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Escherichia coli SecB, SecA, and SecY Proteins Are Required for Expression and Membrane Insertion of the Bacteriocin Release Protein, a Small Lipoprotein

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The SecB, SecA, and SecY dependency of a small outer membrane lipoprotein in *Escherichia coli*, the bacteriocin release protein (BRP), was studied. The detrimental effect of BRP expression on the culture turbidity (quasi-lysis) was strongly reduced in the *sec* mutants. Immunoblotting and radioactive labeling experiments showed that the expression, membrane insertion, and processing of the BRP precursor are dependent on SecB, SecA, and SecY. Labeling experiments with hybrid BRP gene constructs revealed that the mature part of the BRP precursor and not its stable signal sequence is important for its SecB dependency.

Periplasmic and outer membrane proteins of *Escherichia coli* are synthesized as preproteins with an amino-terminal signal peptide which directs their transfer across the cytoplasmic membrane (27, 29, 36). Two classes of precursor proteins can be distinguished with respect to their mechanism of translocation across the cytoplasmic membrane. One class consists of preproteins with a content of more than approximately 70 amino acid residues which require for their transfer the products of several Sec proteins, whereas the other class consists of smaller preproteins that are apparently secreted by a Sec protein-independent mechanism.

The SecB and SecA proteins and the SecY/E protein complex are the best characterized. SecB is a cytoplasmic chaperone that stabilizes many precursor proteins in an export-competent conformation (14, 16). Furthermore, SecB is probably involved in the targeting of preproteins to the translocation sites in or at the cytoplasmic membrane (11). SecA is a peripheral cytoplasmic membrane protein which is able to bind the complex of SecB and preprotein (11). SecA hydrolyzes ATP, thereby generating energy for the first steps of membrane insertion of the protein precursor (17, 30). SecY/E is a complex of two integral cytoplasmic membrane proteins, SecY and SecE, postulated to be directly involved in the transfer of preproteins across the cytoplasmic membrane (2). However, the experimental evidence for this postulated role is still limited.

Some small proteins have been shown to insert into or pass the cytoplasmic membrane by a mechanism which is independent of SecA and SecY. Among this class of preproteins are phage M13 coat protein (37) and prepromellitin (6). It is not clear to which extent the size or other properties of these proteins are responsible for Sec independency of insertion and translocation. Some hybrid proteins consisting of amino-terminal sequences of M13 coat protein or prepromellitin fused to carboxy-terminal sequences of larger secreted proteins did require SecA and SecY for their translocation, whereas others did not (12, 13). Studies with deletion mutants of the outer membrane protein OmpA, which is dependent on SecA and SecY for translocation, revealed a lower size limit of around 70 amino acid residues for export of the generated fragments (10). This might indicate that Sec proteins interact with nascent polypeptide chains because the signal peptide of smaller proteins is still partly buried in the ribosome before translation is completed and therefore not freely accessible for interactions with other components. The export of the small outer membrane lipoprotein of *E. coli* (Lpp) is dependent on functional SecA, SecD, SecE, and SecF proteins but does not require a functional SecB protein (32, 35).

We study the structure and function of a group of small (about 50 amino acid residues) membrane proteins, the bacteriocin release proteins (BRPs), also called colicin lysis proteins. These are lipoproteins involved in the export of bacteriocins across both the cytoplasmic and outer membrane and into the culture medium (9). In contrast to their corresponding bacteriocins, the BRPs are synthesized with an amino-terminal signal peptide. The maturation of certain pre-BRPs is unusual in that it occurs very slowly, rendering a signal peptide which is not degraded and a mature, lipid-modified BRP which is located in both membranes (5, 18, 21, 26). The stable signal peptide of these BRPs was shown to play a role in the release of the bacteriocin and in the detrimental effects of high-level expression of the BRP (20).

The unusual features of BRP targeting and processing raise the question whether BRPs follow a Sec-dependent or a Sec-independent pathway for transfer across the cytoplasmic membrane. Therefore, the effects of the depletion of Sec proteins on the expression, processing, and functioning of the pCloDF13-encoded BRP were investigated.

