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Influence of Impaired Chaperone or Secretion Function on SecB Production in *Escherichia coli*

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The efficient export of proteins through the cytoplasmic membrane of *Escherichia coli* requires chaperones to maintain protein precursors in a translocation-competent conformation. In addition to SecB, the major chaperone facilitating export of particular precursors, heat shock-induced chaperones DnaK-DnaJ and GroEL-GroES are also involved in this process. By use of *secB′-lacZ* gene fusions and immunoprecipitation experiments, SecB production was studied in *E. coli* strains containing conditional lethal mutations in chaperone or *sec* genes. While the loss of heat shock chaperones resulted in an increased production of SecB, mutations in *sec* genes showed only minor effects on SecB synthesis. Neither the plasmid-mediated overexpression of precursors of exoproteins nor the overexpression of *secB* altered the synthesis of SecB. These results suggest that under conditions where chaperones become depleted, *E. coli* responds by raising the expression of *secB*. These data confirm the supposed synergy of different chaperones involved in protein export.

In *Escherichia coli*, exported proteins are synthesized as precursor proteins with an N-terminal signal peptide. A complex of so-called Sec proteins facilitates the translocation of these precursor proteins through the cytoplasmic membrane. SecB, a cytosolic chaperone protein, is required for efficient export of particular precursor proteins (24). It promotes the interaction of precursors with the SecA protein. At the membrane surface, it interacts with the translocase, an integral membrane protein complex formed by SecY, SecE, and SecG (Band 1 or P12). The translocase mediates the ATP- and proton motive force-dependent translocation across the membrane. The final steps of the translocation process are the removal of the signal peptide by leader peptidases (Lep or SPaseII) and the release of the mature protein into the periplasm, promoted by SecD and SecF (for a recent review, see reference 11).

In *E. coli*, synthesis of secretory proteins and translocation through the cytoplasmic membrane are not coupled. Consequently, it is necessary to maintain precursor proteins in an export-competent conformation prior to the interaction with the translocase. SecB, the major chaperone involved in protein export, promotes the translocation of so-called SecB-dependent precursors (8). These proteins are stabilized by forming a stoichiometric complex with SecB (31, 59), whereas other precursor proteins appear to be translocated in a SecB-independent manner (61). In addition to maintaining precursors in a loosely folded conformation, preventing aggregation and nonspecific interactions with the cytoplasmic membrane (60), SecB targets the precursor proteins to the peripheral membrane component of the protein secretion apparatus, SecA (9, 16). Recent studies demonstrate the existence of an alternative pathway that is likely to be involved in preprotein targeting in *E. coli* (32, 35, 43, 44). This pathway utilizes a ribonucleoprotein complex formed by the Ffh protein, a homolog to the 54,000-molecular-weight subunit of the mammalian signal recognition particle, and the 4.5S RNA, which has similar properties to those of the eukaryotic 7S RNA (35). *E. coli* strains depleted of Ffh or 4.5S RNA mainly accumulated SecB-independent precursors (β-lactamase, alkaline phosphatase, or ribose-binding protein), while levels of SecB-dependent precursor molecules like proOmpA remained unaffected (43, 44, 46).

Other molecular chaperones, such as the heat shock proteins DnaK-DnaJ and GroEL-GroES may also be involved in the export of several proteins. It has been shown that GroEL-GroES may participate in the export of certain proteins both in vitro (28) and in vivo (3, 27). DnaK and DnaJ facilitate protein export under conditions where SecB becomes limiting (62). The accumulation of secretory protein precursors, caused by either mutations in *secB* or *secA* or the overproduction of export-defective proteins, results in a two- to fivefold increase in the synthesis of these heat shock chaperones (63). However, overproduction of DnaK or GroEL-GroES did not rescue the export defect caused when SecB was limiting or absent (1). SecB itself is not a heat shock protein (1), and ATP has no known influence on its functional activity (45). The specific affinity of the SecB-precursor complex for SecA is likely to account for the unique role of SecB in translocation (16).

Little is known about the regulation of the Sec components. The only Sec protein known to be regulated is SecA, which regulates its own expression by binding to *secA* mRNA (51). The synthesis of SecA is coordinated with the level of protein secretion. When protein export is blocked genetically by using *sec* mutants or physiologically by high-level production of export-defective hybrid proteins, the SecA protein synthesis level is elevated 10- to 20-fold (49, 52). While the expression of heat shock chaperones has been studied extensively, nothing is known about factors affecting *secB* expression. To analyze the regulation of *secB* expression, the dependence of SecB production on (i) the activity of heat shock chaperones, (ii) the capacity of the secretion apparatus, and (iii) the overproduction of secretory proteins was studied. The results discussed in this report strongly indicate that *E. coli* regulates SecB production in response to the functionality of heat shock-induced chaperones.

MATERIALS AND METHODS

Media and reagents. For growth of bacteria, TY medium (containing 0.8% Bacto Tryptone, 0.5% Bacto Yeast Extract, and 0.5% NaCl) or M63 medium (36), solidified when required with 1.5% agar, was used. M9-1 and M9-2 media, used in labeling *E. coli*, were prepared as described by van Dijl et al. (58).

