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Published in:
Biochemical Journal

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2000

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Interaction of Bacillus subtilis CsaA with SecA and precursor proteins

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CsaA from the Gram-positive bacterium Bacillus subtilis has been identified previously as a suppressor of the growth and protein-export defect of Escherichia coli secA(Ts) mutants. CsaA has chaperone-like activities in vivo and in vitro. To examine the role of CsaA in protein export in B. subtilis, expression of the csaA gene was repressed. While export of most proteins remained unaffected, export of at least two proteins was significantly reduced upon CsaA depletion. CsaA co-immunoprecipitates and co-purifies with the SecA proteins of B. subtilis, and binds the B. subtilis preprotein prePhoB. Purified CsaA stimulates the translocation of prePhoB into E. coli membrane vesicles bearing the B. subtilis translocase, whereas it interferes with the SecB-mediated translocation of proOmpA into membrane vesicles of E. coli. The specific interaction with the SecA translocation ATPase and preproteins suggests that CsaA acts as a chaperone that promotes the export of a subset of preproteins in B. subtilis.

Key words: chaperone, co-immunoprecipitation, co-purification, in vitro translocation, protein translocation.

INTRODUCTION

In Eubacteria, proteins can be translocated across the cytoplasmic membrane and secreted into the medium. These proteins are usually synthesized as precursors with an N-terminal extension, the signal peptide (for review, see [1]), which is essential for directing them into the export pathway (for review, see [2]). Genetically and biochemically the best-characterized bacterial export system is that of Escherichia coli (for reviews, see [2,3]). This system, termed preprotein translocase, consists of the peripheral membrane protein SecA [4] and a multi-subunit membrane-protein complex with SecY, SecE and SecG as subunits [5]. The SecYEG complex acts as a receptor for SecA, and functions as a preprotein-conducting channel [6,7]. During or shortly after the translocation of the preprotein across the membrane, the signal peptide is removed by signal peptidase(s) (for review, see [8]), a prerequisite for the release of the mature protein from the membrane [9]. The integral membrane proteins SecD and SecF [10] are not essential for precursor protein translocation but, when overproduced, stabilize the SecYEG-bound SecA in a membrane-inserted state [11].

Whereas bacterial protein translocation is largely uncoupled from ongoing translation [12], efficient export requires chaperones to maintain preproteins in a translocation-competent state and to target them to the membrane-associated part of the translocase. In E. coli, SecB is an export-dedicated cytosolic chaperone that is required for efficient export of a subset of preproteins [13]. SecB stabilizes preproteins in an unfolded, non-aggregated state [14], and binds post-translationally or at the late co-translational stage to the mature region of these proteins [15]. The SecB–preprotein complex is then targeted to the SecYEG-bound SecA. The preprotein is subsequently transferred from SecB to SecA, and, upon the ATP-dependent initiation of translocation, the SecB is released into the cytosol [3]. In vivo, only a subset of preproteins appears to be translocated in a SecB-dependent manner [2]. A second targeting factor, consisting of the Ffh protein and the 4.5 S RNA, assists the export of SecB-independent exported proteins [16]. Other molecular chaperones, such as the heat-shock proteins DnaK/DnaJ/GrpE [17–19] and GroEL/GroES [20], appear also to be involved under specific conditions.

The central components of the protein-translocation system of B. subtilis are similar to those of E. coli. So far, homologues of Ffh, 4.5 S RNA, SecA, SecY, SecE, SecG, SecDF and several type-I signal peptides have been identified (for review see [21]).

So far, it is unknown if chaperones are involved in protein translocation in B. subtilis. In E. coli, SecB lacks a SecB homologue [22] and this has raised the question of whether other chaperones are involved in protein translocation. Depletion of signal-recognition-peptide (SRP) components impairs protein translocation [23,24], and the B. subtilis Ffh interacts directly with SecA and promotes the formation of soluble SecA–preprotein complexes [25]. This has led to the suggestion that SRP of B. subtilis not only acts as a targeting factor in co-translational translocation, but also stimulates post-translational translocation of preproteins [25]. The B. subtilis CsaA protein specifies chaperone-like activities possibly related to protein translocation [26]. The csaA gene was identified as a suppressor of growth and secretion defects of E. coli secA(Ts) strains [27]. Presence of CsaA stimulated precursor processing in secA, secB, groEL and dnaJ mutant strains of E. coli and it suppressed the growth defects of dnaK, dnaJ and grpe mutants of E. coli. CsaA stimulates the reactivation of heat-denatured firefly luciferase in groEL, groES, dnaK and grpe mutant strains of E. coli, and prevents the aggregation of heat-denatured luciferase in vitro.

