Defining the Role of the Escherichia coli Chaperone SecB Using Comparative Proteomics*§

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To improve understanding and identify novel substrates of the cytoplasmic chaperone SecB in Escherichia coli, we analyzed a secB null mutant using comparative proteomics. The secB null mutation did not affect cell growth but caused significant differences at the proteome level. In the absence of SecB, dynamic protein aggregates containing predominantly secretory proteins accumulated in the cytoplasm. Unprocessed secretory proteins were detected in radio-labeled whole cell lysates. Furthermore, the assembly of a large fraction of the outer membrane proteome was slowed down, whereas its steady state composition was hardly affected. In response to aggregation and delayed sorting of secretory proteins, cytoplasmic chaperones DnaK, GroEL/ES, ClpB, IbpA/B, and HslU were up-regulated severalfold, most likely to stabilize secretory proteins during their delayed translocation and/or rescued aggregated secretory proteins. The SecB/A dependence of 12 secretory proteins affected by the secB null mutation (DegP, FhuA, FkpA, OmpT, OmpX, OmpA, TolB, ToIC, YbgF, YegK, YgiW, and YncE) was confirmed by “classical” pulse-labeling experiments. Our study more than triples the number of known SecB-dependent secretory proteins and shows that the primary role of SecB is to facilitate the targeting of secretory proteins to the Sec-translocase.

The periplasmic and outer membrane proteins in the Gram-negative bacterium Escherichia coli need to cross the cytoplasmic membrane to reach their final destination. The vast majority of these secretory proteins are translocated through the cytoplasmic membrane via the Sec-translocase (1, 2). The core of the Sec-translocase is comprised of integral membrane proteins SecY and SecE, which form a protein conducting channel (3). The peripheral subunit SecA drives poly-peptide chains in an ATP-dependent manner into and through the Sec-translocase (1).

It is generally assumed that secretory proteins in E. coli are targeted to the Sec-translocase by the cytoplasmic protein SecB in a mostly post-translational fashion (4—8). However, direct evidence for SecB dependence is only established for six secretory proteins (PhoE, LamB, MBP, OmpF, GBP, and OmpA), whereas four secretory proteins (PhoA, Lpp, RbsB, and β-lact) do not seem to require SecB (9—12, 55). SecB also has the capacity to assist the chaperone DnaK in the folding of proteins, as shown in vitro with luciferase as a model substrate (12). This indicates that SecB has the potential to assist the folding of cytoplasmic proteins. The successful complementation of a DnaK/trigger factor (TF)2 double mutant strain by overexpression of SecB, and cross-linking of SecB to nascent chains of both secretory and cytoplasmic proteins in SecB-enriched lysates support this notion (13).

SecB does not bind to signal sequences and peptide library screens suggested a very loosely defined SecB binding “motif” (12). This motif, which is ~9 residues long, is enriched in aromatic and basic residues, whereas acidic residues are disfavored. It theoretically occurs every 20—30 residues in both secretory and cytoplasmic proteins and is too unspecific to facilitate genome-wide prediction of SecB substrates (10—12). Thus experimentation is needed to identify novel SecB substrates.

To characterize the role of SecB in more detail and identify additional SecB substrates, we analyzed a secB null mutant using comparative proteomics. This analysis included flow cytometry, pulse labeling combined with cell fractionation, one- and two-dimensional gel electrophoresis, and mass spectrometry (MS), complemented by immunoblotting. The comparative proteomics approach allowed us to investigate protein mistargeting, aggregation, and translocation kinetics, and to determine changes in the proteome composition. Our analysis showed that, although the secB null mutation did not affect cell growth, there are significant differences at the proteome level. Most differences pointed to protein targeting defects, resulting in a protein folding/aggregation problem in the cytoplasm. Careful analysis of the (sub)proteome(s) of the secB null mutant strain combined with a classical pulse-labeling approach enabled us to more than triple the number of known SecB-dependent secretory proteins.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—We used E. coli strain EK413, which is a MC4100 derivative that is ara+ (a kind gift from Ken-ichi Nishiyama), harboring plasmid pE63 as wild-type. Plasmid pE63 harbors the gpsA gene, which encodes for sn-glycerol-3-phosphate dehydrogenase, under control of an arabinose inducible promoter and has a pSC101 origin of replication and a β-lactamase resistance marker (14). Using P1 transduction, we moved the secB null mutation secB8 (15) from strain HS101/pE63 into EK413/pE63, yielding an EK413/pE63-derived secB

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2 The abbreviations used are: TF, trigger factor; MS, mass spectrometry; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; bis-Tris, 2-[bis(2-hydroxyethyl)methylamino]-2-(hydroxymethyl)propane-1,3-diol; Tricine, 2-[2-hydroxy-1,1-bis(hydroxymethyl)amino]ethanesulfonic acid; Ibp, inclusion body associated protein; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; IPG, immobilized pH gradient; SRP, signal recognition particle.

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null mutant strain. As expected, the secB null mutant is unable to form single colonies on LB plates in the absence of arabinose; i.e. when GspA is not expressed (14). Hereafter, we will refer to this EK413/pE63-derived secB null mutant strain and EK413/pE63 as the secB null mutant and the control strains, respectively.