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype[a]	Source or reference
Strains		
MC4100	F⁻ Δ(*argF-lac*)*U169 araD139 relA1 rpsL150 thiA deoC7 ptsF25 rpsR flbB5301*	20
MC1000	F⁻ *araD139* (*ara-leu*)*7697* Δ*lac74 galE galK rpsL*	13
CG1	MC1000 *secD1*(Cs)	13
CK1953	MC4100 *secB*::Tn*5*	25
PR520	MC4100 *secE501 argE*::Tn*10*	48
MM52	MC4100 *secA51*(Ts)	40
MQ5	MC4100 *secA*(Ts)[Tyr-170→Asp]	53
MQ7	MC4100 *secA*(Ts)[Val-126→Glu]	53
MQ9	MC4100 *secA*(Ts)[Thr-122→del]	53
MQ10	MC4100 *secA*(Ts)[Ala-169→Asp]	53
BA13	MC4100 *secA13*(Am) *supF*(Ts) *trp*(Am)	5
MM66	MC4100 *geneX109*(Am) *supF*(Ts)	41
IQ85	MC4100 *secY24*(Ts) *zhd*Tn*10 rpsE*	54
IT41	W3110 *lep9*(Ts) Tc^r	19
BB1458	MC4100 *dnaJ259 thr*::Tn*10*	B. Bukau (56)
BB1048	MC4100 *dnaK756 thr*::Tn*10*	B. Bukau (14)
BB1752	B178 *grpE280 pheA*::Tn*10*	B. Bukau (2)
NRK117	MC4100 *groEL44*(Ts) *zje*::Tn*10*	27
NRK223	MC4100 *groES619*(Ts) *zje*::Tn*10*	27
Plasmids		
pLBL332	pLR300-derived plasmid of *L. lactis* carrying *secB′-lacZ* gene fusion; Em^r Km^r	This study
pACYC184	p15A-derived plasmid; Tc^r Cm^r	7
pBR322	Ap^r Tc^r	4
pUC19	Ap^r	65
pAK330	pBR322-derived plasmid carrying the complete *E. coli secB* gene; Ap^r	26
pSM752	*skc* Tc^r Em^r	33
pAR3	pACYC184-derived plasmid carrying the *araB* promoter operator and the *araC* repressor gene from *S. typhimurium*; Cm^r	42
pAR*malE*	pAR3-derived plasmid carrying *malE* under control of *araB* promoter operator; Cm^r	This study

[a] For example, Tyr-170→Asp indicates a Tyr-to-Asp mutation at position 170. del, deletion.

Ampicillin (40 μg/ml), kanamycin (20 μg/ml), or tetracycline (15 μg/ml) was added as required.

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 2-nitrophenyl-β-D-galactopyranoside (ONPG) were obtained from Serva. [^{35}S]methionine (specific activity, 1,330 Ci/mmol) was from Amersham International, Amersham, United Kingdom.

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Table 1.

Construction of a translational fusion between *secB* and *lacZ*. To monitor expression of *secB*, plasmid pLBL332 was constructed and integrated at the *secB* locus of the *E. coli* chromosome. The plasmid is based on the replication functions of plasmid pWVO2, which are present on a 5,072-bp *Eco*RI-*Sma*I fragment from pLR300 (22). The plasmid also contained a *secB′-lacZ* gene fusion, obtained by ligating a 700-bp *Bam*HI-*Hpa*I fragment from pAK330, which contained the 5′ end of *secB*, to a 3,200-bp *Sma*I-*Msc*I fragment from pMLB1034 (6), which contained the *lacZ* gene. The *secB′-lacZ* fusion contained the first 131 codons of *secB*. In addition, plasmid pLBL332 contained a kanamycin resistance marker from *Streptococcus faecalis*, which was present on an 1,850-bp *Eco*RI-*Bam*HI fragment from pAT21 (57). For chromosomal integration of pLBL332 into *E. coli* strains, electroporation (50) of pLBL332 or transduction with a P1 lysate from MC4100::pLBL332 was used. P1 transductions were carried out as described by Miller (36). Recipient cells were infected with phage P1*vir* grown on MC4100::pLBL332. Integrants were selected for a β-galactosidase-positive and kanamycin-resistant phenotype at the permissive temperature on Luria-Bertani (LB) agar plates containing kanamycin and X-Gal. Correct Campbell-type mode of integration of plasmid pLBL332 into the chromosome of *E. coli* strains was confirmed by Southern hybridization or PCR amplification of a junction DNA fragment reaching from a DNA region upstream of *secB* to *lacZ*. β-Galactosidase specific activity was measured with ONPG as the substrate as described previously (36).

Cloning and inducible expression of *malE*. The *malE* gene coding for the periplasmic maltose-binding protein MBP was amplified from *E. coli* MC4100 chromosomal DNA by PCR with primers MEP1 and MEK2. For amplification, oligonucleotides MEP1 (5′-GTTTTCACCTGCAGTTCACCAACAAGG-3′) and MEK2 (5′-CACGCCGGTACCGGCATTTCACAGC-3′) were used. MEP1 is localized 5′ to the ribosome binding site of *malE* incorporating a *Pst*I restriction site, and oligonucleotide MEK2 is localized 3′ to the *malE* stop codon incorporating a *Kpn*I restriction site. Amplification was carried out with the Expand high-fidelity PCR system (Boehringer Mannheim) with proofreading activity. The amplified fragment was digested with *Pst*I and *Kpn*I, purified from agarose gels, and cloned into *Pst*I- and *Kpn*I-digested pAR3 (42), resulting in plasmid pAR*malE*. On this plasmid, expression of *malE* is mediated by the arabinose-inducible *araB* promoter-operator from the *Salmonella typhimurium* arabinose operon (18).

In the absence of arabinose, almost no MBP could be detected after immunoprecipitation from pulse-labelled MC4100(pAR*malE*) cells grown in M9 medium containing 0.4% glucose. Induction of the *araB* promoter-operator resulted in a 100-fold induction of MBP production, exceeding the molar OmpA level about threefold as determined immunologically (data not shown).

