The carboxyl terminus of the *Bacillus subtilis* SecA is dispensable for protein secretion and viability

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The *Escherichia coli* secretion-dedicated chaperone SecB targets a subset of proteins to the translocase by interacting with the carboxyl (C-) terminus of SecA. This region of SecA is highly conserved in Eubacteria, but despite its presence in the *Bacillus subtilis* SecA, the *B. subtilis* genome does not appear to contain a gene for a clear homologue of SecB. Deletion of the C-terminus of the *B. subtilis* SecA yields cells that have normal viability, but that exhibit a response reminiscent of oxidative stress and the loss of a number of secretory proteins from the culture supernatant. Semi-quantitative RT-PCR demonstrates that these proteins are expressed at lower levels. The C-terminus of SecA fused to glutathione S-transferase (GST) specifically binds a cytosolic protein, termed MrgA. This protein has been reported to function in relation to oxidative stress, but deletion of the *mrgA* gene does not result in a secretion defect nor does it cause an oxidative stress response. It is concluded that the C-terminus of the *B. subtilis* SecA is not essential for secretion and viability.

Keywords: protein secretion, chaperones, SecA, SecB

INTRODUCTION

Bacillus subtilis has become a paradigm for studies on protein secretion in Gram-positive bacteria primarily because bacilli have a high capacity for the production of exoenzymes. Protein secretion across the cytoplasmic membrane of *B. subtilis* is catalysed by a system that is homologous to the precursor protein translocase of *Escherichia coli* (Overhoff *et al.*, 1991; van Wely *et al.*, 1998) that has been studied in detail. In *E. coli*, protein translocation is mediated by cytosolic chaperones, the translocation ATPase SecA, and a large integral membrane protein complex with SecY, SecE, SecG, SecD and SecF as subunits (Driessen, 1996). Only SecA, SecE and SecY are essential for viability, and homologues have been identified genetically in *B. subtilis*. SecA is encoded by the *divA* gene (Sadaie *et al.*, 1991; Asai *et al.*, 1997), and was originally identified in a set of mutants

conditionally defective in division and sporulation. SecY (Suh *et al.*, 1990; Breitling *et al.*, 1994) and SecE (Jeong *et al.*, 1993) were identified after nucleotide sequence analysis of the chromosomal regions that contain the ribosomal *spc* operon and *nusG*, respectively. The analogous regions in *E. coli* contain *secY* and *secE*, respectively. Homologues of the SecG (Swaving *et al.*, 1999; van Wely *et al.*, 1999) and SecD/SecF (Bolhuis *et al.*, 1998) proteins have been identified by sequence analysis of the completed *B. subtilis* chromosome.

Studies in *E. coli* have demonstrated that prior to their interaction with SecA, precursor proteins may interact with the signal recognition particle (SRP) (Powers & Walter, 1997; Bunai *et al.*, 1999) or the export-dedicated molecular chaperone SecB (Kumamoto, 1989, 1991). Both pathways converge at the translocase (Valent *et al.*, 1998; Bunai *et al.*, 1999). The bacterial SRP consists of Ffh (Römisch *et al.*, 1989; Honda *et al.*, 1993) and 4·5S RNA (Struck *et al.*, 1989; Powers & Walter, 1997). In *E. coli*, SRP interacts with hydrophobic signal sequences of nascent precursor proteins, and targets these nascent chain-ribosome complexes to the membrane through the SRP receptor, FtsY (Luirink *et al.*, 1994; Oguro *et al.*, 1995). The *B. subtilis* Ffh specifically binds to precursor proteins (Bunai *et al.*, 1996) and promotes

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Abbreviations: Cam, chloramphenicol; GST, *Schistosoma japonicum* glutathione S-transferase; GST-C, GST fusion bearing the C-terminal 22 amino acids of *B. subtilis* SecA; Phle, phleomycin; SRP, signal recognition particle.