Cells were cultured in standard M9 medium supplemented with thiamine (10 μM), all amino acids but methionine and cysteine, glucose (0.2% w/v), arabinose (0.2% w/v), and ampicillin (100 μg/ml). Overnight cultures were diluted 1:50 in pre-warmed medium and cultured at 37 °C. Growth was monitored by measuring the A_{600} with a Shimadzu UV-1601 spectrophotometer. Under these conditions, we did not observe differences in growth (as monitored by A_{600} measurements) between the secB null mutant and the control (results not shown). For all the experiments, cells were harvested at an A_{600} of 1.0 (i.e. in the early exponential phase).

Flow Cytometry—Analysis of the secB null mutant and the control by means of flow cytometry was done using a FACSCalibur (BD Biosciences) instrument. Cultures of the secB null mutant and the control were immediately diluted in ice-cold phosphate-buffered saline to a final concentration of ~10^6 cells per ml, and analyzed with an average flow rate of 400 events/s. Forward and side scatters were measured and used for comparison of cell morphology of the secB null mutant and control (16). Propidium iodide staining was performed to assess viability (16).

Immunoblot Analysis—The protein accumulation of SecB, SecY, SecE, SecA, FliH, PspA, TF, GroEL, DnaK, and IbpB (it should be noted that the IbpB antiserum cross-reacts with IbpA) in the secB null mutant strain and the control strain were determined by immunoblot analysis. Cells were cultured as described above. Cells (0.2 A_{600} units) or inner membranes (5 μg of protein) isolated by sucrose gradient centrifugation (17, 18) were solubilized in Laemmli solubilization buffer. Proteins were separated by SDS-PAGE. Blotting, immunodecoration, detection, and quantification of blots were done as described previously (19).

Protein Translocation Assays in Vivo—Protein translocation assays were done with 1 ml of culture each. Cells were labeled with [35S]methionine (60 μCi/ml, Ci = 37 GBq) for 45 s and subsequently precipitated in 10% trichloroacetic acid. Trichloroacetic acid-precipitated samples were washed with acetone, resuspended in 10 mM Tris-HCl (pH 7.5), 2% SDS, and immunoprecipitated with antiserum to OmpA and GspA (20). Band-by-band analysis of the immunoprecipitates was performed to assess protein translocation (21). IbpB antiserum cross-reacts with IbpA (20, 21) and was used for comparison of cell morphology of the secB null mutant and the control strain (results not shown). For all the experiments, cells were harvested at an A_{600} of 1.0 (i.e. in the early exponential phase).

Preparation of Radiolabeled Membranes—Cells were cultured to an A_{600} of 2.0 units. The culture was divided into four aliquots. Aliquot A was labeled with [35S]methionine (60 μCi/ml, Ci = 37 GBq) for 1 min. An excess of cold methionine (final concentration 1 mg/ml) was added and cells were collected by centrifugation either directly after labeling or after a 10-min chase. The remaining unlabeled cells were washed and collected by centrifugation. Before breaking the cells, labeled and unlabeled cells from the same culture were pooled back together resulting in a mixture of labeled and unlabeled cells with a ratio of 1:100 that was then used for membrane isolations. Carbonate-washed total membranes (i.e. a mixture of inner and outer membranes) were isolated essentially as described by Molloy et al. (23), with the exception that we used sonication rather than French pressing to break cells. Protein concentrations were determined with the BCA assay (Pierce) according to the instructions of the manufacturer.

Two-dimensional Gel Electrophoresis—The analysis of stained two-dimensional electrophoresis gels of whole cell lysates was first done on gels with a low protein load (0.5 A_{600} units of cells) to avoid saturation and allow analysis of highly abundant proteins, and then on gels with a high protein load (1 A_{600} unit of cells) for the analysis of low abundant proteins. 1 A_{600} unit of cells was used for the analysis of [35S]methionine-labeled whole cell lysates. Whole cell lysates were solubilized in 7 M urea, 4% (w/v) CHAPS, 2 mM tributylphosphine, 0.5% (v/v) Triton X-100, 5% glycerol, 2% (v/v) immobilized pH gradient gel (IPG) buffer for pH 4–7 (Amersham Biosciences) and bromphenol blue. For analysis of the outer membrane proteome, 350 μg of protein was solubilized in 7 M urea, 2 M thiourea, 1% (w/v) ASB-14, 2 M tributylphosphine, 5% glycerol, 2% (v/v) IPG buffer for pH 4–7 (Amersham Biosciences) and bromphenol blue (23). Unsolubilized material was removed by centrifugation at 14,000 × g for 30 min. The clarified protein solution was used to re-swell Immobilon DryStrips, pH 4–7 (Amersham Biosciences), overnight at room temperature. Isoelectric focusing was subsequently performed at 20 °C in a Multiphor II apparatus (Amersham Biosciences); whole cell samples at 80 kVh and membrane samples at 60 kVh at a maximum 3,500 V. Proteins were separated in the second dimension on 10% duracrylamide (Genomic Solutions) gels (10% acrylamide monomer and 1% bisacrylamide) containing 1 M Tris-HCl (pH 8.45), 0.1% (w/v) SDS, and 20% (v/v) glycerol. After focusing, proteins in the IPG strips were reduced and alkylated, as described before (24). The strips were loaded on top of the second dimension gel by submerging the strips in warm agarse solution (1% (w/v) low melting agarose, 0.2% SDS, 150 mM bis-Tris, 80 mM HCl and bromphenol blue). Electrophoresis was performed with Tricine-SDS buffer system (25) in a DALTON tank (Amersham Biosciences) at 30–60 mA/gel for ~48 h, until the dye front reached the bottom of the gel. Gels used for comparative analysis were stained with high sensitivity silver stain (26) and gels containing radiolabeled proteins were dried on filter paper. Preparative gels used for identification of proteins by mass spectrometry were stained with Coomassie Brilliant Blue R-250 or with mass spectrometry compatible silver stain.