Protein pulse-labeling. Pulse-labeling experiments were performed in M9 medium containing 0.4% glucose, as described by van Dijl et al. (58). Bacterial cultures were grown in M9-1 medium containing methionine and cysteine, washed once with methionine- and cysteine-free M9-2 medium, and further cultivated in M9-2 medium for 45 min. Cultures were pulse-labeled with 20 μCi of [^{35}S]methionine for the time indicated followed immediately by precipitation with trichloroacetic acid (0°C).

Immunoprecipitation, SDS-PAGE, fluorography, and quantification of protein. Immunoprecipitation was carried out as described by Edens et al. (12) with specific antisera. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (28). ^{14}C-methylated proteins from Amersham International were used as molecular weight reference markers. Fluorography was performed as described by Skinner and Griswold (55). For quantification of SecB and OmpA, relative amounts of radioactivity were estimated with a PhosphorImager (Molecular Dynamics). The relative level of SecB synthesis was calculated as the ratio of SecB to total OmpA (precursor and mature protein). Completeness of the immunoprecipitation was confirmed by a second immunoprecipitation step of the total cell lysates by using the same antibodies.

RESULTS

Construction of *secB′-lacZ* strains. The expression of *secB* was studied by using a translational *secB′-lacZ* gene fusion, localized on the *Lactococcus lactis* plasmid pLBL332. After the Campbell-type integration of pLBL332 into the *secB* region of the chromosome, the *secB′-lacZ* fusion was under the transcriptional control of the *secB* promoter, thereby facilitating the measurement of *secB* promoter activity (Fig. 1). A second intact copy of *secB* was under the control of the promoter of the tetracycline resistance gene of pBR322. To analyze the level of *secB* expression mediated by the tetracycline promoter, the amount of immunoprecipitable SecB was determined in MC4100 and MC4100::pLBL332. As a control, production of OmpA was determined. In MC4100::pLBL332, the ratio of SecB/OmpA was reduced about 2.5-fold as compared to that in MC4100 (Fig. 2).

In MC4100::pLBL332, the efficiency of processing of pro OmpA was reduced significantly but was still more efficient than in CK1953 *secB*::Tn*5*. To study whether the reduced expression of *secB* or the production of the SecB′-LacZ fusion protein affected the processing of proOmpA, MC4100::pLBL332 was transformed with plasmid pAK330. Plasmid-mediated sixfold overexpression of *secB* in MC4100::pLBL332 (pAK330) restored efficiency of proOmpA processing significantly but not to the wild-type level. In contrast, transformation of MC4100::pLBL332 with pBR322 reduced the processing of proOmpA slightly but reproducibly, compared with that of MC4100::pLBL332 (Fig. 2, compare lanes 1 and 3). Despite this observation, no alteration of growth characteristics or

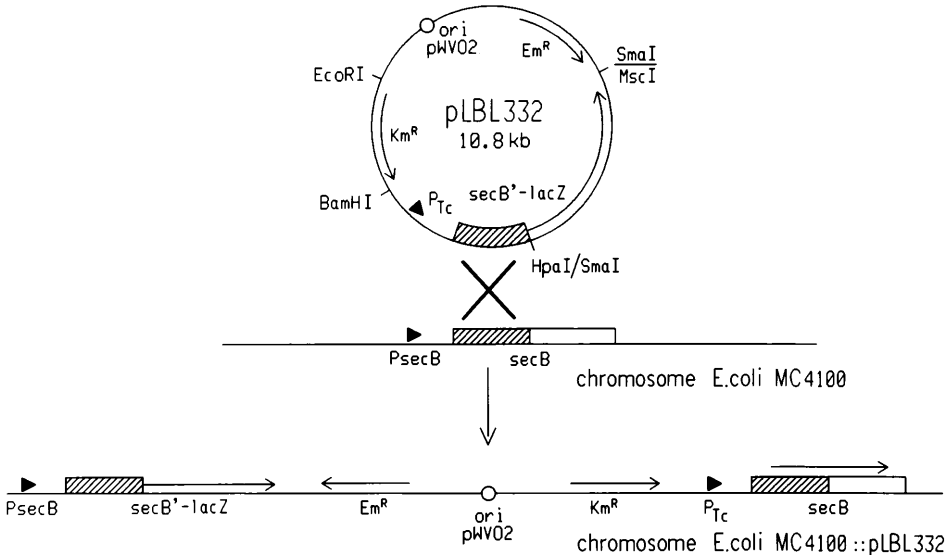

FIG. 1. Schematic representation of plasmid pLBL332 and Campbell-type mode of integration of pLBL332 into the *secB* region of the *E. coli* MC4100 chromosome. In the chromosome of MC4100::pLBL332, the *secB′-lacZ* fusion was under the transcriptional control of the promoter of *secB* (P_{SecB}), and the intact copy of *secB* was under the control of the promoter of the tetracycline resistance gene of pAK330 (P_{Tc}).

retardation of β-lactamase processing could be observed in MC4100::pLBL332(pBR322).

The effect of the introduction of *secB′-lacZ* into MC4100 on the synthesis of the chaperones DnaK and GroEL was also determined. Strains MC4100 and MC4100::pLBL332 were pulse-labeled, and DnaK, GroEL, and total OmpA were quantified by immunoprecipitation. No differences in the ratio of DnaK/OmpA or the ratio of GroEL/OmpA between the two strains, either at 28 or 42°C, could be observed (data not shown).