<i>Ec</i>	AQTGERKVG	GRND	PCPCGSGKKYK	CHGRLQ		901
<i>Va</i>	MVRDERKVG	GRND	PCPCGSGKKYK	CHGKID		908
<i>Hi</i>	EDYSRRIR	GRND	PCPCGSGKKYK	CHGSRVARQ		901
<i>Rc</i>	DPTTWGEP	SRND	PCPCGSGKKYK	CHGRLA		904
<i>Aa</i>	GTEEHKKI	GRND	PCPCGSGKKYK	CHGSRARH		899
<i>Cc</i>	LPEGWDR	TNRN	PCPCGSGKKYK	CHGSLVR		921
<i>Hp</i>	VKAFSTPK	RNRN	PCPCGSGKKYK	CCAKSGPKGLFA		865
<i>Af</i>	GTEEHKKI	GRND	PCPCGSGKKYK	CHGSRARH		899
<i>Bb</i>	VVRSSPKI	GRND	PCYCGSGKKYK	NCHGKS		899
<i>Tp</i>	PIQAGAKV	GRNT	PCPCGSGKKYK	CHGCCR		916
<i>Rp</i>	DPMSWGRV	SRND	PCPCGSGKKYK	CHGVNE		906
<i>Bs</i>	PVRKVVDI	GRNA	PCPCGSGKKYK	NCCGRTE		841
<i>Sc</i>	PVVKDNHI	GRND	PCPCGSGKKYK	NCCGK		844
<i>Sa</i>	PIVKGQV	GRND	PCPCGSGKKYK	NCHGK		843
<i>Lm</i>	PIRKQHI	GRND	PCPCGSGKKYK	NCHGKEA		836
<i>Mt</i>	RAQPRRDL	GRNE	PCPCGSGKKYK	CHLGREALPLAER		478
Consensus	GRND	PCPCGSGKKYK	CHG			
	E	F	C			

Fig. 1. Multiple sequence alignment of the C-terminal domains of bacterial SecA proteins. Black and grey shading indicate a higher or lesser degree of conservation. Organisms are indicated as follows: *Ec*, *Escherichia coli* (PIR:S40402); *Va*, *Vibrio alginolyticus* (EMBL:BAA33403); *Hi*, *Haemophilus influenzae* (PIR:H64068); *Rc*, *Rhodobacter capsulatus* (SWISS-PROT:P52966); *Aa*, *Actinobacillus actinomycetemcomitans* (EMBL:AAD22450); *Cc*, *Caulobacter crescentus* (SWISS-PROT:P38380); *Hp*, *Helicobacter pylori* (SWISS-PROT:O25475); *Af*, *Aquiflex aeolicus* (TREMBLNEW:G2982840); *Bb*, *Borrelia burgdorferi* (EMBL:AAC46347); *Tp*, *Treponema pallidum* (SWISS-PROT:Q83394); *Rp*, *Rickettsia prowazekii* (SWISS-PROT:Q9ZCX7); *Bs*, *Bacillus subtilis* (EMBL:E1186051); *Sc*, *Staphylococcus carnosus* (SWISS-PROT:P47994); *Sa*, *Staphylococcus aureus* (SWISS-PROT:O06446); *Lm*, *Listeria monocytogenes* (SWISS-PROT:P47847); *Mt*, *Mycobacterium tuberculosis* (EMBL:CAB09972). The conserved region of the *M. tuberculosis* SecA represents an internal sequence.

their binding to SecA (Bunai *et al.*, 1999). Depletion of components of the SRP pathway results in a partial block in protein translocation that seems to vary depending on the precursor protein (Oguro *et al.*, 1996, Hirose *et al.*, 2000).

In *E. coli*, SecB supports the translocation of a subset of proteins, mainly precursors of outer-membrane proteins (Kumamoto & Francetić, 1993). SecB binds to nascent precursor proteins (Randall *et al.*, 1997; Behrmann *et al.*, 1998), and holds them in a translocation-competent conformation (Lecker *et al.*, 1989). SecB subsequently targets these proteins to the SecYEG-bound SecA (Hartl *et al.*, 1990; Fekkes *et al.*, 1997). This targeting event is accomplished by the high-affinity binding of SecB to the carboxyl (C-) terminus of the SecYEG-bound SecA, whereas cytosolic SecA interacts with only poor affinity (Den Blaauwen *et al.*, 1997; Fekkes *et al.*, 1997). The interaction is stimulated by the presence of a precursor protein with a functional signal sequence (Fekkes *et al.*, 1998). During the ATP-dependent initiation of protein translocation, SecB is released from the SecYEG–SecA–precursor complex and recycled to the cytosol (Fekkes *et al.*, 1997).

