Chaperone-like activities of the CsaA protein of *Bacillus subtilis*

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The growth and protein export defects of *Escherichia coli* secA51(Ts) strains can be suppressed by the CsaA protein of *Bacillus subtilis*. The present studies indicate that this effect can be attributed to chaperone-like activities of CsaA. First, CsaA stimulated protein export in secB, groES and dnaJ mutant strains of *E. coli*. Second, CsaA suppressed the growth defects of dnaK, dnaJ and grpE mutants of *E. coli*. Third, and most importantly, CsaA exhibited chaperone-like properties by stimulating the reactivation of heat-denatured firefly luciferase in groEL, groES, dnaK and grpE mutant strains of *E. coli*, and by preventing the aggregation of heat-denatured luciferase *in vitro*. Thus, it seems that CsaA suppresses the growth and secretion defects of *E. coli* secA(Ts) strains either by improving the translocation competence of exported pre-proteins, thereby making them better substrates for mutant SecA proteins, or by stimulating the translocation activity of mutant SecA proteins.

Keywords: *Bacillus subtilis*, CsaA, protein export, protein targeting, chaperone

INTRODUCTION

The transport (i.e. export) of proteins across the cytoplasmic membrane of eubacteria depends on properties of the transported protein and the cellular protein export apparatus. Exported proteins are usually synthesized as precursors with an amino-terminal extension, the signal peptide, which directs these proteins into the export pathway (Pugsley, 1993; von Heijne, 1990). Genetically and biochemically the best characterized bacterial protein export apparatus is that of *Escherichia coli*. It consists of cytoplasmic and membrane-bound components (Wickner *et al.*, 1991; Duong *et al.*, 1997; Fekkes & Driessen, 1999).

Cytoplasmic proteins, like SecB (Kumamoto & Beckwith, 1985), GroEL, GroES (Altman *et al.*, 1991; Kusukawa *et al.*, 1989), DnaK, DnaJ and GrpE (Altman *et al.*, 1991; Wild *et al.*, 1992, 1993), denoted as chaperones, are important for the export competence of pre-proteins, either by stabilizing an unfolded conformation, or by preventing their aggregation (Hendrick & Hartl, 1993). In addition, SecB is involved in the targeting of pre-proteins to the membrane-bound pre-protein translocase complex (Fekkes *et al.*, 1997; Hartl *et al.*, 1990). A second targeting factor, which is homologous to the eukaryotic signal recognition particle (SRP), assists the export of a subset of pre-proteins (De

Gier *et al.*, 1997; Luirink *et al.*, 1992; Phillips & Silhavy, 1992; Wolin, 1994).

The pre-protein translocase consists of the peripheral membrane protein SecA (Oliver & Beckwith, 1982), which acts as a force generator for protein translocation, and a complex of the integral membrane proteins SecD, SecE, SecF, SecG, SecY and YajC, which form the translocation channel (for recent reviews, see Fekkes & Driessen, 1999; Duong *et al.*, 1997; Pohlschröder *et al.*, 1997). SecA plays a crucial role in protein translocation. First, it can function as a receptor for pre-protein–SecB complexes (de Cock & Randall, 1998; Fekkes *et al.*, 1997; Hartl *et al.*, 1990). Second, SecA drives protein transport through cycles of pre-protein binding, membrane insertion, pre-protein release and deinsertion from the membrane (Economou *et al.*, 1995; Economou & Wickner, 1994; Kim & Oliver, 1994). The cycling of SecA is regulated by ATP binding and hydrolysis, which causes major conformational changes in this protein (den Blaauwen & Driessen, 1996; van der Does *et al.*, 1998). During or shortly after the translocation of the pre-protein across the membrane, the signal peptide is removed by signal peptidase (also known as leader peptidase), which is a prerequisite for the release of the mature protein from the membrane (Dalbey *et al.*, 1997).

Compared to *E. coli*, the protein export apparatus of *Bacillus subtilis* has been studied in less detail. So far, homologues of GroEL, GroES (Schmidt *et al.*, 1992),

Abbreviation: 6H-CsaA, hexa-histidine-tagged CsaA.