Several proteins were found in multiple spots at different pI values, but with the same molecular weight. This was also observed in the outer membrane maps of E. coli constructed by Molloy et al. (23). Most of
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FIGURE 1. Analysis of the E. coli secB null mutant by flow cytometry and immunoblotting. A, flow cytometric properties of secB null mutant (SecB−) and control (SecB+) cells. In the first 2 panels, the size of the population (forward scatter FSC) is plotted versus granularity (side scatter, SSC) for both SecB+ and SecB−. To facilitate comparison of those 2 parameters for SecB+ and SecB−, histograms for size and granularity are shown in the third and fourth panels. One representative experiment of four is shown. Cells were cultured and flow cytometry was performed as described under “Experimental Procedures.” B, Western blot analysis of SecB and components of the Sec-translocase (SecA, -Y, -E), Ffh, a constituent of the SRP-targeting pathway, and PspA, of which the expression is up-regulated when the electrochemical potential is affected. Left panel, cells (0.2 A600 units) were separated by means of SDS-PAGE and subsequently subjected to immunoblot analysis with antibodies to SecB, SecA, Ffh, and PspA. Right panel, inner membranes (5 μg of protein) were separated by means of SDS-PAGE, and subsequently subjected to immunoblot analysis with antibodies to SecA, SecY, and SecE.

these “trains of spots” are because of modifications induced during sample preparation (27), likely because of stepwise deamidation of residues Asn and Gln, resulting in loss of 1 dalton and net loss of one positive charge.

Image Analysis and Statistics—Stained gels were scanned using a GS-800 densitometer from Bio-Rad. Radiolabeled gels were scanned in a Fuji FLA-3000 phosphorimager. Spots were detected, quantified, matched, and compared using the two-dimensional analysis software PDQuest (Bio-Rad). The analyses of silver-stained and radiolabeled outer membrane proteins were done on the same set of gels. In all cases, each analysis set consists of at least three gels in each replicate group (i.e. secB null mutant and the control). All gels in a set represented independent samples (i.e. samples from different bacterial colonies, cultures, and membrane preparations), which were subjected to two-dimensional electrophoresis and image analysis in parallel, i.e. en group. Spot quantities were normalized using the “total density in gel image” method to compensate for non-expression related variations in spot quantities between gels. The PDQuest software was set to detect differences that compensate for non-expression related variations in spot quantities between gels. The PDQuest software was set to detect differences that compensate for non-expression related variations in spot quantities between gels. The PDQuest software was set to detect differences that compensate for non-expression related variations in spot quantities between gels.

Protein Identification by Mass Spectrometry and Bioinformatics—Stained protein spots or bands were excised, washed, digested with modified trypsin and peptides extracted manually or automatically (ProPic and Progest, Genomic Solutions, Ann Arbor, MI), and peptides were applied to the MALDI target plates as described previously (28). The mass spectra were obtained automatically by MALDI-TOF MS in reflectron mode (Voyager-DE-STR; PerSeptive Biosystems, Framingham, MA), followed by automatic internal calibration using tryptic peptides from autodigestion. The latest version of the NBCI non-redundant data base (downloaded locally) were searched automatically with the resulting peptide mass lists, using the search engine ProFound (29), as part of Knexus (30). Criteria for positive identification by MALDI-TOF MS peptide mass fingerprinting were at least four matching peptides with an error distribution within ±25 ppm and at least 15% sequence coverage. During the search, we only allowed one missed cleavage and partially oxidized methionines. In the more complex samples, the peptides were also analyzed by nano-LC-ESI-MS/MS in automated mode on a quadruple/orthogonal acceleration TOF tandem mass spectrometer (Q-TOF; Micromass, Manchester, UK) (see Ref. 31 for details). The spectra were used to search the SwissProt 42.10 data base with the Mascot search engine. All significant MS/MS identifications by Mascot were manually verified for spectral quality and matching y and b ion series.

Isolation of Protein Aggregates—Protein aggregates were isolated essentially as described (32). 100 ml of culture with an A600 of 1.0 was used for each aggregate isolation. The protein content of total cells and aggregates was determined with the BCA assay according to the instructions of the manufacturer (Pierce). Aggregates were analyzed by SDS-PAGE using 24-cm long 8–16% acrylamide gradient gels. Proteins were stained with Coomassie Brilliant Blue R-250 and identified by mass spectrometry as described before.

For radiolabeling of aggregates, 100 A600 units of cells were labeled with [35S]methionine (2500 Ci/ml, Ci = 25 GBq) for 30 s and chased for 1, 3, and 15 min by addition of an excess of cold methionine (final concentration 1 mg/ml). Aggregates were isolated as described above, solubilized in 10 mM Tris-HCl (pH 7.5), 2% SDS, and subsequently processed using an OmpA antiserum as described under “Protein Translocation Assays” (see above).