SecB production in *E. coli* strains with impaired chaperone function. To study the response of *secB* expression to the depletion of chaperones, plasmid pLBL332 was transformed into the strains NRK117 *groEL44*(Ts) and NRK223 *groES619*(Ts). Attempts to introduce the plasmid into strains BB1752 *grpE280* (Ts), BB1048 *dnaK756*(Ts), or BB1458 *dnaJ259*(Ts) failed, suggesting that the resulting strains were not viable. By use of a P1 lysate obtained from MC4100::pLBL332, kanamycin-resistant, β-galactosidase-positive transductants could be obtained from the *dnaK* and *dnaJ* strains but not from the *grpE* strain. These transductants showed poor growth at 28°C and formed tiny, dark-blue colonies on LB agar plates containing kanamycin and X-Gal which were easily overgrown by faster-growing mutants. Incubation at room temperature had no effect on the stability of the strains.

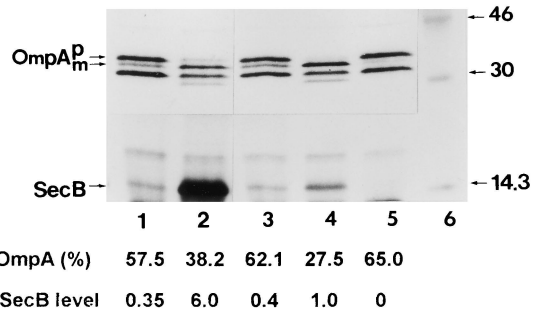

FIG. 2. Production of SecB and processing of proOmpA in *E. coli*. Cells of MC4100::pLBL332 (lane 1), MC4100::pLBL332(pAK330) (lane 2), MC4100::pLBL332(pBR322) (lane 3), MC4100 (lane 4), and CK1953 (lane 5) were grown in M9 medium at 37°C and pulse-labelled for 30 s. Labelling was stopped with trichloroacetic acid, and SecB and OmpA were immunoprecipitated and analyzed by SDS-PAGE. Production of SecB (rel. SecB level) was calculated in relation to production of OmpA. Lane 6, molecular mass reference markers (in kilodaltons); p, precursor; m, mature.

The *groEL44*(Ts) mutant NRK117::pLBL332 and *groES619* (Ts) mutant NRK223::pLBL332 were cultivated in TY medium at 28°C and shifted to 42°C during the exponential phase of growth. Samples were taken and β-galactosidase activity was monitored in comparison with that of the wild-type construct MC4100::pLBL332. At 28°C, the β-galactosidase activity of the *groES* strain was about 50% higher than that of the wild-type while the *groEL* strain showed levels of activity similar to that of the wild-type strain (Fig. 3a). After a temperature shift to 42°C, the level of β-galactosidase activity was significantly increased, two- to threefold in the case of NRK117::pLBL332 and up to fourfold in the case of NRK223::pLBL332, compared with that of *E. coli* MC4100::pLBL332 (Fig. 3a). When grown in M9-1 medium, the chaperone mutants showed a similar stimulation of β-galactosidase activity after shift to the nonpermissive temperature (data not shown).

Heat shock induction of proteins is accompanied by large transient changes in rates of protein synthesis (38). To distinguish between a potential heat shock response rather than the depletion of chaperones from the cells, the β-galactosidase activity was monitored in 10-min intervals after an upshift in temperature. With strains MC4100::pLBL332, NRK117::pLBL332, and NRK223::pLBL332, no induction of β-galactosidase activity could be observed for up to 30 min after the temperature shift (data not shown). In addition to the use of the chromosome-based *secB′-lacZ* gene fusion, the production of SecB in chaperone temperature-sensitive strains was determined by simultaneous immunoprecipitation of pulse-labelled SecB and OmpA. These results are summarized in Table 2. At 28°C, the *groEL*, *groES*, and *dnaJ* strains showed a slightly elevated level of SecB, while the level was increased about 2.5-fold in the *dnaK* strain. After 2 h at 42°C, SecB production in all chaperone temperature-sensitive strains was raised 2- to 4.4-fold as compared with that of the wild-type strain MC4100.