Table 1. Bacterial strains and plasmids used

Strain/plasmid	Characteristic	Source/reference
Strains		
MC4100	F ⁻ <i>araD139 lacU169 rspL thi relA</i>	Ito <i>et al.</i> (1981)
CK1953	MC4100 <i>secB::Tn5</i>	Kumamoto & Beckwith (1985)
MC4100::pLBL332	MC4100 <i>secB'-lacZ</i>	Müller (1996)
MM171.3	MC4100 <i>secA'-lacZ</i>	Schmidt & Oliver (1989)
BB1458	MC4100 <i>dnaJ259 thr::Tn10</i>	B. Bukau*; Sunshine <i>et al.</i> (1977)
BB1048	MC4100 Δ <i>dnaK756 thr::Tn10</i>	B. Bukau*; Georgopoulos (1977)
BB1752	B178 <i>grpE280 pheA::Tn10</i>	B. Bukau*; Ang <i>et al.</i> (1986)
NRK117	MC4100 <i>groEL44(Ts) zje::Tn10</i>	Kusukawa <i>et al.</i> (1989)
NRK223	MC4100 <i>groES619(Ts) zje::Tn10</i>	Kusukawa <i>et al.</i> (1989)
BB1553	MC4100 <i>dnaK52::Cm^r sidB1</i>	Hesterkamp & Bukau (1998)
Plasmids		
pBR322	Ap ^r Tc ^r	Bolivar <i>et al.</i> (1977)
pBNB8	pBR322-derived plasmid carrying the <i>B. subtilis csaA</i> gene, Ap ^r	Müller <i>et al.</i> (1992)
pTZ18R	pUC18-derived expression vector, Ap ^r	Mead <i>et al.</i> (1986)
pTZcsa82	pTZ18R-derived plasmid carrying the <i>B. subtilis csaA</i> gene, Ap ^r	This study
pQE9	Expression vector, allows production of His-tagged fusion proteins, Ap ^r	Qiagen
pQE9 <i>csaA</i>	pQE9-derived expression vector carrying <i>csaA</i> , Ap ^r	This study
pSEL16	pACYC-derived plasmid carrying the <i>luc</i> gene under control of the streptokinase promoter	Gräfe <i>et al.</i> (1996)

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DnaK, DnaJ and GrpE (Wetzstein *et al.*, 1992), SRP (Honda *et al.*, 1993; Struck *et al.*, 1988), SecA (Overhoff *et al.*, 1991; Sadaie *et al.*, 1991), SecY (Nakamura *et al.*, 1990; Suh *et al.*, 1990), SecE (Jeong *et al.*, 1993), SecDF and YajC (Bolhuis *et al.*, 1998) and several signal peptidases have been identified (Bolhuis *et al.*, 1996; Meijer *et al.*, 1995; Tjalsma *et al.*, 1997, 1998; van Dijl *et al.*, 1992). In an early attempt to clone the *B. subtilis secA* gene by complementation of the *E. coli secA51(Ts)* mutation, we identified the *B. subtilis csaA* gene (Müller *et al.*, 1992). The deduced amino acid sequence of CsaA showed no similarities to that of SecA or other known components of the *E. coli* protein export apparatus. Nevertheless, the expression of the complete *csaA* gene resulted in a suppression of the growth and protein export defects associated with the *secA51* mutation. Unlike other previously identified suppressors of *secA51* (Brinkman *et al.*, 1984; Lee & Beckwith, 1986; Oliver, 1985; Overhoff-Freundlieb & Freudl, 1991), *csaA* showed no pleiotropic effects on mutations in other *sec* genes with the exception of the *secB::Tn5* mutation (Müller *et al.*, 1992). The present studies were aimed at the elucidation of the mechanism by which *csaA* suppresses secretion defects in *E. coli*. The results show that the CsaA protein has chaperone-like activities

in vivo and *in vitro*, suggesting that these activities are responsible for the suppression of the *secA51* mutation.

METHODS

Plasmids, bacterial strains and media. Table 1 lists the plasmids and bacterial strains used. TY medium contained Bacto tryptone (1%), Bacto yeast extract (0.5%) and NaCl (1%). M9 media 1 and 2 used in the labelling of *E. coli* were prepared as described by van Dijl *et al.* (1991). If required, ampicillin (40 µg ml⁻¹), erythromycin (150 µg ml⁻¹), kanamycin (20 µg ml⁻¹) or tetracycline (15 µg ml⁻¹) were added.

DNA techniques. Procedures for DNA purification, restriction, ligation, transformation of *E. coli*, and agarose gel electrophoresis, were carried out as described by Sambrook *et al.* (1989). Enzymes were from Roche Molecular Biochemicals.

Purification of hexa-histidine-tagged CsaA. To provide CsaA with an amino-terminal hexa-histidine tag, the *csaA* gene was amplified by PCR with the primers *csaA*-5' (5'-GGAGTT-ATTGGATCCGACAGTTATTGATGAC-3'; containing a *Bam*HI site) and *csaA*-3' (5'-GCCGATCTGCAGGCCTTTACGGCACACACG-3'; containing a *Pst*I site) and cloned into plasmid pQE9, resulting in plasmid pQE9*csaA*. The sequence of the amplified fragment was verified by DNA sequencing. Hexa-histidine-tagged CsaA (6H-CsaA) was purified from *E. coli* M15 (pREP4, pQE9*csaA*) using Ni²⁺-nitrilotriacetic acid

agarose (Qiagen) under non-denaturing conditions, according to the manufacturer's instructions.

