protein under the control of the *lac* promoter were grown at 30°C in LB medium in the presence of 20 μ M (pGEX-1) or 80 μ M (pGST-PrtC) isopropyl- β -D-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation once they reached an OD_{600 nm} of 1.0. GST alone and its fusion protein were purified by glutathione affinity chromatography as follows: the cells were washed once and resuspended at 50 OD_{600 nm}/ml in 100 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were lysed in a French press cell operated at 10 000 p.s.i.; the insoluble material was removed by centrifugation at 50 000 g for 1 h and the supernatant was incubated with glutathione-agarose (~0.5 ml of gel per 1000 OD initial material) at 4°C for 1 h on a rotary shaker. The agarose was collected by low speed centrifugation and washed five times with the same buffer. The bound proteins were then eluted with several 0.5 ml washes of the same buffer containing 10 mM glutathione and precipitated with 20% trichloroacetic acid (TCA); the precipitated proteins were analyzed by gel electrophoresis.

Detection of precursor and mature form of maltose-binding protein

Cells of various strains harboring different recombinant plasmids were grown at 37°C to early log phase (OD₆₀₀ = 0.2–0.4), and the culture split into two. Maltose was added to a final concentration of 0.4% to one of the aliquots and incubation was continued. Cells were harvested after one more generation (OD = 0.4–0.8); 1 mM PMSF was added immediately to inhibit protease cleavage of the MalE precursor. Whole cell lysates were immediately prepared for electrophoresis and run on 8% polyacrylamide gels; anti-MalE antibodies, kindly provided by J.-M. Betton, were used diluted 1/20 000 for immunodetection.

Preparation of supernatants and cell fractions; gel electrophoresis and immunodetection

Supernatants from various cultures were prepared as previously described (Wandersman *et al.*, 1987); proteins inside the cells were immunodetected in the insoluble cell fraction (PrtD, PrtE, PrtF, TolC, HasD) obtained after sonication. SDS-PAGE was routinely carried out on 12% polyacrylamide gels run according to Laemmli (1970) except where mentioned and for the C-terminal fragment of HasA which was detected on tricine gels (Schagger and von Jagow, 1987). In *S. marcescens*, HasA synthesis and secretion were induced by the addition of 0.4 mM (final concentration) of the iron chelator dipyrityl to the culture (Létoffé *et al.*, 1994b). Immunodetection after polyacrylamide gel electrophoresis and blotting on nitrocellulose membrane was carried out as previously described by using a secondary antibody coupled to alkaline phosphatase (Létoffé *et al.*, 1996). Anti-SecB antibodies were kindly provided by K. Kumamoto; the other antibodies have been described previously. In particular the anti-GST antibodies resulted as the product of immunizing rabbits with a fusion of GST and HasD, the ABC component of the Has transporter, and are also used as anti-HasD antibodies. The N-terminal sequence of the 16 kDa protein was determined after affinity purification at the Laboratoire de Microséquence de l'Institut Pasteur using an Applied Biosystem Procise Sequencer HT.

Pulse-chase experiments

Cells of MC4100(pSYCAC1) and MC4100secB::Tn5(pSYCAC1) were grown in M9 minimal medium at 37°C with 0.4% glycerol as a carbon source up to an OD_{600 nm} of 0.1, at which time they were harvested and concentrated to an OD of 2 in the same medium. One ml of cells was labeled for 3 min at 37°C with 10–50 μ Ci of [³⁵S]methionine (Amersham, 1000 Ci/mmol) and then chased with an excess of cold methionine and in the absence of protein synthesis. Aliquots of 50 and 200 μ l were withdrawn at the indicated time points and either directly mixed with TCA (final concentration 10%, total) or separated by a short centrifugation into supernatant and cell fractions, which were then precipitated with TCA. Immunoprecipitation with anti-HasA antibodies was then carried out before gel electrophoresis and autoradiography.

For the co-immunoprecipitation experiment, three cultures were grown in M9 minimal medium, MC4100, MC4100(pSYC134-238) and MC4100secB::Tn5(pSYC134-238), in the same conditions as the previous experiment. Labeling with [³⁵S]methionine was carried out for 30 s at 37°C; protein synthesis was blocked by the addition of kanamycin and the cells were quickly chilled and centrifuged. Spheroplasts were prepared, lysed and the resulting supernatant obtained after centrifugation used for immunoprecipitation with anti-SecB antibodies pre-adsorbed on extracts of MC4100secB::Tn5 and already bound to protein A-Sepharose beads. Immunoprecipitation was carried at 4°C for 1 h in 10 mM Tris-HCl pH 8.0 150 mM NaCl, 0.05% Triton X-100; immunoprecipitates were washed four times with the same buffer before gel electrophoresis and autoradiography.

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