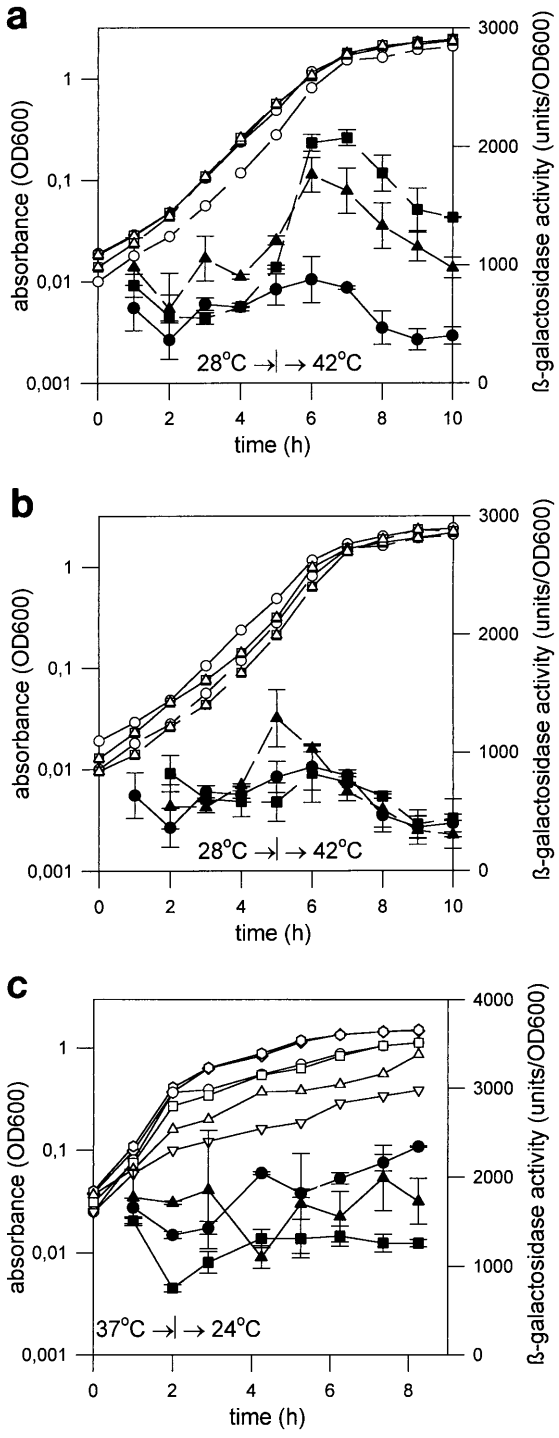

FIG. 3. Analysis of *secB* expression. Time course of growth and the expression of the *secB′-lacZ* gene fusion in cells growing in TY medium. Strains were grown at the permissive temperature for 5 (A and B) or 2 (C) h and then shifted to the nonpermissive temperature as indicated. β-Galactosidase activities (closed symbols) and optical densities (OD) (open symbols) were determined for the wild-type MC4100::pLBL332 (circles) (A to C); the *groEL44*(Ts) mutant NRK117::pLBL332 (squares), and the *groES619*(Ts) mutant NRK223::pLBL332 (triangles) (A); the *secA13*(Am) mutant MM66::pLBL332 (squares) and the *geneX109*(Am) mutant BA13::pLBL332 (triangles) (B); and the *secE501*(Cs) mutant PR520::pLBL332 (squares) and the *secD1*(Cs) mutant CG1::pLBL332 (triangles) (C). Experiments were carried out with duplicate cultures. Error bars indicate standard deviations of β-galactosidase activities.

TABLE 2. SecB levels in *E. coli* chaperone temperature-sensitive strains[a]

Strain	SecB/OmpA ratio[b]	
	28°C	42°C
MC4100	1 ± 0.063	1 ± 0.13
BB1458 *dnaK259*	2.42 ± 0.54	4.4 ± 0.85
BB1048 *dnaJ756*	1.7 ± 0.1	3.2 ± 0.45
BB1752 *grpE280*	ND[c]	2.16 ± 0.65
NRK117 *groEL44*	1.44 ± 0.22	2.6 ± 0.25
NRK223 *groES619*	1.38 ± 0.02	1.95 ± 0.35

[a] Bacterial cultures were grown at 28°C to the exponential phase and shifted to 42°C or further incubated at 28°C. After 2 h, cultures were pulse-labelled for 30 s and subsequently precipitated with trichloroacetic acid. Experiments were carried out with duplicate cultures.

[b] Ratio of incorporated [^{35}S]methionine in SecB to incorporated [^{35}S]methionine in total OmpA found in a given mutant divided by the comparable ratio for MC4100 at 28 or 42°C. Values are means ± standard deviations.

[c] ND, not determined.

The agreement between the results obtained with both the *secB′-lacZ* gene fusion and immunoprecipitation experiments showed that *secB* expression in *E. coli* responds to the malfunction of the heat shock chaperones tested to date in a similar way.

SecB production under impaired secretion capacity. To determine whether the production of SecB is elevated when protein secretion capacity is impaired, *E. coli* strains carrying conditional mutations in *secA*, *secD*, *secE*, *secY*, and *lep* were transformed with plasmid pLBL332. Stable kanamycin-resistant transformants of pLBL332 were isolated from strains BA13 *secA13*(Am) and MM66 *geneX109*(Am). In these strains, amber mutations depleting *secA* expression are suppressed by a temperature-sensitive *supF*. Chromosomal integration of pLBL332 did not result in an alteration of their phenotype at low temperatures (data not shown). No transformants could be obtained from any temperature-sensitive *secA* strain. By transduction of P1 phage grown on MC4100::pLBL332, β-galactosidase-positive and kanamycin-resistant transductants could be obtained from MM52, MQ5, and MQ10 but not from MQ7 or MQ9. At room temperature, these transductants were extremely mucoid and unable to form single colonies like those observed for *secB* null strains (25). Since *secB* null strains grow well on M63 minimal medium, the transductants were cultivated on minimal agar. When M63 minimal agar plates were used, the viability of these strains did not improve. At 28°C and above, fast-appearing β-galactosidase-negative revertants reflected the instability of these strains. At 28°C, kanamycin-resistant transductants of strains IQ85 *secY24*(Ts) and IT41 *lep9*(Ts) grew poorly and were rapidly overgrown by revertants, again reflecting the reduced viability of the *secB′-lacZ* strains. In contrast, plasmid pLBL332 could be introduced stably into the chromosome of the cold-sensitive strains PR520 *secE501* (Cs) and CG1 *secD1*(Cs).