The exact mechanism by which CsaA suppresses the growth and secretion defects of E. coli secA(Ts) strains is unknown. CsaA may either improve the translocation-competence of exported preproteins, thereby making them better substrates for mutant SecA proteins, or stimulate the translocation activity of the

Abbreviations used: NaP, inorganic phosphate, NaH2PO4; His6-CsaA, hexa-histidine-tagged CsaA; His6-SecB, hexa-histidine-tagged SecB; His6-prePhoB, hexa-histidine-tagged prePhoB; IPTG, isopropyl β-D-thiogalactoside; Ni-NTA, Ni2+–nitritoltriacetate; SRP, signal recognition peptide.

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mutant SecA proteins. Expression of CsaA in *E. coli* suppresses synthesis of SecB [26], whereas an impaired function of general chaperones causes an elevation in SecB expression [28]. These data further suggest a chaperone function of CsaA that could possibly be related to protein translocation.

In this paper we have further examined the role of CsaA in protein translocation in *B. subtilis*. CsaA affects the efficient secretion of some preproteins, and interacts specifically with SecA and preproteins. The data suggest that CsaA acts as a chaperone that promotes the export of a subset of preproteins in *B. subtilis*.

**EXPERIMENTAL**

**Bacterial strains, plasmids and media**

Bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown in TY medium (Bacto Tryptone/ Bacto Yeast extract) or on TY plates [28]. Pulse labelling of *B. subtilis* was carried out in defined HPDM medium [29], casamino acids were replaced by amino acids (0.02 mg/ml) excluding methionine and cysteine. If required, ampicillin (80 μg/ml), erythromycin (5 μg/ml) or kanamycin (20 μg/ml) were added.

**Biochemicals**

*B. subtilis* SecA [32], *E. coli* SecA [33] and the *B. subtilis* GroE5 complex [34] were isolated from overproducing strains as described. proOmpA [7] and hexa-histidine-tagged prePhoB (His$_6$-prePhoB) [35] were purified as described. Purified proOmpA, prePhoB and SecA proteins were labelled with carrier-free $^{15}$N according to van Wely et al. [35]. Labelled preproteins were stored frozen in 6 M urea. Polyacrylamide gel was raised against purified hexa-histidine-tagged CsaA (His$_6$-CsaA). Antisera against SecA from *B. subtilis* were from R. Freudl (Forschungszentrum Jülich, Jülich, Germany). Membrane vesicles were prepared from *E. coli* strain SF100 [36] containing plasmid pET605 overproducing SecYEG from *E. coli* [37] or plasmid pET822 overproducing SecYEG from *B. subtilis* DB104 as described in [38]. Membrane vesicles were treated with polyclonal antibodies against *E. coli* SecA to deplete and inactivate the endogenous SecA [38].

### Table 1 Bacterial strains and plasmids, and their genotype/phenotypes

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant genotype/phenotype</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td><em>B. subtilis</em></td>
<td></td>
<td></td>
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<tr>
<td>DB104</td>
<td>his, napE, aprE</td>
<td>[30]</td>
</tr>
<tr>
<td>DB104::pMUTINcsaA$^*$</td>
<td>allows IPTG-inducible expression of csaA</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG1</td>
<td>hsdS/F traD6, proA$^-$B$^+$, Δ(ac-pro) lacIQ, lacZΔM15</td>
<td>[31]</td>
</tr>
<tr>
<td>TG1(pREP4)</td>
<td>Host for overexpression of His-tagged proteins</td>
<td>This study</td>
</tr>
<tr>
<td>TG1(pREP4, pQE9csaA)</td>
<td>TG1 derivative strain, Km$, Apl$, allows IPTG-inducible synthesis of His$_6$-CsaA</td>
<td>This study</td>
</tr>
<tr>
<td>TG1(pREP4, pQE9csaB)</td>
<td>TG1 derivative strain, Km$, Apl$, allows IPTG-inducible synthesis of His$_6$-SecB</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmids</td>
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<tr>
<td>pMUTIN2</td>
<td>pBR322-based integration vector for <em>B. subtilis</em>, containing IPTG-inducible P$_{lac}$ promoter, Apl$, Emr$</td>
<td>[40]</td>
</tr>
<tr>
<td>pMUTINcsaA$^*$</td>
<td>MUTIN2 derivative; carries the 5′ part of csaA</td>
<td></td>
</tr>
<tr>
<td>pREP4</td>
<td>plasmid, containing lacI$^q$ repressor gene, Km$^r$</td>
<td>Qiagen</td>
</tr>
<tr>
<td>pQE9csaA</td>
<td>pQE9 derivative plasmid, Apl$, allows IPTG-inducible synthesis of His$_6$-CsaA</td>
<td>This study</td>
</tr>
<tr>
<td>pQE9secB</td>
<td>TG1 derivative plasmid, Apl$, allows IPTG-inducible synthesis of His$_6$-SecB</td>
<td>This study</td>
</tr>
</tbody>
</table>