SecB seems to be present in Gram-negative bacteria only (Fekkes *et al.*, 1998), in particular in *Enterobacteriaceae*. It is not required for viability of *E. coli* but its gene overlaps with *gpsA*, which encodes a biosynthetic sn-glycerol-3-phosphate dehydrogenase (Shimizu *et al.*,

1997). Disruption of the *gpsA* gene results in a severe growth defect when cells are grown on rich media (Shimizu *et al.*, 1997). On the other hand, the C-terminal SecB-interacting domain of *E. coli* SecA cannot be disrupted (Breukink *et al.*, 1995). SecA lacking this region still promotes protein translocation but no longer binds SecB nor supports SecB-dependent translocation (Fekkes *et al.*, 1997). Since SecB is obsolete, the observation that cells harbouring SecA with a deleted C-terminus are unable to grow is difficult to understand in terms of an impaired SecB function. SecB appears not to be present in Gram-positive bacteria. Strikingly, the C-terminal SecB binding domain is highly conserved among the bacterial SecA proteins (Fig. 1). In the case of *Mycobacterium tuberculosis* SecA, the conserved region is found in the middle of the SecA protein sequence. The conserved cysteine and histidine residues present in this domain are involved in the coordination of a zinc ion that stabilizes a tertiary structure of SecA that is needed for the interaction with SecB (Fekkes *et al.*, 1999).

We have investigated the role of the C-terminus of the *B. subtilis* SecA to determine if this region is involved in the binding of a hitherto unidentified homologue or analogue of the *E. coli* SecB. The data demonstrate that, unlike the situation in *E. coli*, the C-terminus of the *B. subtilis* SecA protein is not needed for viability and protein translocation. Deletion of this region results in aberrant expression of intracellular and secreted proteins and a response reminiscent of oxidative stress.

METHODS

Bacterial strains and growth media. Strains were grown in Luria–Bertani broth or agar. When necessary, the medium was supplemented with relevant antibiotics as indicated. Construction of vectors and expression of GST fusion proteins was done with *E. coli* DH5 α [*supE44* Δ *lacU169* (ϕ 80*lacZ*AM15) *hsdR17* *recA1* *endA1* *gyrA96* *thi-1* *relA1*]. All other experiments were done with *B. subtilis* DB104 [*nprE18* *aprE* Δ 3] (Yang *et al.*, 1984).

Chromosomal disruptions. Deletion of the 22 C-terminal amino acids of SecA (Fig. 1) was accomplished as follows. A gene fragment corresponding to amino acids 670 to 819, followed by a stop codon, was amplified from chromosomal DNA from strain DB104 as a *SacI*–*XbaI* PCR fragment, and cloned into pBluescript SK+. Subsequently, a *XbaI*–*PstI* fragment containing a phleomycin (Phle) resistance marker and the 1.1 kb *PstI*–*NcoI* chromosomal downstream region were cloned downstream of the partial *secA* gene, resulting in plasmid pDEL C. This vector contains the DB104 chromosomal region with the C-terminal 22 amino acids of the *secA* gene replaced by a Phle resistance marker. Vector pDEL C was linearized with *PvuII* to yield a 2.5 kb *secA* Δ C::phleo fragment and subsequently transformed into *B. subtilis* DB104 by natural competence (Young, 1967). Phle-resistant colonies resulting from a double crossover were selected. The correct position of the chromosomal replacement was confirmed by PCR. In the resulting strain, DB104 Δ C, the *secA* gene encodes only the first 819 amino acids of the protein.

The *mrgA* gene of *B. subtilis* DB104 was disrupted as follows. Regions immediately upstream and downstream of *mrgA* were amplified from the chromosome of strain DB104 as *Bam*HI–*XbaI* and *KpnI*–*HincII* cassettes, respectively, and

cloned into pBluescript SK+. Subsequently, a *Bgl*II-*Pvu*II-digested chloramphenicol (Cam) resistance marker was placed between the *Bam*HI and *Hinc*II sites, yielding pDELM. This vector contains the DB104 chromosomal region with the *mrgA* gene replaced by the Cam resistance marker. Vector pDELM was linearized with *Pvu*II to yield a 2.8 kb fragment containing the *mrgA::cam* region and subsequently transformed into *B. subtilis* DB104 by natural competence. Cam-resistant colonies resulting from a double crossover were selected. The correct position of the chromosomal replacement was confirmed by PCR. In the resulting strain, DB104ΔM, the Cam resistance gene replaced the *mrgA* gene while leaving the flanking regions intact. Strain DB104ΔCM, carrying both mutations, was generated by transforming DB104ΔM with chromosomal DNA from strain DB104ΔC and selecting for Cam and Phle resistance. Since the mutations cause a complete deletion, no selective pressure is needed after the initial selection.