CsaA antiserum. A CsaA-based peptide with the sequence KAEEFPEARC (residues 19–27 of CsaA: the last C was added for experimental reasons; Müller *et al.*, 1992) was cross-linked to KLH (Keyhole Limpet Hemocyanin) from Pierce using the *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester, as described by Närvänen (1990). Next, the peptide–KLH conjugate was used to immunize rabbits.

Protein labelling, immunoprecipitation, SDS-PAGE and fluorography. Pulse labelling, or pulse–chase labelling experiments in *E. coli* and subsequent immunoprecipitations were performed as described by van Dijl *et al.* (1991). SDS-PAGE was performed according to Laemmli (1970). [¹⁴C]Methylated molecular mass reference markers were from Amersham. Fluorography was performed as described by Skinner & Griswold (1983). All pulse–chase labelling experiments were repeated at least twice. Relative amounts of radioactivity in gels were determined with a phosphorimager or by scanning of films with a densitometer. Pre-protein processing was calculated as the percentage of the total labelled protein (precursor + mature) present in the mature form at the time of sampling. Differences of more than 10–15% pre-protein processing were reproducibly detected in parallel labelling experiments. Differences in pre-protein processing that are smaller than 10% are, in general, not reproducible.

Immunoblotting. Samples for SDS-PAGE were prepared by boiling of cells (5 min) in sample buffer (Laemmli, 1970). Separated proteins were transferred to a nitrocellulose membrane (BA85; Schleicher & Schuell) as described by Towbin *et al.* (1979). Proteins were detected with specific antisera and ¹²⁵I-labelled protein A. Relative amounts of radioactivity on blots were determined with a phosphorimager.

β -Galactosidase activity. β -Galactosidase activity assays were carried out as described by Miller (1972).

Luciferase activity and aggregation. *In vivo* luciferase activity assays were performed essentially as described by Schröder *et al.* (1993). *E. coli* cells producing luciferase were grown at 30 °C in TY medium. When the cells reached an absorbance (A_{600}) of 0.5, kanamycin (100 μ g ml⁻¹) and chloramphenicol (50 μ g ml⁻¹) were added to stop further protein synthesis, and samples of 1 ml were incubated for 10 min at 42 °C to inactivate luciferase. Next, the samples were incubated at 30 °C and the reactivation of luciferase was determined in duplicate experiments. *In vitro* luciferase aggregation assays were performed as described by Schröder *et al.* (1993).

RESULTS

CsaA affects the expression of *secB*

Due to the temperature sensitivity of the SecA51 protein, *E. coli* MM54 is unable to export proteins at 42 °C. Consequently, the strain is unable to grow at this temperature (Oliver & Beckwith, 1981). We have previously shown that the CsaA protein from *B. subtilis*, encoded by plasmid pBNB8, is able to suppress both the protein export defect and the growth defect of *E. coli* MM54 at 42 °C (Müller *et al.*, 1992). Similar effects were observed when pBNB8 was introduced into other *E. coli* strains with temperature-sensitive SecA proteins (our unpublished results). In addition, the introduction of pBNB8 into *E. coli* CK1953 (*secB*::Tn5) which can not grow on complex media, allowed this strain to form

Fig. 1. Production of CsaA and SecB in *E. coli* MC4100. (a) The production of CsaA in *E. coli* MC4100(pBNB8) (CsaA expression: +) and *E. coli* MC4100(pTZcsa82) (CsaA expression: +++) was analysed by pulse-labelling at 37 °C for 30 s. *E. coli* MC4100(pBR322) was labelled as a control (CsaA expression: -). The production of CsaA was monitored by immunoprecipitation with specific antibodies, SDS-PAGE and fluorography. (b) Production of SecB in *E. coli* MC4100(pBR322) (CsaA expression: -) and *E. coli* MC4100(pTZcsa82) (CsaA expression: +++) was analysed by pulse-labelling at 42 °C for 30 s. Subsequent immunoprecipitation was carried out with antibodies against SecB, β -lactamase (Bla) and OmpA. Bands corresponding to SecB, β -lactamase and OmpA are indicated. The incorporation of ³⁵S-labelled methionine in pulse-labelled cells, indicated as c.p.m., was determined by liquid scintillation counting of 10 μ l aliquots of the labelling mixtures. p, precursor; m, mature.

colonies on complex media (Müller *et al.*, 1992). In contrast, CsaA did not suppress mutations in *secDF* and *secE* (our unpublished results), *secY* and *lep* (Müller *et al.*, 1992). These findings suggested that CsaA, which is a soluble cytoplasmic protein, exerts its effect during the early stages of protein export, involving both SecA and SecB. Thus, CsaA could, in principle, have a function analogous to that of the *E. coli* SecB protein, which is absent from *B. subtilis* (Kunst *et al.*, 1997). As CsaA lacks amino acid sequence similarity with SecB, immunological approaches were used to investigate whether CsaA was related to SecB. To this purpose plasmid pTZcsa82 was constructed by ligation of an 895 bp *SspI*–*HincII* fragment, which contains the complete *csaA* gene, into the high-copy-number pUC18-derived plasmid pTZ18R (Mead *et al.*, 1986). In *E. coli*