The expression of *secB* in the *secA* amber strain BA13::pLBL332, the *geneX* amber strain MM66::pLBL332, and the cold-sensitive *secE* and *secD* strains PR520::pLBL332 and CG1::pLBL332, respectively, was studied by measuring β-galactosidase activity. In none of these strains was a significant alteration of β-galactosidase activity measured, at either the permissive or nonpermissive temperature, compared with that of MC4100::pLBL332 (Fig. 3b and c). The strain carrying the *geneX* amber mutation showed a twofold increase in β-galactosidase activity directly after the upshift to the nonpermissive temperature.

TABLE 3. SecB levels in *E. coli sec* strains[a]

Strain	SecB/OmpA ratio[b]
Temperature sensitive	
MC4100	1 ± 0.12
MM52 *secA51*(Ts)	1.25 ± 0.07
MQ9 *secA*(Ts)	1.86 ± 0.92
MM66 *secA13*(Am)	1.72 ± 0.8
BA13 *geneX109*(Am)	1.5 ± 0.36
IQ85 *secY24*(Ts)	2.05 ± 0.53
Cold sensitive	
MC4100	1 ± 0.085
PR520 *secE501*(Cs)	0.92 ± 0.34
CG1 *secD1*(Cs)	0.92 ± 0.32

[a] Bacterial cultures were grown in M9 medium at the permissive temperature (28 or 37°C) to the exponential phase of growth, shifted to the nonpermissive temperature (42 or 24°C) for 2 h, and then pulse-labeled for 30 s. Experiments were carried out with triplicate cultures.

[b] Ratio of incorporated [^{35}S]methionine in SecB to incorporated [^{35}S]methionine in total OmpA found in a given mutant divided by the comparable ratio for MC4100. Values are means ± standard deviations.

SecB production in the conditional *sec* strains was monitored in parallel with a pulse-label followed by simultaneous immunoprecipitation of SecB and OmpA (Table 3). With the possible exception of IQ85*secY24*(Ts), the analyzed *sec* mutants showed at most a small increase in the SecB/OmpA ratio 2 h after the shift to the nonpermissive temperature (Table 3).

Production of SecB at elevated temperatures. SecB production was studied after prolonged cultivation at 42°C of strains MC4100, MM52 *secA51*(Ts), and NRK117 *groEL44*(Ts). In each case, the ratio of immunoprecipitable SecB to OmpA was monitored at 0, 1, 2, and 3 h after upshift. The wild-type and *secA* strains showed no increase in the relative SecB concentration, while a fivefold induction could be observed in the *groEL* strain (Fig. 4).

SecB production in *E. coli* MC4100::pLBL332 overexpressing secretory proteins. It has been noted previously that expression of the *secA* gene is coregulated with the protein secretion status of the cell, since SecA protein levels are elevated by the induction of high-level production of MalE-LacZ hybrid protein, causing accumulation of native precursor molecules (41). The plasmid-mediated overexpression of exoproteins also increases the pool of precursors with possible consequences to SecB production. MC4100::pLBL332 was transformed with plasmids encoding export proteins, and β-galactosidase activity was measured (Table 4).

TABLE 4. β-Galactosidase activity of *E. coli* MC4100::pLBL332 *secB′-lacZ* containing plasmids coding for exoproteins[a]

Plasmid	Secretory protein	β-Galactosidase activity[b]
None	None	1,479 ± 161
pACYC184	None	1,279 ± 109
pUC19	β-Lactamase	1,200 ± 178
pBR322	β-Lactamase	1,316 ± 176
pSM752	Streptokinase	1,350 ± 188
pAK330 *secB*	β-Lactamase	1,589 ± 83

[a] Bacterial cultures were grown in TY medium with the appropriate antibiotics at 37°C. Samples were taken from exponential-phase cultures. Experiments were carried out with triplicate cultures.

[b] β-Galactosidase activity is indicated in Miller units (means ± standard deviations).

Plasmid-mediated overexpression of the SecB-independent β-lactamase in MC4100::pLBL332 was achieved by using multicopy plasmid pBR322 or pUC19. In the plasmid-containing strains, no significant difference in β-galactosidase activity could be detected compared with that in MC4100::pLBL332 (Table 4). In MC4100::pLBL332(pAR*malE*), induction of plasmid-mediated production of MBP did not result in a significant alteration of β-galactosidase activity (Fig. 5).

It has been shown previously that expression of streptokinase, a gene encoding a secretory protein cloned from *Streptococcus equisimilis*, is detrimental for *E. coli* because of deleterious effects of its signal sequence on the secretion apparatus (37). Streptokinase production in MC4100::pLBL332, mediated by the pACYC184-derived plasmid pSM752, did not result in a noticeable alteration of β-galactosidase activity compared with that of MC4100::pLBL332(pACYC184) (Table 4).

To determine whether the overproduction of SecB affects the activity of the *secB* promoter, MC4100::pLBL332 was transformed with pAK330 encoding *secB*. Its presence in wild-

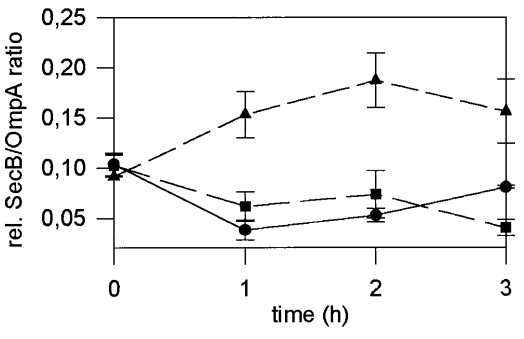

FIG. 4. Analysis of SecB synthesis at 42°C. Cells were grown to a low cell density at 28°C, upshifted to 42°C, and then pulse-labelled after 0, 1, 2, or 3 h. Cultures were labeled for 1 min immediately before temperature upshift or for 30 s at 1, 2, and 3 h after cultivation at 42°C. SecB and OmpA were immunoprecipitated and analyzed by SDS-PAGE. Duplicate cultures of *E. coli* MC4100 (circles), MM52 *secA51* (squares), and NRK117 *groEL44* (triangles) were analyzed. SecB levels are the ratio of SecB to OmpA; standard deviations are indicated by error bars.