A vector expressing a fusion product of *Schistosoma japonicum* glutathione S-transferase (GST) and the C-terminal 22 amino acids of SecA was generated by ligation of a 102 bp *Eco*RV-*Nar*I fragment from pMKL4 (Klose *et al.*, 1993) into *Sma*I-*Acc*I-digested pGEX4T2 (Pharmacia), yielding pET446.

Binding of cytosolic proteins to the C-terminus of SecA. GST and GST-C were purified from cells bearing plasmids pGEX4T2 and pET446, respectively, according to the manufacturer's procedures. After purification, glutathione was removed by dialysis against 50 mM potassium phosphate, pH 7.0, 100 mM NaCl (buffer A). For the preparation of a cytosolic lysate, *B. subtilis* cells were broken by French pressure treatment [three times at 8000 p.s.i. (55 MPa)] and debris was removed by centrifugation at 60000 g for 45 min. For the binding experiments, GST or GST-C (5 mg) was mixed with the supernatant fraction of the lysate (100 mg total protein) in buffer A, and incubated for 30 min on ice. Subsequently, GST or GST-C was reisolated on glutathione Sepharose, washed with 20 column volumes of buffer A, and eluted with one column volume of 25 mM reduced glutathione or alternatively with 50 mM EDTA. The eluted fractions were precipitated with 10% TCA (final concentration), washed twice with cold acetone and analysed by SDS-PAGE.

Analysis of cellular and secreted proteins. *B. subtilis* DB104 and its derivatives were grown at 37 °C in liquid medium. Overnight cultures were diluted 1:50 into fresh medium and grown to the late exponential phase. Cultures were cooled on ice and fractionated into a cellular and a medium fraction by centrifugation. The medium fraction was precipitated with 10% TCA, washed twice with cold acetone and analysed by SDS-PAGE (van Wely *et al.*, 1999). Cellular pellets were resuspended in sample buffer, sonicated and analysed by SDS-PAGE.

Semi-quantitative RT-PCR. Total RNA was isolated from exponentially growing cultures using a total RNA isolation kit (QIAGEN) according to the manufacturer's indications. First-strand synthesis was accomplished in a single reaction using the access RT-PCR system (Promega) and with primer pairs that specify *wapA* (forward, 5'-TGTTAAGTCATGGAAC-TCCGG-3'; backward, 5'-ATCTAATGCCAATTCAGCT-CC-3'), *yweA* (forward, 5'-TCTTACTTCCTTCGGGCCA-AGC-3'; backward, 5'-CCCAAACCGTTTACTACATCGC-C-3') or *yolA* (forward, 5'-TTGCTCTTCTAGCAGTTG-TTGC-3'; backward, 5'-TTTATCCCAGCCTATTACCAC-C-3'). Subsequently, the relative levels of cDNA were determined in a multiplex PCR in the presence of [³²P]dATP with the *gap* (glyceraldehyde-3-phosphate dehydrogenase) primer pair (forward, 5'-TGCACAACAACTGCCTTGC-

GC-3'; backward, 5'-TTTACCATGCTGCCTTCCATAAC-3') alone or in combination with one of the other primer pairs. The PCR products formed after 20 cycles of amplification were separated by electrophoresis on a 6% denaturing acrylamide gel and quantitated by phosphor-imaging and ImageQuant software (Molecular Dynamics). Values were corrected for gel loading.

Miscellaneous methods. Pulse-chase experiments with *B. subtilis* DB104 and DB104ΔC expressing *E. coli* proOmpA or *Bacillus licheniformis* preAmyL were carried out as described before (Meens *et al.*, 1993). Protein concentrations were determined by the method of Lowry, using BSA as standard. For N-terminal sequencing of polypeptides, samples were separated by SDS-PAGE and blotted onto PVDF. Sequencing was done by the NAPS facility at the University of British Columbia (Vancouver, Canada).

RESULTS

The C-terminus of SecA is not essential for viability and protein secretion

The SecB-interacting domain of the *E. coli* SecA is localized in the C-terminal 22 amino acids (Fekkes *et al.*, 1997; Bunai *et al.*, 1999). This domain is highly conserved among the SecA proteins of Gram-negative and most Gram-positive bacteria, including *B. subtilis* SecA (Fig. 1). To define the *in vivo* function of this domain in *B. subtilis*, the last 22 codons of the chromosomal *secA* gene were replaced by a stop codon. In the resulting strain, DB104ΔC, the *secA* gene encodes amino acids 1–819. The truncated SecA protein produced by strain DB104ΔC showed a corresponding higher mobility on SDS-PAGE (Fig. 2). The deletion strain had normal viability and was capable of growing at a rate only slightly slower than the parental strain (data not shown).