Fig. 2. Analysis of the expression of *secB* and *secA*. Time courses of the expression of the *secB'*-*lacZ* gene fusion of *E. coli* MC4100::pLBL332 (a) and the *secA'*-*lacZ* gene fusion of *E. coli* MM171.3 (b) in cells growing on TY medium at 37 °C. β -Galactosidase activities (closed symbols) and optical densities at 600 nm (open symbols) were determined in duplicate for strains containing the plasmids pBR322 (circles), pBNB8 (squares), or pTZcsa82 (triangles).

MC4100, the expression of the pTZcsa82-located *csaA* gene resulted in approximately fourfold overproduction of CsaA, as compared to *E. coli* MC4100(pBNB8) (Fig. 1a). Next, we tried to detect CsaA (12 kDa) with polyclonal antibodies directed against SecB, both by immunoblotting and immunoprecipitation from [35 S]-

Fig. 3. CsaA-stimulated precursor processing in the absence of SecB. The processing of pro-OmpA (a) and pre- β -lactamase (b) in *E. coli* CK1953 *secB*::Tn5 was analysed by pulse-labelling for 30 s at 42 °C. The efficiencies of processing of pro-OmpA and pre- β -lactamase are indicated as the percentage of the mature protein relative to the total amount of protein (precursor + mature protein). Strains tested were: *E. coli* CK1953(pBR322) (lanes 1, CsaA expression: -), *E. coli* CK1953(pBNB8) (lanes 2, CsaA expression: +) and *E. coli* CK1953(pTZcsa82) (lanes 3, CsaA expression: +++). p, precursor; m, mature.

methionine-labelled cells of *E. coli* MC4100(pTZcsa82). The results showed that this was not possible (data not shown), indicating that the antigenic determinants, recognized by the anti-SecB antibodies, are not present in CsaA. Unexpectedly, the amount of SecB immunoprecipitated from *E. coli* MC4100(pTZcsa82) was approximately 10-fold reduced, as compared to that from *E. coli* MC4100(pBR322), whereas similar amounts of the outer-membrane protein A (OmpA) and periplasmic β -lactamase (Bla) were precipitated from both strains (Fig. 1b). Similarly, the presence of pTZcsa82 in *E. coli* MM54 *secA51* resulted in a 10-fold reduction of the synthesis of SecB (data not shown). As cells containing pTZcsa82 or pBR322 incorporated comparable amounts of [35 S]methionine (Fig. 1b), the production of CsaA appeared to interfere specifically with the production of SecB.

To study the effects of CsaA on the production of SecB as a function of growth, *E. coli* MC4100::pLBL332, which contains a translational *secB'*-*lacZ* gene fusion (Müller, 1996), was transformed with pBNB8 or pTZcsa82. As a control, this strain was transformed with pBR322. The resulting strains were grown in TY medium and samples, withdrawn at hourly intervals, were assayed for β -galactosidase activity. Irrespective of the growth phase, the levels of β -galactosidase activity were approximately two- and fivefold reduced in the pBNB8- and pTZcsa82-containing cells, respectively, compared to the control cells (Fig. 2a). To demonstrate that the effect was specific for *secB* and to exclude the possibility that CsaA modulates the activity of β -

Fig. 4. Effects of CsaA production on the growth of chaperone mutant strains. *E. coli* strains MC4100 (wild-type), NRK117 (*groEL44*), NRK223 (*groES619*), BB1048 (*dnaK756*), BB1458 (*dnaJ259*), or BB1752 (*grpE280*) carrying plasmids pBR322 (circles) or pBNB8 (squares) were grown overnight in TY medium at 30 °C and diluted 100-fold in fresh TY medium. After 2 h growth at 30 °C, the temperature was raised to 42 °C. Growth was monitored by optical density readings at 600 nm.

galactosidase, *secA* expression was studied in *E. coli* MM171.3, which contains a chromosomal *secA'*-*lacZ* fusion (Schmidt & Oliver, 1989). Strain MM171.3 was transformed with plasmids pBR322, pBNB8 or pTZcsa82 and β -galactosidase activities were determined. As shown in Fig. 2b, the presence of pBNB8 or pTZcsa82 did not significantly affect the expression of *secA'*-*lacZ* in strain MM171.3. Taken together, these data show that the production of CsaA in *E. coli* (pBNB8 or pTZcsa82) results in a strong reduction of the expression of *secB*.