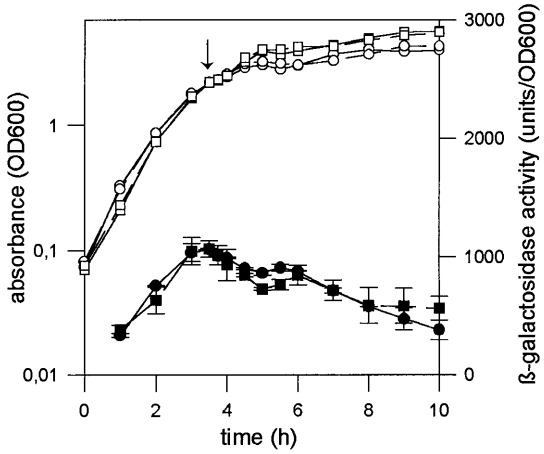

FIG. 5. Expression of the *secB′-lacZ* gene fusion in *E. coli* MC4100::pLBL332(pAR*malE*). Cells were grown in TY medium at 37°C. After 3 h (arrow) in two cultures (squares), expression of *malE* was induced by the addition of arabinose (50 mg/liter), while in two different cultures (circles), the expression of *malE* remained uninduced. β-Galactosidase activities (closed symbols) and optical densities (OD) (open symbols) were determined from duplicate cultures. Error bars indicate standard deviations of β-galactosidase activities.

type strains resulted in a sixfold increase in the production of SecB, as determined immunologically (Fig. 1). The overexpression of *secB* did not alter β-galactosidase activity compared with that of MC4100::pLBL332(pBR322) (Table 4).

DISCUSSION

In this paper, the dependence of *E. coli secB* expression on (i) the presence of cellular heat shock chaperones, (ii) the level of production of exoproteins, and (iii) the capacity of the secretion apparatus was studied. While impaired chaperone function stimulated SecB synthesis two- to fivefold, SecB synthesis was insignificantly altered in mutants carrying cold- or temperature-sensitive *sec* genes grown at nonpermissive temperatures or strains overexpressing genes encoding secretory proteins.

The expression of *secB* was studied by use of a *secB′-lacZ* gene fusion and simultaneous immunoprecipitation of SecB and OmpA of radiolabeled cultures. Relative SecB levels were determined as a ratio of [^{35}S]methionine incorporated in SecB to total (percursor and mature) OmpA. In general, synthesis of OmpA is constitutive (47). Therefore, SecA levels are routinely expressed relative to OmpA (49, 53). However, the stability of *ompA* mRNA is regulated in a growth rate-dependent manner (34, 39). In general, conclusions regarding the possible regulation of *secB* expression are made on the basis of the comparison of relative levels of SecB obtained from *E. coli* mutants and the wild-type strain. The possible alteration of *ompA* expression should affect SecB/OmpA ratios in all strains in a similar way. In addition, the results obtained by calculating the alteration of SecB production as a ratio of radiolabelled SecB to the level of total [^{35}S]methionine incorporation were similar to these obtained with the SecB/OmpA ratio. Finally, the results obtained by use of *secB-lacZ* gene fusions and simultaneous immunoprecipitation of SecB and OmpA showed a similar response to the depletion of chaperones or Sec components.

β-Galactosidase activity from the *secB′-lacZ* strains was determined from cultures grown in rich (TY) medium and synthetic (M9-1) media. Effects were independent of the growth medium used. However, β-galactosidase specific activities were dependent on the medium and cultivation conditions. In synthetic medium, the level of β-galactosidase activity was, in general, lower than that obtained in TY medium. Changes in β-galactosidase activity of a single strain at different temperatures could be caused by altered stability of the SecB′-LacZ fusion protein. In addition, temperature-dependent variation of the ratio of SecB/OmpA might be due to different protein stabilities or altered *ompA* expression. Therefore, no conclusion about the effect of temperature on SecB production could be made.

Integration of plasmid pLBL332 into the chromosome of MC4100 resulted in an approximately 2.5-fold reduction of immunoprecipitable SecB and a drastic retardation of proOmpA processing. Plasmid-mediated overexpression of *secB* restored the precursor processing. The level of heat shock chaperone DnaK or GroEL, the efficient processing of β-lactamase, and the growth characteristics of MC4100::pLBL332 showed no visible alteration compared with that of the wild type. These observations indicate that the accumulation of SecB-dependent proOmpA was caused mainly by the reduced level of SecB and not by the production of the SecB′-LacZ hybrid protein, with potential detrimental effects on the secretion apparatus.

While the integration of pLBL332 into wild-type strain MC4100 had no apparent effects on the growth characteristics, the combination of *secB′-lacZ* with temperature-sensitive mutations in the chaperone genes *dnaK*, *dnaJ*, and *grpE* strongly reduced their viability. This may be due to the inability of the integrants to regulate their *secB* expression. The strains carrying *groEL44*(Ts) and *groES619*(Ts) mutations were able to tolerate the integration of pLBL332. Simultaneous immunoprecipitation experiments showed that at both 28 and 42°C, SecB production in these strains was less affected than that in strains carrying mutations in *dnaK*, *dnaJ*, and *grpE*. The comparable small stimulation of SecB production in the *groEL* and *groES* strains and the toleration of *secB′-lacZ* in the chromosome of these strains indicate a stronger functional relationship of SecB with proteins at the beginning of the chaperone cascade (30) than with those at the end. The induction of heat shock proteins in mutants lacking SecB (63) and the observed general stimulation of *secB* expression in all chaperone mutants tested to date confirm the suggested synergy of cellular chaperones in precursor targeting (17).