To investigate a possible secretion defect, culture supernatants of *B. subtilis* DB104 and DB104ΔC were compared (Fig. 3a). In the culture supernatant fraction of strain DB104ΔC, a considerable number of proteins appeared to be absent (Fig. 3a, compare lanes 1 and 2). Polypeptides present in the culture supernatant of strain DB104, but absent from that of DB104ΔC were blotted and N-terminally sequenced. The resulting sequences QSASIEAK and AEALPLYL correspond to the mature parts of the secretory proteins YweA (molecular mass 16545 Da) and YolA (17086 Da), respectively. These two proteins co-migrate on SDS-PAGE. Since the yield of the two proteins in the N-terminal sequencing showed

Fig. 2. Western blot analysis of SecA protein from strains DB104 (lane 1) and DB104ΔC (lane 2). Equal amounts of cellular protein were separated on SDS-PAGE, blotted, and incubated with antibodies directed against *B. subtilis* SecA.

Fig. 3. Coomassie-stained SDS-PAGE of the culture supernatant (a) and cellular (b) fractions of *B. subtilis* strains DB104 (lane 1), DB104ΔC (lane 2), DB104ΔM (lane 3), and DB104ΔCM (lane 4). Cultures were grown to late exponential phase at 37 °C. The position of the molecular mass markers is indicated on the left. The position of AhpC and the tentatively identified AhpF, KatA and MrgA proteins, and of the aberrantly expressed proteins, is indicated on the right.

Fig. 4. Pulse-chase experiments of preAmyL (a) and proOmpA (b) in *B. subtilis* DB104 and DB104ΔC. Cells were labelled with [³⁵S]methionine as described before (Meens *et al.*, 1993), and chased for the indicated times. Positions of AmyL, proOmpA and OmpA are indicated.

an equimolar distribution and the polypeptide band has disappeared completely from the mutant, we assume both proteins are affected equally. The 28 kDa protein had a peptide sequence of EVYLDPIH, which corresponds to an internal fragment of WapA (molecular mass 258022 Da). The N-terminal sequence of the 45, 50 and 100 kDa proteins could not be determined. In addition, some extra bands appeared in the culture supernatant of strain DB104ΔC, but these bands correspond to cytosolic polypeptides that are expressed to a high level in this strain (see below), indicating that some lysis has occurred (compare Fig. 3a and 3b).

To examine if the SecAΔC protein supports efficient secretion of precursor proteins, translocation of *B. licheniformis* preAmyL (Fig. 4a) and *E. coli* proOmpA (Fig. 4b) was analysed in strains DB104 and DB104ΔC by pulse-chase experiments. ProOmpA was chosen because this protein requires SecB for efficient secretion by *E. coli*. Due to the rapid processing of the precursor, only the mature AmyL protein was detected in either the parental or the mutant strain. Importantly, the mutant strain did not accumulate any precursor, suggesting that translocation is not impaired. With proOmpA, a significant portion of the protein could be detected in the

Fig. 5. Binding of the *E. coli* SecB to the C-terminal domain of the *B. subtilis* SecA. The amount of SecB protein associated with (lane 1) GST or (lane 2) the GST-C fusion protein isolated from *E. coli* was determined by Western blot analysis using polyclonal antibodies against SecB.

precursor form after the first chase time and nearly all protein was processed within 10 min. The processing of proOmpA in strain DB104ΔC was somewhat slower, but still most of the precursor was processed after 10 min. In both the mutant and the parental strain, translocation of proOmpA was accompanied by proteolysis of the translocated protein into the 16 and 18 kDa fragments (data not shown) (Meens *et al.*, 1997). Taken together these data show that strain DB104ΔC is only marginally affected in its secretion abilities, and that the C-terminus of SecA has no function in protein secretion in *B. subtilis*.

Deletion of the C-terminus of SecA suppresses the expression of *wapA* and *yweA* genes

Since the pulse-chase experiments did not show striking differences between strains DB104 and DB104ΔC, the absence of several protein bands in the supernatant of the DB104ΔC cells may not result from a secretion defect but may be caused by an altered expression of the corresponding genes. To obtain an estimate of the relative expression levels of WapA, YweA and YoaA in both strains, semi-quantitative RT-PCR was performed. Taking the level of the *gap* (glyceraldehyde-3-phosphate dehydrogenase) mRNA transcript as 1.0 (the absolute level was identical in DB104 and DB104ΔC), the *wapA* and *yweA* transcript levels were 0.45 and 0.89 for DB104, and undetectable and 0.27 for DB104ΔC. No significant levels of *yoaA* mRNA could be detected in either strain. These data strongly suggest that a lowering of the expression level accounts for the absence of protein bands in the culture supernatant of strain DB104ΔC.