CsaA stimulates protein export in the absence of SecB

The suppression of the growth defect of *E. coli* MM54 *secA51* at 42 °C could either be due to the reduction of the synthesis of SecB, and consequently reduced rates of targeting of SecB-dependent pre-proteins to the SecA51 protein, or to a direct effect of CsaA on protein export. To analyse these possibilities, we investigated the efficiency of processing of pro-OmpA and pre- β -lactamase by signal peptidase in CsaA-producing cells of *E. coli* CK1953, which lack SecB. Compared to the wild-type, *E. coli* CK1953 exports pro-OmpA at a reduced rate (Kumamoto & Beckwith, 1985), whilst the rate of pre- β -lactamase export is not affected (Kumamoto, 1989). As shown in Fig. 3, the production of CsaA resulted in increased rates of pro-OmpA and pre- β -lactamase processing at 42 °C, the highest rates being observed in *E. coli* CK1953(pTZcsa82). At lower

temperatures, no alteration of protein export could be measured in the presence or absence of CsaA (data not shown).

As demonstrated by immunoblotting, the *E. coli* strains MM54 (*secA51*), CK1953 (*secB*::Tn5) and MC4100 (wild-type) produced comparable amounts of the major chaperones DnaK and GroEL, irrespective of the production of CsaA (data not shown). Thus, the effects of CsaA at 42 °C, as described above, were not due to an increased synthesis of DnaK and GroEL, which have been implicated in protein export (Kusukawa *et al.*, 1989; Wild *et al.*, 1992). Furthermore, suppression of the *secA51* and *secB*::Tn5 mutations required the presence of an intact *csaA* gene (Müller *et al.*, 1992; our unpublished results), ruling out the possibility that the effects of CsaA production are due to protein (i.e. CsaA) overproduction *per se*, as previously reported for the suppression of cold-sensitive *sec* mutations through the overproduction of proteins unrelated to the process of protein export (Danese *et al.*, 1995).

Taken together, our data imply that CsaA either has a direct effect on protein export in *E. coli* MM54 *secA51* and CK1953 *secB*::Tn5 at 42 °C, or that CsaA induces the synthesis of as yet unidentified proteins which participate in maintaining pre-proteins in an export-competent conformation, thereby reducing (but not bypassing) the requirements for SecA and/or SecB. In what follows, we show that CsaA can prevent protein aggregation, suggesting that CsaA can have a direct effect on protein export.

Table 2. Growth of *E. coli* chaperone mutant strains containing plasmids pBR322 (control) or pBNB8 as determined by their ability to form single colonies on TY agar plates at 37 °C and 42 °C

A '+' indicates that the number of c.f.u. was identical to the number of c.f.u. at 28 °C; '+/-' indicates that the number of c.f.u. was at least 10- to 100-fold lower than the number of c.f.u. at 28 °C; '-' indicates that the number of c.f.u. was at least 1000-fold reduced compared to the number of c.f.u. at 28 °C.

Strain	Genotype	Growth of pBR322-containing strain		Growth of pBNB8-containing strain	
		37 °C	42 °C	37 °C	42 °C
MC4100	Wild-type	+	+	+	+
BB1458	<i>dnaJ259</i>	-	-	+	-
BB1048	<i>dnaK756</i>	+	-	+	+/-
BB1752	<i>grpE280</i>	+/-	-	+	-
NRK117	<i>groEL44</i>	+	-	+	-
NRK223	<i>groES619</i>	+	+/-	+	-

Fig. 5. Effects of CsaA production on the processing of β -lactamase in chaperone mutant strains. *E. coli* strains MC4100 (wild-type), NRK117 (*groEL44*), NRK223 (*groES619*), BB1048 (*dnaK756*), BB1458 (*dnaJ259*), or BB1752 (*grpE280*) carrying plasmids pBR322 (circles) or pBNB8 (squares) were grown overnight in M9 minimal medium at 30 °C, diluted in fresh M9 medium and incubated at 30 °C until the mid-exponential growth phase. Next, the temperature was raised to 42 °C and growth was continued for 2 h. Cells were pulse-labelled with [35 S]methionine for 30 s and chased with non-radioactive methionine at 42 °C. Samples were withdrawn at the times indicated. Processing of labelled pre- β -lactamase was analysed by immunoprecipitation and SDS-PAGE. The relative amounts of precursor and mature forms of β -lactamase were determined with a phosphorimager. The kinetics of pre- β -lactamase processing are plotted as the percentage of the total β -lactamase (precursor + mature) present in the mature form at the time of sampling.

