Accumulation of Secretory Protein Precursors in *Escherichia coli* Induces the Heat Shock Response

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The accumulation of secretory protein precursors, caused either by mutations in *secB* or *secA* or by the overproduction of export-defective proteins, results in a two- to fivefold increase in the synthesis of heat shock proteins. In such strains, σ^{32} , the alternative sigma factor responsible for transcription of the heat shock genes, is stabilized. The resultant increase in the level of σ^{32} leads to increased transcription of heat shock genes and increased synthesis of heat shock proteins. We have also found that although a *secB* null mutant does not grow on rich medium at a temperature range of 30 to 42°C, it does grow at 44°C. In addition, we found that a *secB* null mutant exhibits greater thermotolerance than the wild-type parental strain. Elevated levels of heat shock proteins, as well as some other non-heat shock proteins, may account for the partial heat resistance of a SecB-lacking strain.

The *Escherichia coli* SecB protein participates in the export of a subset of proteins destined for the periplasm or outer membrane. In its function as a chaperone, it maintains target precursor proteins in a partially unfolded state competent for translocation. Moreover, because of its interaction with SecA, the peripheral membrane domain of the translocase, SecB helps to position precursor proteins at membrane sites containing the integral SecY/E components of translocase (for a review, see references 24, 25, and 30). Strains lacking the SecB chaperone are viable, but they have a number of pleiotropic defects. In such strains, export of the secretory proteins LamB, OmpA, OmpF, PhoE, and maltose-binding protein is defective, leading to an accumulation of precursor forms of these proteins. In addition, although *secB* deletion strains are viable on minimal glycerol medium, they are unable to grow on rich medium (16, 17).

We have recently shown that the DnaK and DnaJ heat shock proteins can partially substitute for the SecB chaperone function. Strains lacking SecB require DnaK and DnaJ both for residual transport of several SecB-dependent proteins and for viability on minimal medium. Furthermore, overproduction of DnaK and DnaJ permits *secB* null mutants to grow on rich medium, albeit at a reduced rate, and enhances the rate of export of some SecB-dependent proteins (31). These observations led us to wonder whether mutants lacking SecB might have a higher level of heat shock proteins than wild-type strains. This seemed possible because the cytoplasmic precursor proteins that accumulate in *secB* deletion strains may be seen as abnormal proteins by the cell. A variety of abnormal proteins, including puromycin fragments (11), induce the heat shock response. In addition, unfolded proteins that are not degraded (23) and the MalE-LacZ hybrid protein (14) also induce the heat shock response. In this report, we show that in a *secB* null mutant and a *secA*(Ts) mutant, synthesis of heat shock proteins is also induced. Moreover, we show that the signal for induction of heat shock protein synthesis is accumulation of cytoplasmic precursors of secretory proteins and that this results in accumulation of σ^{32} , the alternative sigma factor

responsible for directing RNA polymerase to transcribe heat shock genes.

MATERIALS AND METHODS

Strains, plasmids, and bacteriophages. *secB::Tn5* (17) was introduced into MC1061 (6) by P1 transduction to create CAG13461. All labeling experiments were carried out with this strain. To construct the *PhpG-lacZ* operon fusion (pJW2), the *EcoRV* fragment containing the *htpG* promoter (pDC421 laboratory collection) was cloned into the *SmaI* site of pRS415 (26). The *PhpG-lacZ* fusion was transferred in vivo into λ RS45 (26) to give λ JW2. The expression of the *PhpG-lacZ* fusion (see Table 2) was monitored in MC4100 (5) derivatives. pSE87 was constructed by recombining the *lamBSE87* mutation from λ apSE87 (9) into pSE1 (1). pUClamBSE87 was constructed by moving the *EcoRI-StuI lamBSE87* fragment from pSE87 into pUC8 that had been restricted with *EcoRI* and *StuI*. Plasmids pUClamBSE87, pUClamBSE60, and pUClamBSE60 Δ IR are described elsewhere (1).