DNA manipulation/techniques

Procedures for DNA purification, restriction, ligation, transformation of *E. coli* and agarose-gel electrophoresis were carried out as described by Sambrook et al. [39]. The nucleotide sequences of cloned PCR fragments were confirmed by DNA sequencing.

To amplify csaA, oligonucleotides 5′-TCTCGAATTCATAGGGAGAAAGGAGTTGAGT$^{3′}$-3′, localized 5′ to the ribosome-binding site of csaA and incorporating an EcoRI restriction site, and 5′-GGACCAAGGGATCCATTTAATCCGGCG$^{78}$-3′, localized within csaA and incorporating a BamHI restriction site, were used (the numbers refer to those in Müller et al. [27]). The csaA gene was amplified by PCR from *B. subtilis* chromosomal DNA. The amplified fragment was digested with EcoRI and BamHI and cloned into pMUTIN2 [40] digested with the same enzymes. The resulting plasmid pMUTIN csaA$^*$ was integrated into the chromosome of *B. subtilis* DB104 via Campbell-type integration.

For synthesis of His-tagged proteins secB and csaA genes were amplified excluding their ATG start codons and were inserted 3′ to the His-coding region of pQE9. Genes were amplified by PCR using oligonucleotides 5′-CACGGAATTCGGACACAAACACTG$^{38}$-3′, incorporating a BamHI restriction site, and 5′-CCGACTGCTAGTTGAAGCATTACGG$^{38}$-3′, with a PstI restriction site, for secB (the numbers refer to those used in [41]), and oligonucleotides 5′-GGAATTTACAGTTGATCGCTGACGTTA$^{38}$-3′, with a BamHI restriction site, and 5′-GGCAGTCCTGAGCCCTTTACCGCACACAG$^{38}$-3′, incorporating a PstI restriction site, for csaA (the numbers refer to those used in [27]). PCR fragments were purified, digested with BamHI and PstI and inserted into pQE9 digested with the same enzymes. The resulting plasmids pQE9secB and pQE9csaA were transformed into *E. coli* TG1(pREP4).

**Pulse–chase protein labelling and analysis of protein secretion**

Pulse–chase protein-labelling experiments were performed as described by van Dijl et al. [42]. *B. subtilis* was grown in minimal medium to exponential growth in the absence or presence of 1 mM isopropyl β-D-thiogalactoside (IPTG), pulse labelled with $^{[35]$S)methionine for 1 min and subsequently chased. Samples were taken 2 and 5 min post chase time and proteins secreted into the growth medium were collected as described in [43]. SDS/PAGE was performed according to Laemmli [44]. $^{13}$C-Methylated proteins (Amersham International, Amersham, Bucks, U.K.) were used as molecular-size markers. Relative amounts of radioactivity were estimated by using a PhosphoImager (Fuji) and associated image-analysis software.

**Purification of proteins**

His$_6$-CsaA and hexa-histidine-tagged SecB (His$_6$-SecB) were prepared from IPTG-induced *E. coli* TG1(pREP4, pQE9csaA) and TG1(pREP4, pQE9secB) cultures as abundant proteins and purified by Ni$^{2+}$-nitrilotriacetic acid (Ni-NTA) agarose affinity chromatography. Purification was carried out under native
conditions following standard protocols (Qiagen, Hilden, Germany). Cell lysates prepared in 50 mM NaP (inorganic phosphate, NaH$_2$PO$_4$), pH 8.0, and 300 mM NaCl, were supplemented with 10 mM imidazole and applied on to the Ni-NTA column. The column was washed three times with 50 ml of buffer containing 20 mM imidazole. His-tagged proteins were eluted with NaP$_2$ buffer containing 500 mM imidazole.