2 V. Zabrouskov et al., unpublished results.
FIGURE 2. Analysis of whole cell lysates of the secB null mutant by two-dimensional electrophoresis. A, comparative two-dimensional electrophoresis gel analysis of highly abundant proteins in whole cell lysates of the secB null mutant (SecB−) and its control (SecB+). Cells were harvested when the cultures had reached an A600 of 1.0. 0.5 A600 units of cells were solubilized and proteins were separated by two-dimensional electrophoresis. Proteins were visualized by silver stain and differences between secB null mutant and control gels were analyzed using the PDQuest software (Bio-Rad). At least four independent samples from each strain were used for the analysis. Differential protein expression between the secB null mutant and the control was analyzed using the Student’s t test and a 99% level of confidence (see “Experimental Procedures”). Proteins were identified by mass spectrometry from spots excised from gels stained with Coomassie or mass spectrometry compatible silver stain (Table 1 and supplemental Table 1). Annotated spots have been matched onto the silver-stained gels shown here using the PDQuest software (Bio-Rad). The levels of the highly abundant chaperones DnaK and GroEL are ~50% higher in the secB null mutant (supplemental Table 1). In contrast, the level of TF is unaffected in the secB null mutant, whereas the level of TF is unaffected. C, comparative two-dimensional electrophoresis gel analysis of low abundant proteins in whole cell lysates of the secB null mutant (SecB−) and the control (SecB+). Proteins from 1 A600 unit of cells were visualized by silver staining and analyzed as described above. 46 additional spots are significantly changed using the criteria described above (supplemental Table 1). Spots that are down-regulated in the secB null mutant are indicated in the “SecB−” gel and spots that are up-regulated are indicated in the “SecB−” gel. Spots where proteins have been successfully identified are labeled with both the spot number and gene name (Table 1 and supplemental Table 1). The level of GroES, co-chaperone, and regulator of GroEL, is 50% increased in the secB null mutant. The level of the chaperone ClpB is doubled and the level of the chaperone/protease HslU is tripled. D, zooms of two-dimensional electrophoresis gels with radiolabeled whole lysates from the secB null mutant (SecB−) and the control (SecB+) visualized by autoradiography. Processed (m – mature) and precursor (p) forms of the secretory proteins OmpT, OmpA, and OppA are indicated.
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TABLE 1
Proteins identified in differentially regulated spots in two-dimensional electrophoresis gels of whole cell lysates of the secB null mutant and the control

Whole cells were analyzed by two-dimensional electrophoresis (Fig. 2, A and C). Differentially regulated spots were excised from silver- or Coomassie-stained gels. Proteins were identified by MALDI-TOF MS and/or by nlc-LSI-ESI-MS/MS as described under “Experimental Procedures” (supplemental Table I). Spots 1–2 are from whole cell maps loaded with 0.5 A465 units of cells (Fig. 2A), and the remaining spots are from gels loaded with 1 A465 units of cells (Fig. 2C).

| Spot no. | Gene name(s) | Protein name | Localization | Change (secB null mutant/control) |
|----------|--------------|--------------|--------------|-----------------------------------|
| 1        | dnaK, grpE, groP, seg | Chaperone protein DnaK | Cytoplasmic | 1.5 |
| 2        | groEL, groL, mopA | 60-kDa Chaperonin GroEL | Cytoplasmic | 1.6 |
| 3        | ccr, csr, tex, tgs, treD | PTS system, glucose-specific IIa component | Cytoplasmic | 0.3 |
| 5        | ompT | Protease VII | Outer membrane | 0.3 |
| 10       | fhuA, tonA | Ferrichrome-iron receptor | Outer membrane | 0.5 |
| 11       | luxS | S-Ribosylhomocysteine synthetase | Cytoplasmic | 0.5 |
| 14       | deoB, drm, thyR | Phosphotransacetylase | Cytoplasmic | 0.5 |
| 15       | ribH, ribE | Riboflavin synthase β chain | Cytoplasmic | 0.5 |
| 16       | oppA | Periplasmic oligopeptide-binding protein | Periplasmic | 0.5 |
| 18       | oppA | Periplasmic oligopeptide-binding protein | Periplasmic | 0.6 |
| 22       | oppA | Periplasmic oligopeptide-binding protein | Periplasmic | 0.7 |
| 26       | oppA | Periplasmic oligopeptide-binding protein | Periplasmic | 0.7 |
| 27       | kdgA, eda, hga | KHG/KDPG aldolase | Cytoplasmic | 0.7 |
| 27       | ssb, cexB, lexC | Single-strand binding protein, helix-destabilizing protein | Cytoplasmic | 0.7 |
| 31       | groES, mopB | 10-kDa Chaperonin, GroES protein | Cytoplasmic | 1.5 |
| 33       | rplL | 50 S ribosomal protein L7/L12, L8 | Cytoplasmic | 1.7 |
| 35       | clpB | Chaperone ClpB | Cytoplasmic | 2.3 |
| 38       | halU, htpI | ATP-dependent hsl protease ATP-binding subunit HalU | Cytoplasmic | 3.8 |
| 44       | yfF | Hypothetical protein YfF | PSORT: cytoplasmic | >100 |
| 46       | yfR | Hypothetical protein YfR | PSORT: cytoplasmic | >100 |
| 47       | yfR | Hypothetical protein YfR | PSORT: cytoplasmic | >100 |

*The numbering corresponds to the spots in two-dimensional electrophoresis gel images shown in Fig. 2, A and C.*

*Names in bold are used to label the corresponding spots in the gel shown in Fig. 2, A and C.*

*Localization according to the SwissProt database. The localization of unknown proteins was predicted using PSORT.*

*The PDQuest software was used to detect and calculate the fold change, i.e. the ratio of the average intensity of spots in secB null mutant gels to the average intensity of matched spots in the control gels (supplemental Table I).*