Effects of *sec* mutations on quasi-lysis of cells expressing BRP. Strong induction of the pCloDF13-encoded BRP results in a sharp decline in culture turbidity, also called quasi-lysis, and cell death due to an accumulation of the stable BRP signal peptide and mature BRP (20). To test whether the BRP-induced quasi-lysis is dependent on the SecB, SecA, and SecY proteins, various *E. coli* strains defective in one of these proteins were transformed (7) with pJL7 isolated by the method of Birnboim and Doly (1). This plasmid contains the pCloDF13-encoded BRP gene downstream of the inducible *lpp-lac* promoter-operator (19, 22)

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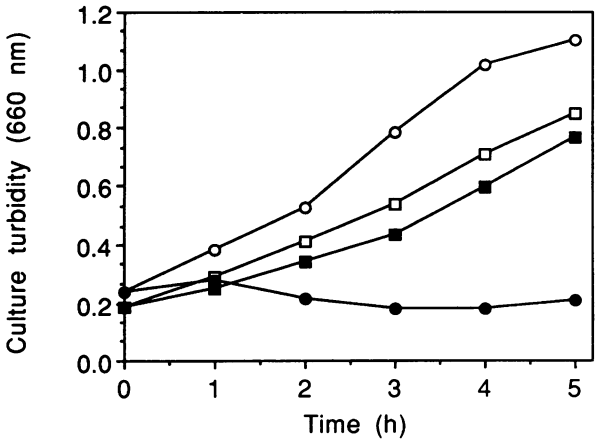

FIG. 1. BRP-induced quasi-lysis of *E. coli* CK1953 *secB*(pJL7) and *E. coli* MC4100(pJL7) control cells. Cells were cultured at 37°C in M9 minimal medium containing thiamine (0.1 mg/ml) and 0.4% glucose (23). IPTG was added to part of the cultures at zero hour. Symbols: ○, MC4100(pJL7), no IPTG; ●, MC4100(pJL7) + 1 mM IPTG; □, CK1953 *secB*(pJL7), no IPTG; ■, CK1953 *secB*(pJL7) + 1 mM IPTG.

and the genes for cloacin DF13 under control of the mitomycin-inducible CloDF13 promoter (19). The transformed cells were cultured in the absence and presence of the inducer isopropyl-β-D-thiogalactopyranoside (IPTG), and the culture turbidity was determined at several time points during growth (Fig. 1, 2, and 3). At noninduced conditions, cells of *E. coli* CK1953 *secB*::Tn*5* Kan^r (15) carrying pJL7 grew steadily at 37°C albeit a bit more slowly than cells of its parent strain MC4100 F$^-$ *lacU169 relA rpsL thi araD139* (24) carrying pJL7. Induction with 1 mM IPTG resulted in a rapid inhibition of growth and in a decline in culture turbidity of the control strain MC4100(pJL7) but hardly affected the growth of the *secB* mutant cells (Fig. 1). Apparently, the absence of SecB resulted in a nonfunctional expression of the BRP (no quasi-lysis).

The effect of SecA on the functioning of the BRP was tested in the conditional lethal mutant strain MM52 *secA52*(Ts) (24). At the permissive temperature strong induction of cells of MM52(pJL7) resulted in significant quasi-lysis. Although the observed decline in culture turbidity was somewhat smaller than that of strongly induced control cells (Fig. 2A), the result still suggested a functional BRP protein at permissive temperature in this mutant. At nonpermissive conditions (42°C) the decline in turbidity was less pronounced than in control cells (Fig. 2B). Apparently, the SecA protein also is required for a functional expression and/or secretion of the BRP. Comparable results were obtained with the conditional lethal *secA* mutant strain BA13 *secA13*(Am) (3, 25) and its parent strain MM65 *leu*::Tn*5* Tn*10* *sup3*(Ts) *trp*(Am) Tet^r (25).

The effect of depletion of SecY on induction and functioning of the BRP was tested by using the conditional lethal mutant strain IQ85 *secY24*(Ts) Tet^r (31) and its nonmutant parent strain IQ86 (31) (Fig. 3A and B). At the permissive temperature (Fig. 3A), induction of the BRP resulted in a decline of culture turbidity. This decline was not as large as in control cells, indicating that the SecY mutant is leaky. At the nonpermissive temperature, induction of the BRP resulted in an even smaller decline in culture turbidity (Fig. 3B), which suggested that SecY is required for BRP expression.