CsaA from _B. subtilis_ was purified immunologically. Affinity-purified rabbit antibodies against CsaA were covalently linked to CNBr-activated Sepharose. Exponentially grown _B. subtilis_ DB104 cells were harvested and extracts were prepared from lysozyme-treated cells via sonication. Cleared cell extracts were loaded on to the anti-CsaA-Sepharose column at room temperature. Anti-CsaA-Sepharose was washed with 30 ml of phosphate buffer (50 mM, pH 7.0) containing 0.5 M NaCl until no detectable protein was eluted from the column. CsaA was eluted with 0.1 M acetic acid, pH 3.0, and subsequently lyophilized. To obtain SecA-free CsaA, _B. subtilis_ cell extracts were purified by preparative isoelectric focusing in Sephadex gel with a pH gradient from pH 4.0 to 9.0 for 18 h. After focusing, the Sephadex gel was cleaved to fractions and CsaA-containing fractions (as determined immunologically) were analysed by thin-layer polyacrylamide gel isoelectric focusing with an ampholyte solution (pH 3.5–10.0) and SDS/PAGE. The CsaA was precipitated with ammonium sulphate to a final concentration of 80% saturation. The precipitated protein was dissolved, dialysed against H$_2$O and lyophilized.

**Western blotting**

Proteins isolated from exponentially growing cultures were assayed by Western blotting [45]. Cell lysates were prepared by boiling (5 min) in sample buffer and subjecting to SDS/PAGE [44]. Separated proteins were transferred to a nitrocellulose membrane (Schleicher & Schüll), as described by Towbin et al. [45]. CsaA and SecA proteins were detected with specific polyclonal antibodies and alkaline phosphatase-conjugated goat anti-rabbit antibodies (Bio-Rad) according to the manufacturer’s instructions. Similar amounts of total cell protein were loaded on to each lane.

**Binding studies**

Binding of $[^{35}S]$His$_2$-prePhoB or $[^{35}S]$SecA to CsaA was measured as follows: 1 µg of $[^{35}S]$-labelled protein and 1 µg of CsaA were incubated at room temperature in 50 µl of Heps buffer (50 mM, pH 7.6, additional buffer components as indicated). After 60 min, 5 µl of CsaA-specific antiserum pre-complexed with 5 µg of Protein A–Sepharose was added and the mixture was further incubated for 60 min with regular vortexing. Subsequently, the Protein A–Sepharose beads were washed five times with 500 µl of Heps buffer, pH 7.6. $[^{35}S]$-Labelled proteins bound to Protein A–Sepharose beads were counted in Optima Gold scintillation liquid (Packard Instruments B.V., Groningen, The Netherlands).

**Translocation assay**

The efficiency of translocation _in vitro_ of $[^{35}S]$proOmpA and $[^{35}S]$prePhoB into _E. coli_ membrane vesicles was assayed by treatment with proteinase K [46]. When indicated, 1.0 µg of purified _E. coli_ or _B. subtilis_ SecA protein, 2.0 µg of _E. coli_ SecB or 4 µg of purified _B. subtilis_ GroESL or _B. subtilis_ CsaA were added. Reactions were started by the addition of 1 µl of $[^{35}S]$-labelled precursor protein (0.5 µg of protein). For preincubation experiments, mixtures were incubated for 30 min at 37 °C without membrane vesicles prior to the translocation reactions. After 30 min of translocation at 37 °C samples were chilled on ice, treated with proteinase K (0.5 mg/ml) for 15 min, precipitated with 7.5% trichloroacetic acid, washed with acetone, dissolved in SDS/PAGE sample buffer, and analysed by SDS/PAGE (10% gel) and autoradiography.