CsaA-mediated suppression of the temperature-sensitive growth of *dnaK*, *dnaJ* and *grpE* mutants

Since the observed effects of CsaA on protein export are in accordance with the hypothesis that CsaA itself could

have a chaperone-like activity (Müller *et al.*, 1992), we tested whether this protein can complement the temperature-sensitive growth phenotype of *E. coli* strains carrying mutations in various chaperone-encoding genes. To this purpose, strains containing the

Fig. 6. CsaA stimulated reactivation of thermally inactivated luciferase in chaperone mutant strains. *E. coli* strains MC4100 (wild-type), NRK117 (*groEL44*), NRK223 (*groES619*), BB1048 (*dnaK756*), BB1458 (*dnaJ259*), or BB1752 (*grpE280*) carrying plasmids pBR322 (circles) or pBNB8 (squares) were transformed with plasmid pSEL16, which carries the *luc* gene for firefly luciferase, and grown in TY medium at 30 °C. To inactivate the luciferase present in the cells and to prevent *de novo* luciferase synthesis, the cells were incubated at 42 °C for 10 min in the presence of kanamycin and chloramphenicol. Next, the cells were reincubated at 30 °C ($t = 0$) and luciferase activities were determined in duplicate as a function of the incubation time. The kinetics of luciferase reactivation were plotted as the percentage of the luciferase activity before thermal inactivation at 42 °C.

groEL44, *groES619*, *dnaK756*, *dnaJ259* or *grpE280* mutations were transformed with plasmids pBNB8, pTZcsa82 or pBR322 (control). As essentially the same results were obtained with strains containing pBNB8 or pTZcsa82, only the results obtained with strains containing pBNB8 are documented here. As shown in Fig. 4, at 42 °C, the post-exponential growth in TY medium of strains containing the *groEL44* or *groES619* mutations was slightly, but reproducibly, impaired due to the expression of CsaA, similar to the wild-type strain MC4100. In contrast, the post-exponential growth of strains containing the *dnaK756*, *dnaJ259* or *grpE280* mutations was stimulated by the expression of CsaA, the growth of the *dnaJ* mutant strain being stimulated most strongly (Fig. 4). To verify these effects of CsaA, the growth of chaperone mutant strains expressing *csaA* was analysed on plates (Table 2). Whilst the expression of *csaA* neither exacerbated nor improved the ability of strains containing the *groEL44* or *groES619* mutations to form single colonies at 42 °C, a stimulatory effect could be observed in strains containing the *dnaK756*, *dnaJ259* or *grpE280* mutations (Table 2). Furthermore, the expression of CsaA strongly reduced the formation of long filamentous cells of strains containing the *dnaK756*, *dnaJ259* or *grpE280* mutations (data not shown). These findings show that CsaA can suppress growth defects of strains with impaired activity of the

DnaK–DnaJ–GrpE chaperone machinery. Nevertheless, CsaA was unable to replace DnaK as the transformation of *E. coli* BB1553 Δ *dnaK52*, which lacks the *dnaK* gene (Hesterkamp & Bukau, 1998), with pBNB8 or pTZcsa82 did not result in a suppression of the growth defects of this strain at 42 °C (data not shown). Furthermore, at 37 °C and 42 °C, the impaired replication of bacteriophage in the above strains with defective DnaK–DnaJ–GrpE or GroEL–GroES chaperone machineries (see Polissi *et al.*, 1995) was not restored by CsaA (data not shown). These observations demonstrate that CsaA can not complement for the malfunction of the major chaperone machineries of *E. coli*.

CsaA-stimulated export of β -lactamase in *groES* and *dnaJ* mutant strains

It was previously shown that mutations in chaperone-encoding genes affect the export of pre-proteins which are exported independently of SecB (Kusukawa *et al.*, 1989; Wild *et al.*, 1992, 1996). Therefore, we investigated whether the expression of CsaA in chaperone mutant strains containing pBNB8 affected the export of β -lactamase, which is one of the proteins not depending on SecB for its export. As shown in Fig. 5, at 42 °C, processing of pre- β -lactamase to the mature form in the *groEL44*, *dnaK756* or *grpE280* mutant strains was not

significantly affected by CsaA, like in the wild-type strain MC4100. In contrast, processing of pre- β -lactamase was stimulated by the expression of CsaA in strains containing the *groES619* or *dnaJ259* mutations (Fig. 5). These findings show that CsaA can, at least in part, suppress protein export defects of strains with mutations in the GroES and DnaJ chaperones.

CsaA-stimulated reactivation of heat-denatured luciferase in *groEL*, *groES*, *dnaK* and *grpE* mutant strains