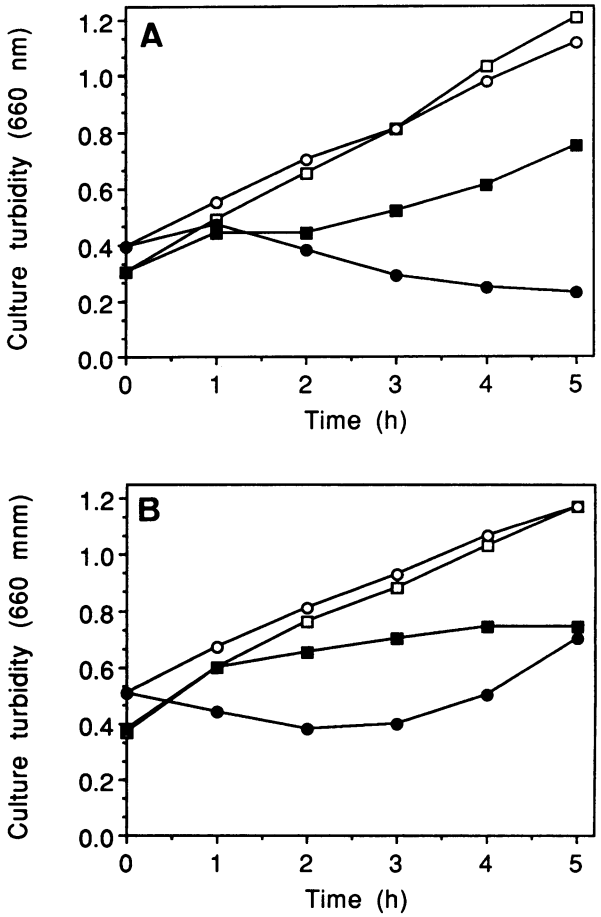

FIG. 2. Effect of BRP induction on quasi-lysis of *E. coli* MM52 *secA*(pJL7) and *E. coli* MC4100 (pJL7) control cells. Cells were cultured in Lab-Lemco broth supplemented with 0.5% sodium lactate (19) and induced with IPTG at time zero. (A) Growth and induction at permissive temperature, 30°C. ○, MC4100, no IPTG; ●, MC4100 + 1 mM IPTG; □, MM52 *secA*, no IPTG; ■, MM52 *secA* + 1 mM IPTG. (B) Cells were first grown at 30°C and then shifted to 42°C (nonpermissive temperature). One hour after the shift, 1 mM IPTG was added to part of the cultures. Symbols are as in panel A.

Expression of BRP in *secB* mutant cells. The expression of the BRP in CK1953 *secB*(pJL7) was studied by using highly cross-linked 16.5% polyacrylamide gels in tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine-SDS-PAGE) (28) and immunoblotting using nitrocellulose filter paper with a small pore size (33). Immunoblots were developed with a monoclonal antibody which recognizes the mature BRP, its precursor containing the 21-amino-acid-residue signal sequence, and its lipid-modified precursor (18, 20). In induced control cells (Fig. 4, lane 3) and in induced cells of the *secB* parent strain MC4100 (pJL7) (Fig. 4, lanes 1 and 2), the three forms of the BRP were clearly detected. Most of the BRP was present in a lipidated precursor form at 1 h and also at 3 h after induction, indicating a slow processing of the precursor after lipid modification into its mature form and stable signal sequence which was not detectable on the blots (18, 20). One hour after induction of CK1953 *secB*(pJL7), the BRP and BRP precursor were hardly detectable. The lipidated precursor form of the BRP