Growth conditions, labeling, and assays. Cells were grown at 30°C in M9 minimal medium supplemented with 0.5% glycerol and all amino acids except L-methionine and L-cysteine. To measure rates of protein synthesis, 1-ml samples of exponentially growing cells were pulse-labeled for 1 min with 20 μ Ci of Tran ³⁵S-label per ml (or 80 μ Ci/ml for σ^{32} synthesis), chased for 1 min with 200 μ g of nonradioactive L-methionine and L-cysteine per ml, and transferred to trichloroacetic acid. To examine the global heat shock response, samples were analyzed by two-dimensional gel electrophoresis as described previously (12) with 0.1% sodium dodecyl sulfate–13.75% polyacrylamide for the second dimension. The synthesis rates of selected heat shock proteins were determined by immunoprecipitating ³⁵S-labeled protein with the corresponding antibodies, using L-[³H] leucine-labeled cells to correct for losses during sample preparation (13). Synthesis of σ^{32} was determined by an immunoprecipitation which was standardized by coimmunoprecipitation of a truncated σ^{32} (28). To measure σ^{32} stability, 1-ml aliquots were removed to trichloroacetic acid at the indicated times following addition of excess unlabeled

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FIG. 1. Two-dimensional electrophoretic analysis of proteins synthesized in the *secB* null and *secB*⁺ strains. CAG13461 (*secB*::Tn5) grown at 30°C (B) and MC1061 (*secB*⁺) grown at 30°C (A) and then shifted for 5 min to 42°C (C) were labeled with Tran ³⁵S label, processed, and electrophoresed in two dimensions as described in Materials and Methods. Protein spots are marked as follows: ○, heat shock proteins (1, DnaK; 2, GroEL; 3, HtpG; 4, F84.1; 5, GrpE; 6, GroES); □, control proteins (7, EF-G; 8, EF-Tu); and △, non-heat shock proteins specifically induced in the *secB*::Tn5 mutant.

L-methionine and L-cysteine to the pulse-labeled samples, immunoprecipitated, and processed as described previously (28). Radioactivity was quantified with an Ambis radioanalytic imaging system (AMBIS Systems, San Diego, Calif.) interfaced with an IBM computer. The level of σ^{32} was determined by Western blot (immunoblot) analysis as described previously (28). Total protein extracts were prepared from the cell samples, and the volume of cell extracts loaded onto gels was adjusted to account for differences in cell density at the time of sampling. To quantify Western blots, a

Hoefer Scientific GS300 Scanning Densitometer interfaced with a Macintosh computer was employed. The rich medium used was Luria-Bertani medium described previously (20). β -Galactosidase assays were performed as described previously (20).

Thermotolerance assay. Cells grown in M9 glycerol medium to an A_{450} of 0.3 were diluted 10⁴-fold to a final volume of 10 ml and shifted to 50°C. Samples of 0.1 ml taken immediately before the shift to 50°C and at various times after the shift were plated on M9 glycerol plates and incubated at 30°C. To induce thermotolerance, cells were preincubated for 15 min at 42°C before the 50°C temperature challenge.

RESULTS

Heat shock protein synthesis is induced in the *secB* null mutant. To determine whether the synthesis of heat shock proteins is elevated in the *secB*::Tn5 mutant, we compared the rate of the heat shock protein synthesis in a *secB* null mutant with that of its wild-type parent by two-dimensional gel electrophoresis. The heat shock proteins (including DnaK, GroEL, HtpG, F84.1, GrpE, and GroES) were synthesized in increased amounts at 30°C in the *secB* null mutant (Fig. 1B). We have also found that in the *secB*::Tn5 strain at least seven additional non-heat-shock proteins are synthesized at a higher rate than in the isogenic *secB*⁺ strain grown at either 30 or 42°C (compare Fig. 1B with Fig. 1A and C).

To quantify the magnitude of induction of the heat shock protein synthesis, we compared the synthesis rates of DnaK, GroEL, and GrpE at 30°C in the *secB*::Tn5 and wild-type strains by using a pulse-chase, immunoprecipitation protocol (see Materials and Methods). The synthesis of all three proteins at 30°C is two- to fivefold higher in the *secB* null mutant than in the isogenic *secB*⁺ control (Table 1). Interestingly, expression of DnaK and GrpE seems to be maximally induced at 30°C, whereas GroEL is induced further after temperature upshift.