**RESULTS**

Repression of CsaA expression results in alteration of protein secretion

In _B. subtilis_ strain DB104::pMUTIN2csaA, _csaA_ is localized downstream of the P$_{spac}$ promoter, allowing the IPTG-inducible expression of the CsaA protein. Cells grown in the absence of IPTG were normally viable but Western blotting analysis showed that they contained a dramatically reduced cellular level of CsaA. Induction of the P$_{spac}$ promoter resulted in a production level of CsaA that was about 2-fold higher than in the wild type (Figure 1A). To study the function of CsaA in protein secretion, the pattern of proteins secreted by _B. subtilis_ DB104::pMUTIN2csaA grown in the absence or presence of IPTG was determined. While the pattern of secreted proteins of the strain grown in presence of IPTG was indistinguishable from that of the wild type, repression of _csaA_ expression resulted in a decrease in at least two proteins with apparent molecular masses of 19 and 36 kDa (Figure 1B). In particular, the 36-kDa protein

![Figure 1](image-url)  
Repression of _csaA_ in _B. subtilis_ affects protein export

_A_ _B. subtilis_ strain DB104 (lane 1) and strain DB104::pMUTIN2csaA (lanes 2 and 3) were grown in TY medium in the absence (lanes 1 and 2) or presence (lane 3) of 1 mM IPTG. Cell extracts were analysed by SDS/PAGE and Western blotting using CsaA-specific antibodies. _B_ Protein secretion of _B. subtilis_ DB104::pMUTIN2csaA was analysed by pulse labelling of cells grown in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of IPTG. Secreted proteins were collected by filtration and analysed by SDS/PAGE and autoradiography.
Figure 2  *E. coli* SecA interacts with His$_6$-CsaA and His$_6$-SecB

*E. coli* strains TG1(pREP4, pQE9csaA) (A, B), TG1(pREP4, pQE9secB) (C) and TG1(pREP4, pQE9) (D) were cultivated in TY medium to exponential growth and the phage T5 promoter was derepressed by adding IPTG (final concentration, 2 mM) to the culture medium. After 2 h, cells were harvested and lysed by sonication, and the cleared cell extracts were loaded on to Ni-NTA columns. Aliquots of non-bound fraction (FT, lane 1), wash (W1–W3, lanes 2–4) and elution fractions (E1–E8, lanes 5–12) were analysed by SDS/PAGE and Western blotting using antibodies directed against CsaA (A) and *E. coli* SecA (B–D). The asterisk indicates the position of dimeric CsaA. 

Figure 3  *B. subtilis* SecA co-purifies with CsaA

A cell lysate of *B. subtilis* DB104 was loaded on to an anti-CsaA–Sepharose column, washed with NaCl buffer (0.5 M) and the immunologically bound CsaA was eluted with 0.1 M acetic acid. The eluate was analysed by SDS/PAGE and Coomassie Brilliant Blue staining (lane 1) or Western blotting using polyclonal antibodies (pAb) directed against the *B. subtilis* SecA (lane 2) or CsaA (lane 4). The asterisk indicates the localization of dimeric CsaA. As a control, a lysate of *B. subtilis* DB104::pMUTIN2csaA grown in the absence of IPTG was loaded on to the anti-CsaA–Sepharose column, and the fraction eluted with 0.1 M acetic acid was stained immunologically with antibodies directed against the *B. subtilis* SecA (lane 3).

SecA antibodies revealed that SecA co-eluted with CsaA from the column (Figure 2B). To exclude the possibility that SecA non-specifically binds to the Ni-NTA resin, a lysate of *E. coli* TG1(pREP4) cells that do not express the His-tagged CsaA was loaded on to an Ni-NTA column (Figure 2D). Under those conditions, no SecA was retained by the column, suggesting an interaction between CsaA and SecA. Since SecA is known to interact with SecB in *E. coli* [6,7,47], His$_6$-SecB was purified from *E. coli* TG1(pREP4, pQE9secB) under conditions used for purification of His$_6$-CsaA. SecA was found to co-elute with His$_6$-SecB (Figure 2C). These results indicate that SecA was retained in the Ni-NTA column via specific binding to His-tagged CsaA or SecB.