RESULTS

Characterization of the secB Null Mutant Strain—Using P1 transduction we moved the secB null mutation secB8 (15) from strain HS101/pE63 (14) into strain EK413/pE63 (a M4100 derivative that is ara +, a kind gift from Ken-ichi Nishiyama), yielding an EK413/pE63-derived secB null mutant strain. Hereafter, we will refer to the secB null mutant strain and EK413/pE63 as the secB null mutant and control, respectively. The secB null mutant and control were cultured aerobically in M9 minimal medium. Under these conditions, we did not observe any differences in growth, as monitored by A600 measurements. In addition, propidium iodide staining (16) did not point to differences in viability between the mutant and the control (results not shown). Early log-phase cells were used in all the experiments described in this study. The morphology of cells was analyzed by means of flow cytometry (16, 33). Interestingly, we detected a small increase of both the forward scatter and side scatter of secB null mutant cells (Fig. 1A). This indicates that secB null mutant cells are slightly bigger than control cells and most likely contain extra internal structures (i.e. extra membranes and/or protein aggregates).

To verify the phenotype of the secB null mutant strain, we monitored the targeting of the established SecB-dependent outer membrane protein OmpA (14) and SecB-independent periplasmic protein β-lactamase (34), using pulse-chase radiolabeling experiments in combination with immunoprecipitations. As expected, the translocation of OmpA was impaired in the secB null mutant, as evidenced by accumulation of precursor protein, whereas the translocation of β-lactamase was not affected (results not shown). The levels of SecA, -Y, and -E were determined by Western blotting for the secB null mutant and control, because SecB delivers proteins to the SecYE protein-conducting channel through interaction with SecA (1). Protein levels of the SecAYE-translocase components did not change in the absence of SecB (Fig. 1B). Ffh is a core component of the SRP targeting pathway, which mainly targets inner membrane proteins to the Sec-translocase but may have some overlap with the SecB targeting pathway (20, 35–37). It is not known if the SRP targeting pathway can compensate for the absence of SecB. Immunoblot analysis of secB null mutant and control showed that Ffh levels were unchanged in the absence of SecB. It has been shown that expression of PspA is up-regulated when the electrochemical potential is affected (38). Because the electrochemical potential plays an important role in protein translocation we analyzed the levels of the PspA protein by immunoblot analysis. In contrast to several other Sec mutants (38), there is no PspA response in the secB null mutant (Fig. 1B).

Analysis of Whole Cell Lysates of the secB Null Mutant by Two-Dimensional Electrophoresis—To identify potential SecB substrates and compensatory mechanisms and/or stress responses in the secB null mutant, we used a proteomics approach. Whole cell lysates of the mutant and the control were analyzed by two-dimensional electrophoresis, using IEP strips with a pI range from 4 to 7. To allow for quantitative analysis of highly abundant proteins, gels were loaded with limited amounts of protein, such that staining of highly abundant proteins was not saturated. The comparative analysis was based on 4 gels per strain (an independent culture was used for each gel). Gels were stained with silver, scanned, and images were analyzed and compared using the PDQuest software (Bio-Rad). Significance was determined using Student’s t test (for details see “Experimental Procedures”). This analysis demonstrated that the levels of both DnaK and GroEL were increased by about 50% (p < 0.01) in the secB null mutant (Fig. 2A, Table 1, and supplemental Table 1). Higher levels of DnaK and GroEL are consistent with increased synthesis rates of these proteins in a SecB knock-out strain (39). The level of TF, the first cytoplasmic chaperone that interacts with ribosome-associated nascent peptides, was not affected. These results were confirmed by immunoblot analysis (Fig. 2B).
To detect differences in accumulation of low abundant proteins, we repeated the two-dimensional electrophoresis proteome analysis with higher protein loading (Fig. 2C). In total 48 spots were found to be significantly (with \( p < 0.01 \)) altered. Although most of these spots were weakly stained, we were able to identify 16 non-redundant proteins by MS (Table 1; image analysis and MS data are summarized in supplementary Table 1). Some proteins were identified in multiple spots located in "trains" next to each other and in total 20 spots could be annotated. In addition to DnaK and GroEL, the levels of the chaperones GroES, ClpB, and HslU were increased by approximately 400%. GroES is the regulator and co-chaperone of GroEL and the GroEL/ES chaperone system is essential for proper folding and maturation of proteins in the cytoplasm (40). The chaperone ClpB has been shown to act in concert with DnaK and inclusion body proteins (Ibp) to extract and refold proteins from aggregates (41). The protein HslU can function either on its own as a chaperone (42) or in a complex together with HslV (ClpY) as a protease (43). However, we did not find any spots that were differentially regulated in the region of the gel where HslV should migrate. Notably, the genes encoding the DnaK, GroEL/ES, ClpB, and HslU proteins are all regulated by transcription factor \( \sigma^{32} \) (44, 45). The \( \sigma^{32} \)-induced response, better known as the "heat shock response," is activated in response to protein misfolding/aggregation in the cytoplasm (44, 45).

The levels of the processed forms of the outer membrane proteins ferrichrome-iron receptor (FhuA), the protease OmpT, and the periplasmic oligopeptide-binding protein (OppA) were decreased by ~50%, ~70%, and ~30%, respectively, in the secB null mutant strain. Interestingly, the homolog of OppA in Salmonella typhimurium has been shown to bind tightly to E. coli SecB in vitro (46). No precursors of secretory proteins were identified in the silver-stained gels of whole cell lysates.