We used a transcriptional fusion of the *htpG* heat shock promoter to *lacZ* to determine whether increased synthesis of heat shock proteins results from increased transcription of the heat shock genes. The synthesis of β -galactosidase directed from the *htpG* promoter is fivefold higher in the *secB*::Tn5 mutant than in its *secB*⁺ parent (Table 2). Thus, a strain lacking the SecB chaperone exhibits increased tran-

TABLE 1. Heat shock protein synthesis is enhanced in the *secB* null mutant^a

Protein	Temperature (°C)	Synthesis with the following strain:	
		<i>secB</i> ⁺	<i>secB</i> ::Tn5
DnaK	30	1.0	4.9
	42	5.8	5.9
GroEL	30	1.0	2.2
	42	5.1	5.3
GrpE	30	1.0	2.3
	42	2.6	3.0

^a Synthesis rates were determined in pulse-chase-labeled cells of MC1061 (*secB*⁺) or CAG13461 (*secB*::Tn5) by immunoprecipitation as described in Materials and Methods. The synthesis rates for each protein are normalized to that of the wild-type strain grown at 30°C. The values are averages of triplicate determinations.

TABLE 2. Accumulation of secretory protein precursors increases expression from the heat shock promoter^a

Strain	Medium	Relative activity of β -galactosidase
WT	M	1.0
<i>secB::Tn5</i>	M	5.1
WT	R	1.0
<i>secA(Ts)</i>	R	9.8
WT with pUClamBSE87	R	4.3
WT with pUClamBSE60	R	3.8
WT with pUClamBSE60 Δ IR	R	2.9

^a Expression of the *hspG* heat shock promoter was monitored by assaying the β -galactosidase activity of the fused *lacZ* reporter gene. The wild-type (WT), *secB::Tn5*, and *secA(Ts)* strains derived in MC4100, and lysogenized with λ JW2 carrying the operon fusion *PhtpG-lacZ*, were grown at 30°C in minimal glycerol (M) or rich (R) medium (see Materials and Methods). The activity of β -galactosidase calculated in Miller units (20) was normalized to that of the wild-type strain grown in the indicated medium. Plasmids pUClamBSE87, pUClamBSE60, and pUClamBSE60 Δ IR are described elsewhere (1). The values are averages of triplicate determinations.

scription from heat shock promoters, which leads to increased synthesis of heat shock proteins.

Accumulation of secretory protein precursors generates a signal for induction of heat shock proteins. In strains lacking the SecB chaperone, translocation of SecB-dependent secretory proteins is defective, resulting in accumulation of their precursors (17). It was reasonable to assume that these precursor forms of secretory proteins might be recognized by the cell as abnormal proteins and thus generate a signal for heat shock protein induction. We performed two experiments to test whether the accumulation of precursor proteins rather than some other feature of the *secB* null mutant strain was actually the signal. First, we showed that a *secA(Ts)* mutant, which also accumulates precursor proteins (22), also shows increased expression of the *hspG* heat shock promoter. The activity of β -galactosidase expressed from the *PhtpG-lacZ* fusion was almost 10-fold higher in the *secA(Ts)* strain than in the *secA*⁺ strain (Table 2). Second, we showed that increased expression of the *hspG* heat shock promoter also occurred if export-defective proteins were overproduced via a multicopy plasmid. Plasmids pUClamBSE60 and pUClamBSE87 (1), which overproduce LamB protein harboring a defective signal sequence, as well as plasmid pJF32 (7), overproducing a signal sequence-

FIG. 2. The level of σ^{32} is increased in the *secB* null mutant. The level of σ^{32} in CAG13461 (*secB::Tn5*) and MC1061 (*secB*⁺) grown at 30°C and the level after temperature shift were determined by Western blotting of samples adjusted to equal cell densities as described in Materials and Methods. 3' and 15', 3 and 15 min, respectively.

FIG. 3. σ^{32} is more stable in a strain lacking SecB. CAG13461 (*secB::Tn5*) (○) and MC1061 (*secB*⁺) (●) grown at 30°C were labeled for 1 min with Tran ³⁵S label and chased with nonradioactive L-methionine and L-cysteine for the indicated times. Samples were processed and immunoprecipitated with σ^{32} antibodies as described in Materials and Methods. The σ^{32} /standard ratio was normalized to the value at the 0-min chase. $t_{1/2}$ is given in minutes.

defective maltose-binding protein, cause an increase in the amount of β -galactosidase synthesized from the *PhtpG-lacZ* fusion (Table 2). Because pUClamBSE60, pUClamBSE87, and pJF32 elicit an interference phenomenon in which other SecB-dependent proteins accumulate because of the limitation of the SecB protein (1, 4, 7), expression of the *PhtpG-lacZ* fusion was also examined in pUClamBSE60 Δ IR (1). This plasmid overproduces an export-defective LamBS60 protein that does not elicit the interference phenomenon (1) because its binding affinity for SecB is only 4% of that of wild-type LamB protein (2). Production of LamBS60 Δ IR still induces synthesis of β -galactosidase from the *PhtpG-lacZ* fusion (Table 2) even though it does not block the secretory apparatus. Collectively, these data support the notion that it is an accumulation of secretory protein precursors that induces the synthesis of heat shock proteins in the strain lacking SecB.