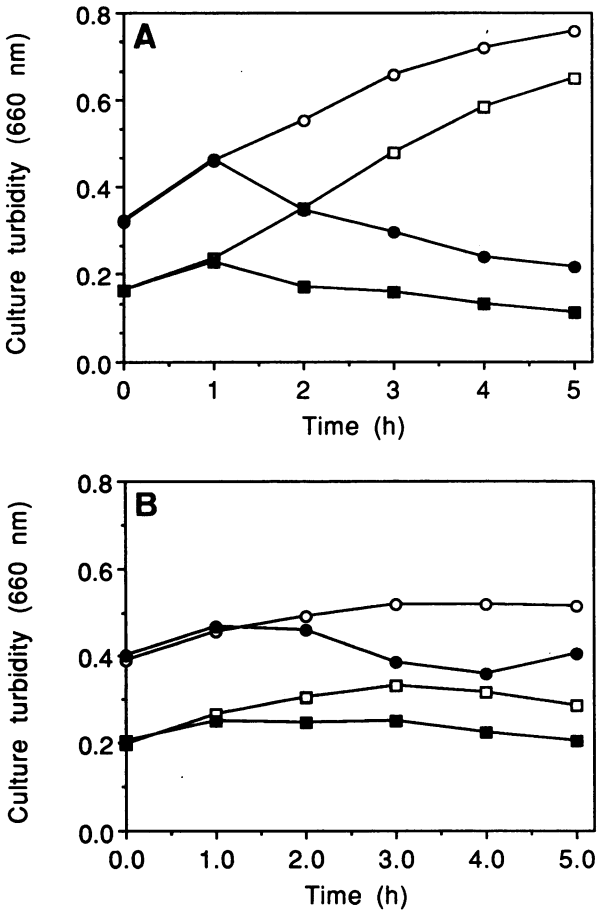

FIG. 3. Effect of BRP induction on the growth of cultures of *E. coli* IQ85 *secY*(pJL7) and *E. coli* IQ86(pJL7) control cells. Conditions were as described in the legend to Fig. 2. Symbols: ○, IQ86, no IPTG; ●, IQ86 + 1 mM IPTG; □, IQ85 *secY*, no IPTG; ■, IQ85 *secY* + 1 mM IPTG.

was not detectable at all. Three hours after induction, even less BRP was detected (Fig. 4, lanes 4 and 5). Apparently, in *secB* cells the BRP precursor is either poorly synthesized or rapidly degraded after synthesis.

Cells of the *secB* mutant strain containing pJL7 and MC4100(pJL7) control cells were also induced with 1 mM IPTG and, after 1 h, pulse-labeled for 5 min with a mixture of ^{3}H-amino acids and chased for 40 min. Samples were analyzed by tricine-SDS-PAGE and fluorography essentially as described previously (18, 20). After the pulse-labeling, small amounts of the mature BRP and its stable signal peptides were detected. After a chase period of 40 min, these products could hardly be detected. These results indicated that in the absence of SecB, the BRP gene product is rapidly degraded (results not shown). Apparently, SecB, which acts as a cytoplasmic chaperone (11), is required for binding the BRP precursor, thereby protecting this polypeptide against proteolytic degradation and maintaining its ability to enter further steps in the secretion pathway. A few BRP precursors may be inserted into the cytoplasmic membrane by a SecB-independent route, which would explain the small effect of BRP induction on culture turbidity.

Expression of BRP in conditional lethal *secA* and *secY* mutants. To study the expression of the BRP gene in *secA* and *secY* mutant strains, cells of *E. coli* MM52 *secA*(pJL7)

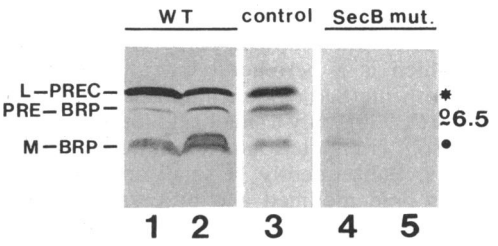

FIG. 4. Immunoblot showing the expression of BRP in the *secB* mutant *E. coli* CK1953(pJL7) strain (lanes 4 and 5) and in *E. coli* MC4100(pJL7) control cells (lanes 1 and 2). Cells were cultured in M9 minimal medium at 37°C and induced with 1 mM IPTG in the early exponential phase of growth (optical density at 66 nm [OD_{66}] ≈ 0.2). At 1 and 3 h after induction, cells were collected and solubilized in solubilization mixture. Aliquots equivalent to 0.1 OD_{660} unit were analyzed. Lane 1, MC4100(pJL7) cells, 1 h after induction; lane 2, MC4100(pJL7) cells, 3 h after induction; lane 3, control cells of *E. coli* FTP 4170(pJL17) expressing BRP (18, 20); lane 4, CK1953(pJL7) cells, 1 h after induction; lane 5, CK1953(pJL7) cells, 3 h after induction. M-BRP, mature form of the bacteriocin release protein (●); PRE-BRP, unmodified precursor form of the BRP (○); L-PREC, lipid-modified precursor of the BRP (*). The molecular mass marker is indicated at the right (in kilodaltons).

and IQ85 *secY*(pJL7) were first cultured at a permissive temperature and then shifted to a nonpermissive temperature and induced for BRP synthesis. Samples were taken 1 h after induction and analyzed by immunoblotting (Fig. 5). At the permissive temperature MM52 *secA*(pJL7) cells produced all three forms of the BRP as their control parent cells (lanes 1 and 3, respectively). However, the amount of the lipid-modified BRP precursor in the mutant cells was reduced compared with that in the parent cells. At the nonpermissive temperature, greatly reduced amounts of mature BRP and of the lipid-modified BRP precursor were found in the mutant cells compared with the control cells. The lipid-modified form of the BRP precursor was not