To study kinetic effects of the secB null mutation, we repeated the comparative proteome analysis with cells labeled with \([^{35}\text{S}]\)methionine. In these radioactive gels, 37 radiolabeled spots were significantly changed (\( p < 0.01 \)) in the secB null mutant. Interestingly, several of these spots were not present in the silver-stained gels. Based on pI and molecular weight they match the precursors of secretory proteins, such as OmpA, OmpT, and OppA (Fig. 2D).

**Isolation and Characterization of Protein Aggregates from the secB Null Mutant**—The heat shock response in the secB null mutant pointed to a problem of protein folding and aggregation in the cytoplasm of cells lacking SecB. In addition, the flow cytometry experiments suggested the presence of extra internal structures, i.e. extra internal membranes and/or protein aggregates, in the secB null mutant. Indeed, protein aggregates containing around 0.5% of total cellular protein could be isolated from the secB null mutant, but were virtually absent in the control strain (Fig. 3A). The aggregates were dissolved in Laemmli solubilization buffer, and proteins were separated by SDS-PAGE, followed by identification using nano-LC electrospray tandem mass spectrometry (nano-LC-ESI-MS/MS). Fourteen secretory proteins and five cytoplasmic proteins were identified (Fig. 3A, Table 2, and supplemental Table 2). The inclusion body protein IbpA, also part of the heat shock regulon, was among the cytoplasmic proteins identified in the aggregates (Table 2) (41, 47). Immunoblotting of total cell extracts showed that the levels of the chaperones IbpA/B are indeed strongly up-regulated in the secB null mutant (Fig. 3B). In contrast, immunoblotting showed that the levels of the periplasmic chaperone Skp and protease DegP are not changed in the secB null mutant (data not shown). This indicates that no significant protein misfolding/aggregation occurred in the periplasm/outer membrane (38, 48).

![FIGURE 3. Characterization of aggregates isolated from E. coli secB null mutant.](image)

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Furthermore, MS/MS data revealed that at least two of the secretory proteins, OmpA and the murein lipoprotein (Lpp or MulI), identified in the aggregates contained an uncleaved signal sequence (results not shown), again pointing to aggregation of proteins in the cytoplasm rather than in the periplasm.

To study the localization and dynamics of the aggregates in more detail, we isolated OmpA by immunoprecipitation from aggregates iso-
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TABLE 2
Identification of proteins in aggregates isolated from the secB null mutant and the control

| Band no. | Gene name(s) | Protein name(s) | Localization |
|----------|--------------|-----------------|--------------|
| 1        | glgB         | 1,4-α-Glucan branching enzyme | Cytoplasmic |
| 2        | tolC, mtcB, mukA, refl | Outer membrane protein TolC | Outer membrane |
| 3        | glgA         | Glycogen synthase | Cytoplasmic |
| 4        | degP, htrA   | Heat shock protein HtrA | Periplasmic |
| 5        | yncE         | ToBI protein | Periplasmic |
| 6        | ompA, tolG, tut, con | Outer membrane protein A | Outer membrane |
| 7        | yncE         | Hypothetical protein YncE | Unkown, PSORT predicts periplasmic or outer membrane |
| 8        | fkpA         | FKP-like peptidyl-prolyl cis-trans isomerase FkpA | Periplasmic |
| 9        | ybgF         | Hypothetical protein YbgF | Unkown, PSORT predicts periplasmic or outer membrane |
| 10       | ompA, tolG, tut, con | Outer membrane protein A, Outer membrane protein IbA | Outer membrane |
| 11       | lpp, null, mlpA | Major outer membrane lipoprotein precursor (murein-lipoprotein) | Outer membrane lipoprotein |
| 12       | infC         | Initiation factor 3 | Cytoplasmic |
| 13       | ompX         | Outer membrane protein X | Cytoplasmic |
| 14       | dps          | Stavolization-inducible DNA-binding protein | Cytoplasmic |
| 15       | ilpA, hsiT, htpN | 16-kDa heat shock protein A | Cytoplasmic |
| 16       | ygiW         | Hypothetical Protein YgiW | Unknown, PSORT predicts periplasmic or outer membrane |
| 17       | lpp, null, mlpA | Major outer membrane lipoprotein precursor, murein-lipoprotein | Outer membrane lipoprotein |
| 18       | ycgK         | Hypothetical protein YcgK | Unknown, PSORT predicts periplasmic or outer membrane |

The numbering corresponds to the bands in the one-dimensional electrophoresis gel shown in Fig. 3A. The gene names and synonyms. Names in bold are used to label the corresponding bands in the gel shown in Fig. 3A. Localization according to the SwissProt database. The localization of unknown proteins was predicted using PSORT.

| Gene name(s) | | | |
|--------------|--------------|-----------------|--------------|
| Hypothetical protein YcgK Unknown, PSORT predicts periplasmic or outer membrane |
| Hypothetical protein YbgF | | | |
| Hypothetical protein YncE | | | |
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| Hypothetical protein YncE | | | |
can only be detected after a 10-min chase rather than directly after labeling as in the control strain.

Identification of Novel SecB-dependent Secretory Proteins—SecB dependence of 12 potential SecB substrates identified in the protein aggregates or in the two-dimensional electrophoresis gels was directly monitored using a pulse-labeling approach (Fig. 5 and supplemental Table 4).