Stabilization of σ^{32} , leading to its increased concentration, accounts for the enhanced heat shock protein synthesis in the *secB* null mutant. The induction of heat shock protein synthesis observed after temperature upshift is controlled by an increase in the intracellular concentration of σ^{32} (28). To determine whether an increase in the concentration of σ^{32} accounts for the heat shock protein induction in the *secB* null mutant, we measured the level of σ^{32} at 30°C and after temperature upshift in the mutant and its parental strain. In agreement with previous findings, the σ^{32} level is hardly detectable in the wild-type cells grown at 30°C and increases

FIG. 4. The *secB::Tn5* mutant grows in rich medium at high temperature. Cells of CAG13461 (*secB::Tn5*) grown overnight at 30°C in M9 glycerol medium were diluted 10⁶-fold, plated on Luria-Bertani plates, and incubated at indicated temperatures.

eightfold upon the shift to 42°C (Fig. 2). In the *secB::Tn5* mutant, the level of σ^{32} at 30°C is already elevated fourfold and does not change significantly upon the shift to the higher temperature (Fig. 2). Thus, the increase in the amount of σ^{32} due to its stabilization is sufficient to explain the increased synthesis of heat shock proteins in the *secB::Tn5* mutant.

The increased concentration of σ^{32} observed after temperature upshift results both from increased synthesis and increased stability of σ^{32} (28). We determined whether either process was altered in the *secB* null strain grown at 30°C. The rate of σ^{32} synthesis determined in ³⁵S-labeled cells by immunoprecipitation with anti- σ^{32} antibodies was similar in *secB::Tn5* and *secB*⁺ strains (data not shown), ruling out increased synthesis as an explanation for higher levels of this protein in the *secB* null mutant. However, the stability of σ^{32} was increased about threefold in the *secB::Tn5* strain (Fig. 3). Thus, accumulation of σ^{32} in the *secB* null mutant strain results solely from the stabilization of σ^{32} .

High temperature suppresses the growth defect of the *secB* null mutant. The *secB* gene is not essential, but absence of its product results in lethality on rich medium. This rich medium growth defect of SecB-lacking strains is suppressed when heat shock proteins are overproduced by increasing expression of σ^{32} (3). Since temperature upshift results in increased expression of heat shock proteins, it might also suppress the rich medium growth defect of *secB* null mutants. This proves to be the case. The *secB* null mutant plated with 100% efficiency on rich medium when incubated at 44°C, although it did not grow at 37 or 42°C (Fig. 4). Apparently, at very high temperatures, the levels of heat shock proteins are high enough to compensate for the lack of the SecB chaperone manifested as the lethality on rich medium.

The *secB* null mutant exhibits thermotolerance. When cells are transiently exposed to mild heat treatment, they develop thermotolerance to the subsequent, more severe heat exposures. Although an increase in the level of heat shock proteins alone is not sufficient for cells to acquire thermotolerance (29), these proteins may still play a role in mediating this response. Since the *secB* null mutant exhibits elevated synthesis of heat shock proteins, we tested its inherent thermotolerance upon a direct shift to 50°C. The *secB* null mutant was more thermotolerant than wild-type cells shifted directly to 50°C (Fig. 5). Whereas 50% of the

SecB-lacking cells survived a treatment of 30 min at 50°C, only 20% of wild-type cells remained viable after this treatment. However, the mutant was not more thermotolerant than the preadapted wild-type strain. Preadapted cells of both the mutant and the wild type each plated with 85% efficiency after being treated for 30 min at 50°C (Fig. 5).