*E. coli* SecA co-purifies with His$_6$-CsaA and His$_6$-SecB

His-tagged CsaA protein was overproduced in *E. coli* TG1 (pREP4, pQE9csaA) and purified by Ni-NTA-column chromatography. The imidazole-eluted protein fraction predominantly contained the 13-kDa CsaA protein, and a protein with an apparent molecular mass of about 30 kDa that stained with antibodies directed against CsaA (Figure 2A). The latter probably represents some dimeric CsaA. Immunodetection with SecA antibodies revealed that SecA co-eluted with CsaA from the column (Figure 2B). To exclude the possibility that SecA non-specifically binds to the Ni-NTA resin, a lysate of *E. coli* TG1(pREP4) cells that do not express the His-tagged CsaA was loaded on to an Ni-NTA column (Figure 2D). Under those conditions, no SecA was retained by the column, suggesting an interaction between CsaA and SecA. Since SecA is known to interact with SecB in *E. coli* [6,7,47], His$_6$-SecB was purified from *E. coli* TG1(pREP4, pQE9secB) under conditions used for purification of His$_6$-CsaA. SecA was found to co-elute with His$_6$-SecB (Figure 2C). These results indicate that SecA was retained in the Ni-NTA column via specific binding to His-tagged CsaA or SecB.

*B. subtilis* SecA interacts with CsaA

To establish whether the interaction of CsaA with SecA can be observed in *B. subtilis*, CsaA was purified from *B. subtilis* DB104. Cell extracts were loaded on to an anti-CsaA–Sepharose column, washed and eluted as described in the Experimental section. The eluate was analysed by SDS/PAGE and Coomassie Brilliant Blue staining (Figure 3, lane 1). In addition to CsaA, a protein co-eluted with an apparent molecular mass of about 90 kDa. The latter could be immunostained with an antibody directed against the *B. subtilis* SecA (Figure 3, lane 2), suggesting that the *B. subtilis* SecA associates with CsaA. As a control, a lysate of *B. subtilis* DB104::pMUTIN2csaA grown in the absence of IPTG was used. Under those conditions, CsaA was not expressed (Figure 1A, lane 2) and no SecA was found to bind to the anti-CsaA–Sepharose column (Figure 3, lane 3).

Since the data *in vivo* suggest a specific interaction between SecA and CsaA, the interaction was examined further using...
purified proteins. CsaA was co-incubated with \(^{125}\text{I}\)-labelled \(E.\ coli\) and \(B.\ subtilis\) SecA. Complex formation was analysed by immunoprecipitation of CsaA. To differentiate between CsaA-mediated and non-specific binding of SecA to Protein A–Sepharose, CsaA and/or antiserum against CsaA were excluded from the reaction mixture. In the presence of SecA, both the \(E.\ coli\) and \(B.\ subtilis\) SecA proteins could be immunoprecipitated by the CsaA antibodies (Table 2). Background levels of immunoprecipitation were observed when either CsaA (Table 2) or the CsaA antibodies were excluded from the reaction mixture. These results suggest that SecA and CsaA are interacting proteins.

### CsaA binds the preprotein prePhoB and stimulates its translocation in vitro

CsaA interacts with unfolded proteins [26]. To determine if CsaA also interacts with unfolded preproteins, urea-denatured \(B.\ subtilis\) preprotein \[^{125}\text{I}\]\(\text{His}_8\)-prePhoB at 1 mg/ml was diluted 100-fold into a buffer containing 10 mM Tris·HCl (pH 7.6), supplemented with 60 mM urea and 0.125% octylglucoside. After 60 min of incubation at room temperature, CsaA or \(E.\ coli\) or \(B.\ subtilis\) SecA-specific antibodies pre-complexed with Protein A–Sepharose were added and the incubation was continued for another 60 min. Immunoprecipitates were collected by centrifugation and the amount of bound SecA was quantified by liquid scintillation counting. Amounts of radiolabelled SecA (means ± S.D. from two independent experiments) are shown in c.p.m. pAb, polyclonal antibody.

<table>
<thead>
<tr>
<th>CsaA</th>
<th>CsaA pAb</th>
<th>PhoB pAb</th>
<th>Bound [^{125}\text{I}](\text{PhoB}) (c.p.m.)</th>
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<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>–</td>
<td>1199</td>
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### CsaA interferes with SecB for translocation of \[^{125}\text{I}\]\(\text{His}_8\)-prePhoB