Cells with and without SecB were labeled with [35S]methionine and the precursor/processed forms of the secretory proteins tested (DegP, FhuA, FkpA, OmpT, OmpX, OppA, TolB, TolC, YbgF, YcgK, YgiW and YncE) were subsequently immunoprecipitated and analyzed by SDS-PAGE and autoradiography. Strikingly, translocation of all these secretory proteins was hampered in the secB null mutant, as shown by the accumulation of precursors compared with control. This shows that all these proteins indeed need SecB for efficient translocation across the cytoplasmic membrane. In addition, similar pulse-labeling experiments in the presence of the SecA inhibitor azide showed that translocation of these 12 proteins is also SecA dependent (results not shown).

**DISCUSSION**

To characterize the role of the chaperone SecB in E. coli in more detail, we studied a secB null mutant strain using a comparative proteomics approach complemented with Western blotting and pulse-chase experiments. The secB null mutation did not significantly affect growth, but flow cytometry experiments showed that the secB null mutation did affect cell morphology; cells are slightly bigger and seem to contain internal structures.

The comparative proteome analysis of the secB null mutant resulted in three main observations and conclusions: 1) absence of SecB results in aggregation of secretory proteins and increased levels of cytoplasmic chaperones; 2) SecB is not required for targeting and translocation of secretory proteins per se, but rather is needed to improve efficiency of the cytoplasmic targeting process and delivery to the Sec-translocase; and 3) SecB dependence was established for an additional 12 secretory proteins. Below we explain and discuss these main conclusions and observations in more detail.

**Absence of SecB Affects Protein Homeostasis in the Cytoplasm**—The two-dimensional electrophoresis analysis of radiolabeled whole cell lysates showed that precursors of secretory proteins accumulate in the secB null mutant, as shown by the accumulation of precursors compared with control. This shows that all these proteins indeed need SecB for efficient translocation across the cytoplasmic membrane. In addition, similar pulse-labeling experiments in the presence of the SecA inhibitor azide showed that translocation of these 12 proteins is also SecA dependent (results not shown).

Indeed, cytoplasmic protein aggregates, which contained mainly secretory proteins, were for the first time isolated from secB null mutant cells. The amount of aggregated proteins in secB null mutant cells (around 0.5% of the total protein) is about half as much as in single mutant cells that lack cytoplasmic chaperones like DnaK and TF (32, 49, 50). This suggests that DnaK and TF play a different role than SecB in protein homeostasis.
Quantification of silver-stained and $[^{35}S]$methionine-labeled two-dimensional electrophoresis outer membrane maps

Methionine-labeled membranes from the secB null mutant and the control were analyzed by two-dimensional electrophoresis (Fig. 4, A and B). Silver-stained and $[^{35}S]$methionine-labeled spots were matched and quantified as described under "Experimental Procedures." Proteins were identified by MALDI-TOF mass spectrometry from Coomassie-stained protein spots. Details are given in supplementary Table 3.

| Gene name(s) $^a$ | Protein name | Localization $^b$ | Ratio silver stain (secB null mutant/control) $^c$ | Ratio $[^{35}S]$Met (secB null mutant/control) $^c$ |
|------------------|--------------|------------------|-----------------------------------------------|-----------------------------------------------|
| acrA, mtcA, lir  | Acriflavine resistance protein A | Inner membrane lipoprotein | No significant change | No significant change |
| btuB, bfe, cer, dcrC | Vitamin B12 receptor | Outer membrane | No significant change | 0.23 |
| cirA, cir, cerA | Colicin I receptor | Outer membrane | No significant change | No significant change |
| fadL, fadD, fadM | Long chain fatty acid transport protein | Outer membrane | No significant change | 0.27 |
| fepA, fep, fepB | Ferritin-ferrooxin receptor | Outer membrane | 2.16 | 1.28 |
| fluA, tonA | Ferrichrome-iron receptor | Outer membrane | 0.46 | 0.15 |
| fluE | FluE receptor | Outer membrane | 0.52 | 0.16 |
| ftsZ, fts, sulB | Cell division protein FtsZ | Cytoplasm (attaches to the inner membrane during cell division) | No significant change | No significant change |
| metQ | $[^{35}S]$-Methionine-binding lipoprotein metQ | Probably attached to membrane by lipid anchor | No significant change | Not detected |
| nlpA | Lipoprotein-28 | Inner membrane lipoprotein | No significant change | No significant change |
| nlpB, nlpX | Lipoprotein-34 | Outer membrane lipoprotein | No significant change | No significant change |
| ompA, tolA, tolB, tolC | Outer membrane protein A | Outer membrane | No significant change | No significant change |
| ompC, meoA, par | Outer membrane protein C | Outer membrane | No significant change | No significant change |
| ompT | OmpT, Ompin, Protease A | Outer membrane | No significant change | 0.34 |
| ompX | Outer membrane protein X | Outer membrane | No significant change | 0.28 |
| ostA, imp | Organic solvent tolerance protein | Outer membrane | No significant change | Not detected |
| pal, excC | Peptidoglycan-associated lipoprotein | Outer membrane lipoprotein | 0.49 | No significant change |
| ppiD | Peptidyl-prolyl cis-trans isomerase D | Inner membrane | No significant change | Not detected |
| tolC, mtcB, mukA, refl | TolC | Outer membrane | No significant change | No significant change |
| txa, nupA | Nucleoside-specific channel-forming protein txA | Outer membrane | No significant change | No significant change |
| yaeT | Outer membrane protein assembly factor YaeT | Outer membrane | No significant change | No significant change |
| vacJ | VacJ lipoprotein | Outer membrane lipoprotein | No significant change | No significant change |
| ybcC | Hypothetical protein in bioA 5 region, Putative lipoprotein YbcC | Probably attached to the OM with lipid anchor | No significant change | Not detected |
| ybiL | Probable tonB-dependent receptor YbiL | Potentially outer membrane | No significant change | No significant change |
| yfgM | Hypothetical protein YfgM | PSORT predicts inner membrane | No significant change | No significant change |

$^a$ The gene names and synonyms. Names in bold are used to label the corresponding spots in the two-dimensional electrophoresis gel images in Fig. 4, A and B.