Under physiological conditions, SecB does not appear to exhibit significant association with cytoplasmic proteins (23). The selectivity of SecB for its ligands can be explained in part by a kinetic partitioning (15). Under conditions in which the general chaperone function of the cell becomes impaired, the folding pathway of newly synthesized polypeptides is affected, resulting in probable unphysiological specificities of SecB binding. The unspecific binding of SecB to nonsecretory proteins would raise the demand of the cell for SecB, with possible consequences for its synthesis rate.

All strains carrying temperature-sensitive mutations in genes coding for components of the secretion apparatus showed a markedly reduced viability after integration of *secB′-lacZ*. The inhibition of protein export at the permissive (low) temperature has been shown for strains containing *secA51*(Ts) or *secY24*(Ts) (21). At the low temperature, the combination of the reduced secretion capacity of the *sec* strains and the reduced production of the SecB might be detrimental for the cell. Only the *secA* amber strains MM66 and BA13 tolerated the integration of plasmid pLBL332. The presence of the temperature-sensitive suppressor SupF in MM66 and BA13 obviously circumvented any phenotypic alteration at 28°C. In contrast, plasmid pLBL332 could be integrated into the chromosome of the strains PR520 *secE501*(Cs) and CG1 *secD1*(Cs) and stably maintained at 37°C.

By using the *secB′-lacZ* gene fusion in all *sec* strains tested, no notable differences in *secB* expression could be observed compared with that of the wild type. However, immunoprecipitation of radiolabelled SecB revealed minor [in the case of the *secY*(Ts) strain IQ85, up to twofold] increases in SecB production in response to the depletion of Sec proteins. Since the impaired capacity of the secretary complex of the *E. coli sec*(Ts) strains results in accumulation of protein precursors in the cytoplasm, extensive growth at the nonpermissive temperature results in a range of other physiological alterations (10). The absence of an evident SecB response could, for instance, be due to the raised levels of heat shock-induced proteins as well (63).

Expression of *secB* was studied after plasmid-mediated overexpression of SecB-dependent and SecB-independent precursor proteins. Neither the constitutive expression of streptokinase or SecB-independent β-lactamase nor the induction of SecB-dependent MBP resulted in a serious alteration of *secB′-lacZ* expression. It should be noted that it is not known whether streptokinase is translocated in a SecB-dependent or -independent way. Processing kinetics of the streptokinase precursor were comparable in MC4100 and CK1593 *secB*::Tn*5*, suggesting that SecB is not required for the translocation of the precursor (unpublished results). Induction of high-level produc-

tion of MBP resulted in accumulation of proOmpA (data not shown). This indicated a saturation of the SecB-dependent secretion route of the cell.

The data discussed above, therefore, suggest that cells defective in functional chaperone activity respond by induction of the production of SecB. Conversely, *E. coli* strains lacking SecB respond by producing five- to sixfold-higher concentrations of DnaK and DnaJ, compared with the isogenic wild-type strain (63). This provides them with a mechanism to overcome the bottleneck in the cytoplasmic part of the secretion process (62).

Heat shock induction of proteins is accompanied by large transient changes in rates of protein synthesis (38). No increase of the SecB/OmpA ratio could be observed for up to 30 min after temperature shift to 42°C. Only the prolonged incubation of chaperone temperature-sensitive strains at the nonpermissive temperature induced SecB production permanently, presumably as a result of the depletion of functional chaperone activity with time. This observation confirms that the expression of *secB* is not regulated similarly to that of heat shock proteins. It should be noted that it is not known whether the shift to the nonpermissive temperature inactivates the mutated chaperone proteins synthesized before temperature upshift or if functional chaperones are depleted after prolonged growth at the nonpermissive temperature. The gradual fall in growth accompanying the gradual increase in *secB* expression indicates the latter. Since the plasmid-mediated overexpression of *secB* in MC4100::pLBL332(pAK330) did not result in altered β-galactosidase activity, it appears that the *secB* gene product is not affecting its own expression. Currently, this line of research is being pursued to obtain further clues on how the expression of *secB* is regulated genetically.

Previous studies showed that induction of the heat shock transcription factor sigma^{32} does not influence *secB* expression, and it was therefore concluded that SecB was not a heat shock protein (1). ATP has no known effect on its functional activity (45). Therefore, it is unlikely that SecB is able to substitute for the malfunction of general chaperones. Since the depletion of general chaperones results in increased SecB production, the data presented in this paper provide further evidence that the general chaperones mediate the folding of protein precursors (27, 29, 62). It is not clear yet whether all of the newly synthesized precursors of exoproteins recognized by general chaperones or only a subset of precursor molecules, namely, SecB-independent exoproteins, keeps their export-competent state via interaction with DnaK-DnaJ and GroEL-GroES. Several models have been suggested to explain the possible involvement of general chaperones, SecB, and the signal recognition particle-like ribonucleoprotein complex in chaperoning and targeting proteins for export (17, 32, 64). Further studies need to be done to study the functional relationship between the different cytosolic components involved in bacterial protein export.

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