The C-terminus of *B. subtilis* SecA binds *E. coli* SecB and *B. subtilis* MrgA

When fused to GST, the C-terminal 22 amino acids of the *E. coli* SecA have been shown to constitute a genuine SecB-binding site (Fekkes *et al.*, 1997). To identify a possible binding partner in *B. subtilis*, the C-terminal 22 amino acids of *B. subtilis* SecA were fused to GST to yield GST-C. Both GST and GST-C were expressed in *E. coli* and purified by glutathione-affinity chromatography. *E. coli* SecB specifically co-purified with the GST-C fusion protein as detected by Coomassie-stained SDS-PAGE (data not shown) and Western blotting (Fig. 5). The same binding was detected before with the GST

fusion protein harbouring the C-terminal 22 amino acids of the *E. coli* SecA (Fekkes *et al.*, 1997). Binding studies with purified *E. coli* SecB confirmed the interaction with the GST-C fusion protein harbouring the *B. subtilis* sequence (data not shown). It is, therefore, concluded that the C-terminus of the *B. subtilis* SecA provides a valid binding site for SecB.

Next, cytosol of *B. subtilis* DB104 was incubated with GST and GST-C, and bound polypeptides were analysed by SDS-PAGE. A larger number of proteins associate non-specifically with both GST and GST-C. However, one polypeptide with an apparent molecular mass of 18 kDa was found to specifically copurify with GST-C. Particularly high amounts of this protein bound to GST-C when cytosol derived from strain DB104ΔC was used (Fig. 6), although other proteins are expressed to much higher levels in this strain (Fig. 3b). Blotting and subsequent N-terminal amino acid sequencing identified the 18 kDa polypeptide as the MrgA protein. MrgA could be eluted from its GST-C bound form by 50 mM EDTA under conditions where GST-C remains attached to the column material.

Deletion of the C-terminus of SecA results in an oxidative stress response

Since the deletion of the C-terminus of SecA appears to affect protein secretion in an indirect manner, we also compared the cellular polypeptide patterns of *B. subtilis* DB104 and DB104ΔC (Fig. 3b). Striking differences between DB104ΔC and its parental strain were also evident in the cellular fractions. Strain DB104ΔC showed a dramatic increase in the level of proteins with apparent molecular masses of 25, 55 and 120 kDa, and a decreased level of a protein with an apparent molecular mass of 44 kDa (Fig. 3b). The 25 kDa protein had an N-terminal sequence of MSLIGKEV, which corresponds to AhpC (20482 Da), a protein involved in oxidative and other forms of stress (Völker *et al.*, 1994; Antelmann *et al.*, 1996). It was not possible to identify the 55 and 120 kDa proteins by N-terminal sequencing. However, they probably correspond to AhpF (54705 Da) and KatA (54567 Da) running together, and MrgA, respectively. These proteins are expressed together with AhpC in a coordinated fashion (Chen *et al.*, 1995; Antelmann *et al.*, 1996), and their calculated molecular mass is in perfect agreement with the observed polypeptide bands. The protein band at 44 kDa was not investigated further.

AhpF functions together with AhpC in the reduction of alkyl hydroperoxides (Chen *et al.*, 1995), whereas KatA is involved in the reduction of hydrogen peroxide (Bsat *et al.*, 1996). MrgA is synthesized as a 17.3 kDa polypeptide, but has been reported to exist in a high-molecular-mass complex that does not completely dissociate even after boiling in SDS (Chen & Helmann, 1995). The high-molecular-mass form of MrgA confers resistance to oxidative challenge, whereas the form bound by GST-C seems to exclusively represent the monomeric form (Fig. 6). These data indicate that the

Fig. 6. Specific binding of MrgA to the C-terminus of the *B. subtilis* SecA. A buffer control (lanes 1 and 4), or a cytosolic lysate of *B. subtilis* DB104 (lanes 2 and 5) and DB104ΔC (lanes 3 and 6) was loaded on a glutathione Sepharose column with bound GST (lanes 1–3) or GST-C fusion protein (lanes 4–6), washed, and the bound proteins were eluted with reduced glutathione (a) or EDTA (b). Samples were subsequently analysed on a Coomassie-stained SDS-PAGE gel. The position of the molecular mass markers and the MrgA protein are indicated on the left and right, respectively.

deletion of the C-terminus of SecA elicits a strong oxidative stress response.