The reactivation of thermally inactivated firefly luciferase at 30 °C is impaired in certain chaperone mutant strains (Schröder *et al.*, 1993). To further characterize the activity of CsaA in *E. coli*, its effects on the reactivation of luciferase were studied. To this purpose, wild-type and chaperone mutant strains containing plasmids pBNB8 or pBR322 were transformed with the compatible plasmid pSEL16. The latter plasmid contains the *luc* gene for luciferase under the control of a constitutive promoter (Gräfe *et al.*, 1996). As shown in Fig. 6, CsaA restored the reactivation of thermally inactivated luciferase in *dnaK756* and *grpE280* mutant strains. Interestingly, even though the reactivation of thermally inactivated luciferase was not impaired in strains containing the *groEL44* or *groES619* mutations, CsaA expression in these strains resulted in significantly increased levels of luciferase activity, suggesting that some luciferase was already in an inactive conformation before the heat treatment and that this luciferase was also reactivated by CsaA (Fig. 6). In contrast, CsaA did not restore the reactivation of luciferase in the *dnaJ259* mutant strain, and it interfered with the reactivation of luciferase in the wild-type strain (Fig. 6). As in the *dnaJ259* mutant strain, CsaA did not restore the reactivation of luciferase in the Δ *dnaK52* mutant strain (data not shown). As verified by Western blotting, cells containing pBR322 or pBNB8 produced comparable amounts of luciferase (data not shown), which is important because the reactivation kinetics of luciferase depend on its expression level. Thus, it seems that the expression of CsaA in strains containing different chaperone mutations has different effects on the reactivation of luciferase. In particular, the CsaA-mediated reactivation of thermally inactivated luciferase in *dnaK756* and *grpE280* mutant strains, and the enhanced reactivation of luciferase in *groEL* and *groES* mutant strains indicate that CsaA has a chaperone-like activity.

CsaA prevents the aggregation of luciferase *in vitro*

To obtain direct evidence for a chaperone-like activity of CsaA, its effects on the aggregation of luciferase at 42 °C and the reactivation of heat-denatured luciferase were determined *in vitro*, as previously described for DnaK, DnaJ and GrpE (Schröder *et al.*, 1993). To this purpose, purified 6H-CsaA was used. As revealed by light-scattering experiments, the presence of 6H-CsaA prevented the aggregation of luciferase at 42 °C. In

Fig. 7. CsaA prevents the aggregation of luciferase. Luciferase (100 nM) was equilibrated at room temperature in renaturation buffer (Schröder *et al.*, 1993) containing 1 mM ATP and 6H-CsaA (750 nM, squares), bovine serum albumin (750 nM, triangles), or no further additions (circles). Next, the mixtures were incubated at 42 °C and light scattering due to luciferase aggregation was monitored at 320 nm in a thermostated cuvette. Aggregation of luciferase in the absence of other proteins was almost complete after 30 min incubation at 42 °C and was set as 100%. The kinetics of luciferase aggregation are plotted as the percentage of this value.

contrast, luciferase aggregated in the presence of bovine serum albumin, or if no other protein was added to the reaction mixture (Fig. 7). Even though 6H-CsaA neither stimulated the reactivation of heat-denatured luciferase nor prevented its inactivation *in vitro* (data not shown), the observation that CsaA can prevent luciferase aggregation shows that it has a chaperone-like activity.

DISCUSSION

As evidenced by *in vivo* and *in vitro* studies, the CsaA protein of *B. subtilis* has chaperone-like activities. First, CsaA stimulated protein export in *secB*, *groES* and *dnaJ* mutant strains of *E. coli*. Second, CsaA suppressed the growth defects of *dnaK*, *dnaJ* and *grpE* mutants of *E. coli*. Third, CsaA stimulated the reactivation of heat-denatured firefly luciferase in *groEL*, *groES*, *dnaK* and *grpE* mutant strains of *E. coli*. Finally, CsaA prevented the aggregation of luciferase *in vitro*. How can we use these observations to explain the suppression of the growth and protein export defects of *secA* mutations through the production of CsaA? Other previously identified suppressors of mutations in *secA* seem to act pleiotropically by reducing the rate of protein synthesis in the cell (Brickman *et al.*, 1984; Oliver, 1985; Overhoff-Freundlieb & Freudl, 1991), or by the overproduction of GroEL (Van Dyk *et al.*, 1989). Since the production of CsaA did not affect the rate of protein synthesis, it seems that the suppression of the defects of *secA* mutant strains containing the complete *csaA* gene (Müller *et al.*, 1992; unpublished observations) must be attributed to the chaperone-like activities of CsaA. At least four mechanisms can be envisaged. First, CsaA could improve the translocation-competence of exported pre-

proteins, for example by preventing their aggregation, thereby making them better substrates for the mutant SecA protein. This view would be supported by the observation that CsaA was able to prevent the aggregation of luciferase *in vitro*. Second, CsaA could stimulate the translocation activity of mutant SecA proteins. In fact, CsaA could have a similar stimulating effect on the DnaK756 mutant protein, as evidenced by the observation that the production of CsaA caused a strong reactivation of heat-inactivated luciferase in the *dnaK756* mutant strain, but not in the Δ *dnaK52* strain, lacking the *dnaK* gene. Third, CsaA could complement SecA function, which seems to be unlikely considering the pivotal role of SecA in the translocation process. Fourth, CsaA could reduce the detrimental effects of *secA* mutations through a reduction of SecB synthesis and, consequently, through reduced rates of targeting of SecB-dependent pre-proteins to the translocase complex in the membrane. The latter explanation is, however, in apparent conflict with the observation that CsaA stimulated export of the SecB-dependent protein OmpA in the absence of SecB.