$^b$ Localization according to the SwissProt database. The localization of unknown proteins was predicted using PSORT.

$^c$ -Fold change of significantly ($p < 0.01$ in silver-stained gels and $p < 0.05$ in $[^{35}S]$methionine-labeled gels) changed spots calculated as the ratio of the average intensity of spots in secB null mutant gels to the average intensity of matched spots in the control gels. Note that not all identified proteins were detected in $[^{35}S]$methionine-labeled gels.
Defining the Role of E. coli SecB

The pro-OmpA isolated from [35S]methionine-labeled aggregates disappears in a chase, showing that the protein aggregates are dynamic. Proteins extracted from aggregates may either be degraded or get a second chance to be translocated. Based on recent observations that Lbp/CipB/DnaK-mediated reactivation of aggregated proteins plays an important role in viability of the E. coli cell (41, 51), we suggest that the aggregates in the secB null mutant are actively reactivated for translocation rather than being degraded.

The composition of the aggregates and whole cell lysates of the secB null mutant do not point to a significant role of SecB in the folding of cytoplasmic proteins. However, a recent study suggests that SecB can play a significant role in the folding of cytoplasmic proteins under specialized conditions, e.g. in the absence of both the chaperones DnaK and TF (13).

SecB Improves Secretion Efficiency but Is Not Required for Secretion per se—The majority of the proteins we identified in the aggregates isolated from the secB null mutant are secretory proteins, and, correspondingly, the two-dimensional electrophoresis analysis of whole cell lysates indicated that deletion of secB affects the targeting kinetics of secretory proteins. The secB null mutation did not cause any changes in the levels of the Sec-translocase core components SecA, -Y, or -E, indicating that the translocase capacity was not compromised. Furthermore, the secB null mutation did not induce a PspA response, indicating that the electrochemical potential, which plays an important role in protein secretion, is not affected by the absence of SecB. Thus the phenotype of the secB null mutation is a direct consequence of the absence of SecB. This urged us to take a closer look at the secretome of the secB null mutant.

Our analysis of the outer membrane proteome showed that the steady state levels of most proteins were unaffected. However, assembly of a considerable number of outer membrane proteins into the outer membrane was delayed in the secB null mutant. Recently, an outer membrane protein complex consisting of the proteins YeaT, NlpB, YfgL, and YfuO has been shown to act as an insertion machinery for outer membrane proteins (52, 53). In our outer membrane two-dimensional electrophoresis gels, we identified YeaT and lipoprotein NlpB. The steady state levels of these components were unaffected in the secB null strain. The analysis of radiolabeled proteins showed no significant differences in the levels of YeaT and NlpB. This suggests that the outer membrane protein insertion capacity is not affected in the secB null mutant, which is consistent with the absence of cell envelope stress responses. The characterization of the outer membrane proteome of the secB null mutant along with the observation that the secB null mutation does not cause any significant protein misfolding/aggregation in the periplasm/out membrane indicates that the bottleneck created by the absence of SecB is at the level of sorting of outer membrane proteins across the inner membrane rather than their sorting in the cell envelope, and that SecB is not essential for targeting of these proteins to the Sec-translocase but rather facilitates their targeting.

The levels of a few processed secretory proteins (FhuA, FhuE, OmpT, and OppA) go drastically down in the absence of SecB. Notably, none of these proteins were detected in the aggregates. It is tempting to speculate that in the absence of SecB the precursor forms of these proteins are more prone to proteolysis, thereby lowering their levels.

Comparative Proteome Analysis as a Platform for the Identification of SecB Substrates—We used a pulse-labeling approach to directly monitor the SecB dependence of a subset of aggregated secretory proteins, as well as secretory proteins that were affected in two-dimensional electrophoresis maps of whole cell lysates and the outer membrane of the secB null mutant. Strikingly, translocation of all tested potential SecB substrates was indeed SecB-dependent. This clearly demonstrates that the comparative proteomics approach is an excellent platform for the identification of SecB-dependent secretory proteins. Thus far, bioinformatic analysis of these novel and previously established SecB substrates has not lead to the identification of a common denominator,4 stressing the importance of experimentation in protein targeting research.

Recently, pulse-labeling experiments showed that the SRP pathway is required for efficient targeting of the murein lipoprotein Lpp to the Sec-translocase (20). However, the identification of the precursor form of Lpp in the aggregates in the current study strongly suggests that targeting of at least a small fraction of Lpp (which is the most highly expressed protein in E. coli) also depends on SecB. Our observations thus further strengthen the idea that selected proteins can be targeted by both the SRP and SecB targeting pathways (20, 35–37).

Conclusions—The analysis of a secB null mutant using comparative proteomics clearly points to a primary role of the chaperone SecB in facilitating targeting of secretory proteins to the Sec-translocase, and has enabled us to more than triple the number of known SecB substrates. This shows for the first time that comparative proteomics is a

4 L. Baars and J. W. de Gier, unpublished results.
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very powerful tool to study protein targeting pathways and their sub-
strates in E. coli.

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