Since CsaA interacts with preproteins and with SecA, an activity reminiscent of SecB, we determined if CsaA is able to substitute for SecB in the translocation of \[^{125}\text{I}\]\(\text{His}_8\)-prePhoB by \(E.\ coli\) membrane vesicles bearing SecYEG and SecA from \(B.\ subtilis\). To examine using the proteinase digestion assay. While in the absence of SecA (Figure 4, lane 1) or ATP (results not shown) hardly any translocation of \[^{125}\text{I}\]\(\text{His}_8\)-prePhoB occurred, efficient translocation was observed when the membrane vesicles were supplemented with purified \(B.\ subtilis\) SecA (Figure 4, lane 2). Addition of \(B.\ subtilis\) GroEL/S complex or increasing amounts of CsaA (Figure 4, lanes 4–6) further stimulated the translocation of \[^{125}\text{I}\]\(\text{His}_8\)-prePhoB. To establish if the stimulatory effect of CsaA is related to maintenance of the translocation competence of \[^{125}\text{I}\]\(\text{His}_8\)-prePhoB, experiments were performed in which \[^{125}\text{I}\]\(\text{His}_8\)-prePhoB was diluted 50-fold into buffer in the absence and presence of CsaA, followed by a 30-min preincubation at 37 °C. However, under these conditions prePhoB hardly exhibited a loss in translocation competence (results not shown), suggesting that CsaA may stimulate translocation by another mechanism.

### Figure 4  Effect of CsaA on the SecA-mediated translocation of prePhoB into membrane vesicles of \(E.\ coli\) containing \(B.\ subtilis\) SecYEG

Translocation of \[^{125}\text{I}\]\(\text{His}_8\)-prePhoB in membrane vesicles was carried out in the absence (lane 1) and presence (lanes 2–6) of exogenous SecA, and the presence of GroEL/S complex (1 μg, lane 3) or CsaA (0.5 μg, lane 4; 2 μg, lane 5; 5 μg, lane 6). After translocation, samples were treated with proteinase K and analysed by SDS/PAGE and autoradiography. p, precursor; m, mature protein.

<table>
<thead>
<tr>
<th>Lane</th>
<th>SecA</th>
<th>GroEL/S</th>
<th>CsaA</th>
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<tbody>
<tr>
<td>1</td>
<td>–</td>
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<td>–</td>
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<td>2</td>
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<td>5</td>
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<td>+</td>
<td>++</td>
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<tr>
<td>6</td>
<td>+</td>
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<thead>
<tr>
<th>Bound [^{125}\text{I}](\text{SecA}) (c.p.m.)</th>
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<tbody>
<tr>
<td>CsaA pAb</td>
</tr>
<tr>
<td>+</td>
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The ATP-dependent translocation of urea-denatured \[^{125}\text{I}\]\(\text{His}_8\)-prePhoB by \(E.\ coli\) membrane vesicles bearing SecYEG and SecA from \(B.\ subtilis\) was examined using the proteinase digestion assay. While in the absence of SecA (Figure 4, lane 1) or ATP (results not shown) hardly any translocation of \[^{125}\text{I}\]\(\text{His}_8\)-prePhoB occurred, efficient translocation was observed when the membrane vesicles were supplemented with purified \(B.\ subtilis\) SecA (Figure 4, lane 2). Addition of \(B.\ subtilis\) GroEL/S complex or increasing amounts of CsaA (Figure 4, lanes 4–6) further stimulated the translocation of \[^{125}\text{I}\]\(\text{His}_8\)-prePhoB. To establish if the stimulatory effect of CsaA is related to maintenance of the translocation competence of \[^{125}\text{I}\]\(\text{His}_8\)-prePhoB, experiments were performed in which \[^{125}\text{I}\]\(\text{His}_8\)-prePhoB was diluted 50-fold into buffer in the absence and presence of CsaA, followed by a 30-min preincubation at 37 °C. However, under these conditions prePhoB hardly exhibited a loss in translocation competence (results not shown), suggesting that CsaA may stimulate translocation by another mechanism.
CsaA in protein export in *B. subtilis*, protein export was analysed in response to *csaA* suppression. Earlier extensive gene-disruption experiments failed [27], suggesting that *csaA* is an essential gene of *B. subtilis*. Therefore, *csaA* was placed under transcriptional control of the *P_{mut}*, promoter using the integrative plasmid pMUTIN2 [40]. Despite the fact that CsaA could not be detected in the absence of IPTG (Figure 1A), no alteration of growth was observed for *B. subtilis* DB104::pMUTIN2csaA‘ when grown in complex or defined medium. The possibility that minimal expression of CsaA, beyond the detection limit, suffices to sustain growth of this strain in the absence of IPTG cannot be excluded. Whereas in the presence of IPTG, protein secretion was essentially similar to the parental strain, repression of *csaA* resulted in a reduced export of at least two proteins at the end of the exponential growth phase.