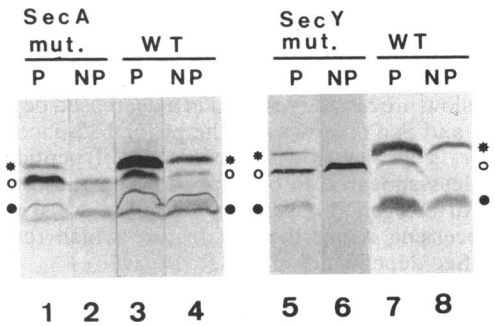

FIG. 5. Immunoblotting showing the expression and processing of the BRP gene product in *E. coli* MM52 *secA*(pJL7) cells (lanes 1 and 2), MC4100 (pJL7) control cells (lanes 3 and 4), IQ85 *secY*(pJL7) cells (lanes 5 and 6), and IQ86 (pJL7) control cells (lanes 7 and 8). Cells were cultured in broth at the permissive (P) temperature of 30°C (lanes 1, 3, 5, and 7) and induced with 1 mM IPTG or shifted to the nonpermissive (NP) temperature (42°C) and induced with 1 mM IPTG 1 h after the temperature shift (lanes 2, 4, 6, and 8). Samples were collected 1 h after induction and analyzed. Symbols: ●, mature BRP; ○, BRP precursor; *, lipid-modified BRP precursor. Note: when massive amounts of the mature and lipid-modified BRP are analyzed on blots, the band often does not stain homogeneously; often a dark-stained edge is observed. The reason for this artifact is not clear.

detected at all (lanes 2 and 4). Similar results were found at 3 h after induction (not shown). Apparently, in the absence of SecA, which is a peripheral inner membrane protein associated with the SecY-SecE inner membrane translocation complex (11, 30), only a small amount of the synthesized BRP precursors is inserted into the inner membrane translocation sites. Subsequently, a small amount of the BRP precursors is lipid modified and processed, giving only a relatively small amount of mature BRP. The remainder of the BRP precursors are probably degraded, which would explain the reduced overall expression of the BRP. Comparable results were found by pulse-labeling and pulse-chase experiments (results not shown). These experiments also showed that the *secA* mutation was effective since other secreted proteins, like the murein lipoprotein and the OmpA outer membrane protein, were clearly affected at the nonpermissive temperature. Comparable labeling experiments with *secY* mutant cells gave the same indications.

IQ85 *secY*(pJL7) cells as well as IQ86(pJL7) control cells contained all three BRP-derived polypeptides when analyzed 1 h after induction at permissive conditions (Fig. 5). However, in the *secY* cells the amounts of the mature BRP and of its lipid-modified precursor were reduced compared with those in the control cells. The *secY* mutation may be partly present at permissive conditions, as already suggested above (see growth experiments). At the nonpermissive temperature, accumulation of the BRP precursor was observed in IQ85 *secY*(pJL7) cells, the lipid-modified BRP precursor could not be detected, and only a minor amount of mature BRP was found (Fig. 5, lane 6). In contrast, at 42°C IQ86(pJL7) control cells mainly produced mature BRP and the lipid-modified BRP precursor but only a minor amount of BRP precursor. Apparently, in this strain at 42°C, the BRP precursor is rapidly lipid modified but poorly processed. Comparable results were obtained by immunoblotting analysis 3 h after induction and by label experiments (not shown). These results indicated that SecY is required for translocation of the BRP precursor to the outside of the inner membrane because at that location lipid modification and processing can occur (32, 35).

Cavard (4) showed that two other small *E. coli* lipoproteins, Cal and CelB, are affected by *secA* and *secY* mutations. CelB expression appeared to be more affected in the mutant strains than Cal expression. It was suggested that Cal is a slowly processed polypeptide, in contrast to CelB, and that this slow processing resulted in a decreased dependency on SecA and SecY. However, the pCloDF13-encoded BRP precursor is also a slowly processed polypeptide, but it nevertheless appeared to be dependent on these two components of the general secretory pathway. Most probably, slow processing is not the key feature which results in a reduced Sec dependency of Cal.