MrgA is not involved in protein secretion

The proposed function of MrgA is to bind to DNA under conditions of oxidative stress and to protect the DNA against damage (Chen & Helmann, 1995). To gain insight into the possible functional relation between SecA and MrgA, the *mrgA* gene was deleted from the chromosome in both DB104 and DB104ΔC, yielding strains DB104ΔM and DB104ΔCM, respectively. These strains had normal viability under the conditions tested. Inactivation of the *mrgA* gene in the DB104ΔC strain indeed resulted in the loss of the 120 kDa polypeptide, confirming that this protein band corresponds to a high-molecular-mass complex of MrgA (Fig. 3b, lane 4). The DB104ΔCM strain, however, still overproduced the AhpC and AhpF proteins to the level observed in strain DB104ΔC (Fig. 3b). Comparison of the culture supernatant of the parental strains and *mrgA* deletion mutants did not reveal any specific differences in the polypeptide pattern except that the overall level of secreted proteins was somewhat reduced (Fig. 3a, lane 4). This demonstrates that MrgA is not directly involved in protein secretion, nor needed for the oxidative stress response induced by the loss of the C-terminus of SecA.

DISCUSSION

In *Enterobacteriaceae*, SecB functions as a molecular chaperone dedicated to protein secretion. SecB targets precursor proteins to SecA by binding to a highly conserved sequence in the C-terminus of SecA. With the recent completion of the *B. subtilis* genome sequence (Kunst *et al.*, 1997), it has become clear that a true SecB homologue is not present in this organism. However, the C-terminus of *B. subtilis* SecA shows a striking homology to the SecB-binding domain of the *E. coli* SecA (Fig. 1). In this study, we have analysed a possible function of this conserved domain in protein secretion in *B. subtilis* SecA. Our study shows that in contrast to *E. coli* (Breukink *et al.*, 1995), a chromosomal deletion *B. subtilis* mutant of SecA lacking the putative SecB-binding domain has normal viability and is secretion proficient. Moreover, *in vitro* binding studies using the C-terminus of the *B. subtilis* SecA as bait, revealed no cytosolic homologue or analogue of SecB as binding partner. The C-terminus of the *B. subtilis* SecA is, however, capable of binding the heterologous *E. coli* SecB protein, as suggested previously by *in vivo* studies (Collier, 1994b). In *E. coli*, translocation of the maltose-binding protein (MBP) strongly depends on SecB. In *B. subtilis*, heterologously expressed preMBP is inefficiently secreted cotranslationally (Collier, 1994a).

Additional co-expression of SecB indeed caused a shift to post-translational translocation, implying that the *B. subtilis* SecA is capable of binding the heterologous SecB protein. When preMBP was equipped with the signal peptide of *B. subtilis* preAprE, processing of preMBP was no longer stimulated by the co-expression of SecB, and secretion was rather effective (Collier, 1994b). Our experiments show that the export of proOmpA, which in *E. coli* depends on SecB, is only slightly lowered upon deletion of the putative SecB-binding domain of SecA from *B. subtilis*. In conclusion, SecB may influence the secretion of some heterologous precursors when expressed in *B. subtilis*, but its function seems not to be required for the secretion of homologous precursor proteins.

The screen with the C-terminus of *B. subtilis* SecA as bait points to the MrgA protein as a cytosolic binding partner. The interaction between the *E. coli* SecA and SecB is stabilized by a zinc ion (Fekkes *et al.*, 1999). By analogy, chelators could disrupt the observed binding of MrgA to the C-terminus of the *B. subtilis* SecA. MrgA is not an analogue of SecB, since the deletion of the *mrgA* gene from the chromosome did not result in any noticeable secretion defect. The chromosomal replacement of the *B. subtilis* SecA by a truncate that lacks the putative SecB-binding domain resulted in the loss of specific proteins from the culture supernatant. In combination with the disruption of the *mrgA* gene, only a slight overall reduction of secretion was observed, but no additional loss of protein bands from the culture supernatant fraction. Since the specific loss of protein bands could be accounted for by a reduction of expression levels, it appears that the interaction of the C-terminus of SecA with MrgA is not directly related to protein secretion.