DISCUSSION

We show that strains lacking SecB have increased levels of heat shock proteins. The previous work of Ito and coworkers (14) indicated that induction of a MalE-LacZ hybrid protein defective in export led both to accumulation of normal precursor proteins destined for export and to induction of the heat shock response. Since induction of a mutant MalE-LacZ fusion protein that did not accumulate precursors also induced the heat shock response, it was not clear whether accumulation of precursor proteins alone was sufficient for induction. Our demonstration that the heat shock response is induced in both *secB* and *secA* mutant cells as well as in cells with an accumulated secretory protein precursor that does not titrate out any components of the secretory apparatus strongly suggests that accumulated secretory protein precursors do induce synthesis of heat shock proteins. Interestingly, accumulation of secretory precursors in the endoplasmic reticulum induces KAR2, the *S. cerevisiae* homolog of mammalian BiP (21). Thus, accumulation of precursors of secretory proteins may function as a general stimulus for inducing heat shock protein synthesis.

We have proposed that the levels of free DnaK (and the associated DnaJ and GrpE heat shock proteins) could serve as a cellular thermometer (8). DnaK, together with DnaJ and GrpE, negatively regulates the stability and synthesis of σ^{32} (27). Accumulation of substrates for DnaK (10, 19) would titrate this protein from its negative regulatory role, thus permitting the accumulation of σ^{32} and overproduction of the heat shock proteins. After sufficient levels of DnaK had been attained to restore the free pool of the protein, DnaK would resume negative regulation of σ^{32} and prevent further increases in synthesis of the heat shock proteins. In cells lacking SecB, accumulation of secretory protein precursors, presumed substrates for DnaK, would be expected to titrate out free DnaK and relieve negative regulation of σ^{32} stability and synthesis. We find that this is indeed the case, as increased amounts of σ^{32} are observed in cells lacking SecB.

However, the cell does respond differently to the lack of SecB and to a temperature upshift. SecB limitation stabilizes σ^{32} without inducing its synthesis, whereas temperature upshift does both, stabilizing σ^{32} and increasing its synthesis. Since the effects of the lack of SecB were tested at 30°C, this difference is consistent with our previous suggestion that DnaK affects the stability of σ^{32} at all temperatures and the synthesis of σ^{32} only at high temperatures (27). Alternatively, the accumulated precursor proteins in cells depleted of SecB might signal σ^{32} stabilization without affecting σ^{32} synthesis. In this case, the pathways signaling synthesis and stabilization of σ^{32} may be distinct. Resolving this question requires growing a steady-state culture at 42°C. Although *secB::Tn5* cells grow on minimal glycerol plates at 42°C, in liquid minimal glycerol medium they grow only for several generations after a shift from 30 to 42°C, and they cannot be maintained at steady-state growth at 42°C in this liquid medium. Examination of the effects of a number of inducing signals on σ^{32} synthesis and stability will be required to see whether the signaling pathways are distinct.

The fact that cells lacking SecB are more thermotolerant

FIG. 5. Survival of 50°C temperature challenge. CAG13461 (*secB::Tn5*) (○ and ●) and MC1061 (*secB*⁺) (△ and ▲) were grown to $A_{450} = 0.3$. Then, aliquots of cells were diluted and transferred to 50°C for the indicated period of time (open symbols). Parallel samples were withdrawn and preincubated for 15 min at 42°C prior to dilution and exposure to 50°C (closed symbols).

than their wild-type counterparts adds to the confusing literature on this subject. It has been reported that increasing the level of σ^{32} in wild-type strains at low temperature to induce the synthesis of heat shock proteins does not increase thermotolerance (29). We have independently confirmed this result in a wild-type strain isogenic to our *secB* null mutant. On the other hand, the level of heat shock proteins may be involved in this phenomenon. The *secB* null mutant, exhibiting an increased level of heat shock proteins, does show increased thermotolerance, and conversely, Δ *rpoH* cells lacking σ^{32} and thus not synthesizing most heat shock proteins are extremely sensitive to the high-temperature exposure (15). Finally, our recently reported dominant *dnaK* mutants (32) are more sensitive to the heat exposure than the wild-type strain and do not develop thermotolerance (30a). This evidence that heat shock proteins are involved in thermotolerance is consistent with the observation that eukaryotic cells expressing higher levels of the Hsp70 protein have increased tolerance to thermal stress (18). Heat shock proteins may be prerequisite but not sufficient for the acquisition of heat resistance. The thermotolerance of the *secB::Tn5* strain may not result solely from the overproduction of heat shock proteins. We have noted additional changes in gene expression in the *secB::Tn5* cells. Perhaps some of these changes contribute to the increased thermotolerance exhibited by this strain.

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