Release of cloacin DF13. The release of cloacin DF13 is strongly dependent on a high-level and functional expression and proper membrane localization of the BRP (9). The effect of BRP expression in the various *sec* mutants on cloacin DF13 release into the culture supernatant fraction was tested after induction with various concentrations of IPTG (for BRP induction) and 500 ng of mitomycin (for cloacin DF13 induction). The amount of cloacin DF13 in culture supernatant fractions and in cell lysates was determined by enzyme-linked immunosorbent assay as described previously (18, 20). At 37°C, the *secB* mutant cells produced large quantities of cloacin DF13 but practically all the bacteriocin remained in the cytoplasm in a soluble form. At nonpermissive temperatures, similar results were obtained for the *secA* and *secY* mutant cells. Apparently, in the mutant cells not enough BRP is expressed and functionally inserted into the membranes to provoke the release of the bacteriocin.

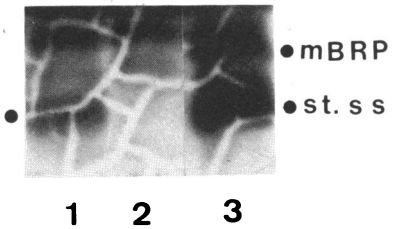

FIG. 6. Fluorography of the hybrid BRP-LPP and Lpp-BRP proteins in *E. coli* CK1953 *secB* cells. Cells harboring pJL41 (signal peptide BRP-mature Lpp; lane 1) or pJL32-T1 (signal peptide Lpp-mature BRP; lane 2) were cultured and induced with IPTG as described previously (18, 20, 31). One hour after induction, cells were labeled with ^{3}H-amino acids (18, 20, 34). Labeled proteins were then analyzed by tricine-SDS-PAGE and fluorography. Only the portion of the gel showing the mature BRP (mBRP) and the stable signal peptide (st.ss) is shown. Lane 3, control cells expressing BRP (mature BRP and accumulated signal peptide). Since all three types of cells used in this experiment produce Lpp, the additional expression of mature Lpp in lane 1 is visible only as a slightly darker Lpp band.

SecB interacts with the mature part of the BRP precursor. The results clearly showed that the small lipoprotein BRP is dependent on SecB, SecA, and SecY. The dependency on SecB is surprising since another relatively small lipoprotein, the *lpp* gene product (murein lipoprotein), is secreted independently of this cytoplasmic chaperone (35). To further investigate the interaction of the BRP precursor with SecB, two different hybrid gene constructs were used. The first construct, plasmid pJL41, encoded a hybrid protein composed of the BRP signal peptide coupled to the mature part of Lpp. The second construct consisted of the Lpp signal peptide coupled to the mature portion of the BRP (plasmid pJL32-T1). Both plasmids have been described and the hybrid products are well expressed in (SecB-positive) *E. coli* strains (20, 34). The BRP signal peptide remains stable after cleavage, whereas the Lpp signal peptide is rapidly degraded (20). The expression of the two hybrid gene products was studied in *E. coli* CK1953 *secB* by labeling of the cells with ^{3}H-amino acids and analysis of the labeled products by tricine-SDS-PAGE (Fig. 6). In control cells not defective in SecB and containing plasmid pJL7, the mature BRP and its accumulated, stable signal peptide were clearly detectable (Fig. 6, lane 3). When cells of *E. coli* CK1953 *secB* harboring pJL41 were analyzed, the stable BRP signal peptide was detectable (Fig. 6, lane 1). In contrast, the wild-type BRP is not expressed in a *secB* background (see results above). This result showed that the hybrid BRP-Lpp construct is normally expressed in a *secB* background. This hybrid protein is, as a consequence, expressed independently of SecB, like Lpp. This also strongly indicates that SecB recognizes the mature part of the BRP precursor. This indication was confirmed by analysis of *E. coli* CK1953 *secB*(pJL31-T1) cells. The Lpp-BRP hybrid was not detectable at all in these *secB* cells (Fig. 6, lane 2), indicating that it is not stably expressed. This hybrid construct, thus, appeared to be dependent on SecB. In conclusion, SecB interacts with the mature portion of the BRP precursor and not with its stable signal sequence.

Recently, De Cock et al. (8) showed that SecB binds at multiple binding sites present in the mature portion of the

precursor of the *E. coli* outer membrane protein PhoE. It was suggested that *secB* might interact with antiparallel β-sheet structures present in the partly folded precursor of this outer membrane protein. The BRP is mainly located in the outer membrane. Interestingly, computer analysis indicated that the central part of the mature BRP contains a rather long stretch of β-structure (18 to 20 amino acid residues) with a 4-amino-acid coil structure in the middle. This central segment of the BRP might constitute the β-sheet structure which interacts with SecB.

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