The *B. subtilis* chromosome (Kunst *et al.*, 1997) contains another protein, YccF, that bears at its N-terminus an almost identical sequence as the C-terminus of SecA. The function of YccF is unknown, but its deletion from the chromosome has no notable effect on the growth of *B. subtilis* (J. M. van Dijk, personal communication). The conserved sequence present in YccF may also represent a zinc-binding site that is involved in the binding of another protein or even MrgA. Since YccF has not been implicated in protein translocation, we have not analysed this possibility further.

A striking observation is the highly elevated expression of the cytosolic protein AhpC (and tentatively the AhpF and KatA proteins) in the SecAΔC strain. AhpC and AhpF mediate the enzymic reduction of various alkyl hydroperoxides, whereas KatA mainly reduces hydrogen peroxide (Bsat *et al.*, 1996). MrgA confers resistance to oxidative stress by the protection of DNA when present in a high-molecular-mass complex that is stable in SDS-PAGE (Chen & Helmann, 1995). Together, *ahpCF*, *katA* and *mrgA* form the peroxide regulon. The peroxide regulon is normally induced during oxidative stress or metal limitation (Völker *et al.*, 1994; Chen *et al.*, 1995; Bsat *et al.*, 1996), although AhpC is induced

under various other conditions (Völker *et al.*, 1994; Antelmann *et al.*, 1996). Even though oxidative stress is the strongest known inducer of the peroxide regulon, the expression of *ahpCF*, *mrgA* and *katA* is under control of a complex regulatory regime and is influenced by other factors. Expression of these proteins is growth-phase dependent, i.e. induced upon entry into stationary phase (Bol & Yasbin, 1994; Chen *et al.*, 1995). Mutation of AhpC leads to an increased expression of the other proteins in the regulon (Antelmann *et al.*, 1996; Bsat *et al.*, 1996). Finally, the growth-phase-dependent but not the peroxide-induced expression of KatA is abolished by mutation of *spoOA* (Bol & Yasbin, 1994). The exact mechanism by which deletion of the C-terminus of SecA induces expression of the peroxide regulon is unknown. The same mutation however results in the loss of a number of distinct polypeptides in the culture supernatant. One of these proteins is WapA, which is secreted as a very large (2334 amino acids) precursor that is subsequently processed into a number of smaller polypeptides with various functions (Foster, 1993). The loss of these proteins from the supernatant of strain DB104ΔC probably results from a regulatory process. Synthesis of preWapA is controlled by the DegS–DegU system (Dartois *et al.*, 1998) and affected by salt stress like that of many other secretory proteins (Kunst & Rapoport, 1995). WapA, as well as YweA and Yola, may also be repressed by the stress response that is evoked in strain DB104ΔC. Mutations in the *secA* gene of *B. subtilis* have been shown to affect transcription from *spoOA*-dependent promoters (Asai *et al.*, 1997, 1998), and thus probably affect transcription of the peroxide regulon (Bol & Yasbin, 1994). In the case of SecAΔC, a similar common mechanism could cause the stress response and the repression of a set of secretory proteins.

The question arises whether there is a need for molecular chaperones such as SecB in protein secretion in *B. subtilis*. Although this question cannot be answered at this time, there are a number of notable differences between *E. coli* and *B. subtilis*. Most of the secretory proteins that interact with SecB in *E. coli* are outer-membrane proteins (Kumamoto & Francetić, 1993). Due to their hydrophobic nature, these proteins tend to aggregate when not stabilized by chaperones in solution. *B. subtilis* completely lacks such proteins, and mainly produces soluble exoenzymes that fold rapidly only outside the cell when in contact with calcium (Leloup *et al.*, 1997). A recent report indicates that most exported proteins in *B. subtilis* require Ffh for secretion (Hirose *et al.*, 2000). The *B. subtilis* Ffh has been shown to bind specifically to precursor proteins and stimulates their association with the soluble form of SecA (Bunai *et al.*, 1996, 1999). This has led to the suggestion that Ffh fulfils a general chaperone role in the export of secretory protein in *B. subtilis*, unlike the *E. coli* Ffh that is primarily needed for the targeting of nascent membrane proteins to the translocase (Valent *et al.*, 1997, 1998).

In conclusion, our data suggest that the conserved C-terminal domain of *B. subtilis* SecA is not essential for

protein secretion and viability, and indicate a regulatory function rather than a role in chaperone binding.

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