Interestingly, the production of CsaA antagonized the expression of the *secB* gene specifically, as shown by immunoprecipitation of pulse-labelled SecB and determination of β -galactosidase activities in a *secB'*-*lacZ* strain producing different levels of CsaA. As CsaA has chaperone-like activities, this observation is in accordance with the observation that the expression of *secB* is regulated in response to the cellular chaperone levels: *secB* expression was increased in *groES*, *groEL*, *dnaK*, *dnaJ* and *grpE* mutant strains (Müller, 1996) and reduced in strains overexpressing DnaK and DnaJ, or GroEL and GroES (our unpublished observations). Our present findings indicate that *secB* expression is not only regulated in response to the levels of homologous chaperones, but also in response to the production of a heterologous protein with chaperone-like activities. Notably, the expression of DnaK and GroEL was not affected by the production of CsaA, indicating that these chaperones are not involved in the CsaA-mediated repression of *secB*. Recently, it has been demonstrated that *secB* expression is under the control of catabolic repression by cAMP receptor protein–cAMP complexes at the transcriptional level (Seoh & Tai, 1997, 1999). However, in contrast to catabolic repression, which caused a 1.4-fold reduction of *secB* expression, the presence of CsaA affected the expression levels of *secB* about two- to fivefold, depending on the level of CsaA production. This suggests that CsaA does not exert its effects on *secB* expression via catabolic repression, but we are presently unable to exclude this possibility.

The phenotypes observed upon the production of CsaA in *groEL44*, *groES619*, *dnaK756*, *dnaJ259* and *grpE280* mutant strains indicate that CsaA can suppress a variety of different defects caused by the malfunction of the corresponding mutant chaperones. Two key observations may, at least in part, provide explanations for the effects of CsaA production in the latter strains. First, 6H-CsaA was shown to prevent the aggregation of

luciferase *in vitro*, but it was unable to reactivate heat-denatured luciferase or to prevent its inactivation. This indicates that CsaA has an aggregation-preventing rather than a folding-promoting chaperone-like function. Second, CsaA was unable to replace DnaK in the Δ *dnaK52* mutant strain with respect to temperature-sensitive growth and the *in vivo* reactivation of heat-inactivated luciferase. In contrast, CsaA was able to suppress these defects in the *dnaK756* mutant strain. Taken together, the latter findings show that the presence of CsaA supported the activity of the mutant DnaK756 protein. This could be a direct effect of CsaA on the DnaK756 protein (see above). Alternatively, an indirect effect through the prevention of protein aggregation by CsaA would also be a plausible explanation for these findings, the *in vivo* reactivation of heat-inactivated luciferase in particular. In fact, if CsaA can indeed prevent the *in vivo* aggregation of denatured luciferase, as it does *in vitro*, CsaA would keep this denatured protein in a proper condition for refolding/reactivation by the DnaK–DnaJ–GrpE and/or GroEL–GroES chaperone machineries. This would explain why CsaA showed a pleiotropic effect with respect to the reactivation of heat-inactivated luciferase in *dnaK756*, *grpE289*, *groEL44* and *groES619* mutant strains. Notably, the production of CsaA did not induce a general stress response as evidenced by the lack of effect of CsaA on the expression of DnaK and GroEL. In fact, CsaA activity seemed to interfere to some extent with chaperone activity in wild-type cells, as evidenced by the reduced reactivation of inactivated luciferase in cells of *E. coli* MC4100 producing CsaA.

Interestingly, as reflected by the processing of pre- β -lactamase to the mature form, CsaA only had a significant stimulating effect on β -lactamase export in those chaperone mutant strains in which the rate of export was drastically slowed down (i.e. *groES619* and *dnaJ259*). Consistent with the above hypothesis that CsaA keeps unfolded luciferase in a proper condition for *in vivo* refolding by general chaperones, our observations suggest that CsaA keeps pre- β -lactamase in a proper condition for translocation, for example by preventing its folding and/or aggregation in the cytoplasm. If so, this implies that CsaA has a pre-protein folding-preventing activity, analogous to that of SecB (Topping & Randall, 1997), which would explain the CsaA-mediated suppression of protein export defects in *secA51* and *secB::Tn5* mutant strains as well (see above). This view is consistent with our unpublished observation that purified SecB prevented the aggregation of denatured luciferase *in vitro* in a similar manner to CsaA.

Finally, the role of CsaA in *B. subtilis* remains to be defined. First results indicate that CsaA is required for the efficient secretion of at least a subset of proteins and that it interacts specifically with SecA and pre-proteins, suggesting that it could have protein-secretion-specific chaperone-like activities. This is an intriguing hypothesis in view of the fact that *B. subtilis* lacks a SecB homologue (Kunst *et al.*, 1997).

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