An interaction between CsaA and *E. coli* or *B. subtilis* SecA could be demonstrated by various techniques. *E. coli* SecA co-purifies with His₆-tagged CsaA on an Ni-NTA affinity resin, while the *B. subtilis* SecA was found to specifically co-purify with CsaA by means of an immuno-purification technique. Likewise, *E. coli* SecA co-purifies with His₆-SecB using the Ni-NTA-affinity column-chromatography technique. Since the latter interaction has been established previously [6], it suggests that the CsaA-SecA complex is also stable. No other proteins were copurified in detectable amounts. The markedly lower retention of SecA in the presence of His₆-CsaA compared with the retention by His₆-SecB might have been due to the heterologous character of CsaA when expressed in *E. coli*. The interaction between CsaA and SecA could be demonstrated further in *vitro* by a co-immunoprecipitation of [³⁵S]labelled purified proteins. In *E. coli*, SecB binds to a well-conserved C-terminal region of SecA [7]. When fused to glutathione S-transferase, the 20 C-terminal amino acids of *E. coli* SecA represent an authentic SecB-binding domain. This glutathione S-transferase-fusion protein, however, does not interact with CsaA (J. P. Müller, J. Swaving, K. H. M. van Wely and A. J. M. Driessen, unpublished work), indicating that other domains of SecA are involved in this binding reaction. Interaction of CsaA with unfolded preproteins could be demonstrated by co-immunoprecipitation of His₆-prePhoB via CsaA. Similar experiments with radiolabelled proOmpA suggest that CsaA also interacts with this precursor (J. P. Müller, J. Swaving, K. H. M. van Wely and A. J. M. Driessen, unpublished work). Interaction of CsaA with these unfolded precursors is not unexpected since CsaA can also interact with other unfolded proteins [26].

The exact mechanism of suppression of impaired activity of the temperature-sensitive SecA proteins has not yet been established. CsaA may act by improving the translocation-competence of exported preproteins, thereby making them better substrates for the malfunctioning SecA proteins, or it may act directly on the activity of SecA [25]. Our current data suggest that it may stimulate translocation by improving the targeting to SecA as it binds SecA specifically.

In *E. coli*, chaperones are needed to support translocation of preproteins in *vitro*. SecB is required for a subset of preproteins [14,48], while other chaperones such as GroES/GroEL or DnaK/DnaJ/GrpE support translocation under certain conditions only [49]. In *B. subtilis*, no chaperones have thus far been found that stimulate preprotein translocation, although this function may be fulfilled by SRP, which has been shown to interact with SecA, thereby promoting the formation of soluble SecA-preprotein complexes [24]. Here we have shown that CsaA stimulates the translocation *in vitro* of prePhoB into membrane vesicles by about 2–3-fold. It is important to note that prePhoB only slowly loses its translocation competence when diluted from
urea, suggesting that the stimulation is due to targeting. More striking is the observation that CsaA competes with SecB for proOmpA translocation. When proOmpA is preincubated with a mixture of SecB and CsaA, SecB is no longer able to stimulate the translocation of proOmpA. In this context, it is important to note that CsaA itself does not stimulate the translocation of proOmpA, nor is it able to maintain the translocation competence of proOmpA. Since the inhibitory effect of CsaA is less pronounced when proOmpA is first preincubated with SecB, it seems that competition is mainly at the level of binding of the precursor, thus preventing SecB to target proOmpA to the translocation sites while maintaining its translocation competence. Elucidation of the mechanism of inhibition may provide further insight into the function of CsaA.

Taken together, the results in vivo and in vitro indicate that CsaA is able to bind to both B. subtilis preproteins and the SecA translocation ATPase, suggesting an export-related function of CsaA in B. subtilis. Depletion of CsaA in B. subtilis gives rise to an export defect of a set of proteins. Analysis of the translocation of these proteins in B. subtilis will further define the function of CsaA in protein secretion.

These investigations were supported by a short-term EMBO fellowship to J.P.M., by grants from the Deutsche Forschungsgemeinschaft, and by grants to A.J.M.D. from CEC Biotech nos. BIO2 CT 930254 and BIO4 CT 960097.

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Received 4 January 2000/6 March 2000; accepted 27 March 2000