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Carbon Source-Dependent Synthesis of SecB, a Cytosolic Chaperone Involved in Protein Translocation across *Escherichia coli* Membranes

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SecB is a cytosolic chaperone involved in protein translocation across cytoplasmic membranes in *Escherichia coli*. It has been shown to be required for efficient translocation of a subset of precursor proteins but is not essential for cell viability. This study investigated whether synthesis of SecB is growth rate dependent. Interestingly, the total amount of SecB synthesized in the cells was relatively small. Moreover, the levels of SecB were found to be carbon source dependent since more SecB was produced in cells grown in glycerol media than in cells grown in glucose media, regardless of the growth rate. This is in contrast to the other Sec proteins, whose synthesis is growth rate dependent and not related to glucose as a carbon source. In addition, cyclic AMP (cAMP) partially relieves the lower levels of SecB observed in glucose medium, a compensatory effect that depends on the presence of both *cya* and *crp* gene products. Thus, the glucose-dependent synthesis of SecB may be related to the cAMP-cAMP receptor protein complex-mediated activation.

In response to the available environmental nutrients, bacterial cells produce essential components for cell growth in a growth rate-dependent manner (8, 41). In richer media, more of these components are synthesized in order to meet the faster rate of cell growth. These include the so-called housekeeping components, such as rRNA, ribosomal proteins, translational and transcriptional factors, molecular chaperones (8, 32), and presumably proteins involved in secretion of proteins essential for cell growth.

The SecB protein is unique in that it is a chaperone protein dedicated only to protein secretion (19, 21). The *secB* gene was first identified by screening a mutant strain of *Escherichia coli* for pleiotropic defects in protein localization (19). These *secB* strains exhibit severe defects in export of many proteins. However, not all secretory proteins are affected; the precursors of ribose binding protein and alkaline phosphatase are only minimally affected (20). Purified SecB enhances the protein translocation of a subset of precursor proteins across cytoplasmic membrane vesicles in vitro (22, 43). Furthermore, SecB specifically binds to these precursor proteins, forming complexes (24, 42); this presumably retards premature folding, thereby keeping the proteins in a suitably unfolded, competent form for subsequent export (5, 35). SecB seems to be the only cytosolic chaperone specifically involved in protein export through the signal sequence-dependent general protein secretion pathway (the Sec system or Sec pathway). It has been suggested that SecB functions as a pilot protein, mediating the entry of precursors into the export machinery via concerted or sequential interactions with other Sec proteins (33). For example, SecA forms a soluble complex with SecB (14) and the C-terminal end of SecA interacts with SecB (2). However, a mutation in this region of SecA can partially compensate for the absence of SecB (27).

Some major chaperone proteins, i.e., GroEL/ES and DnaK, seem to provide SecB-like functions in protein export (23, 24). However, these functions may be due to their general involvement in the modulation of protein folding. Moreover, trigger factor was originally identified as a chaperone factor for protein translocation (6) but was later suggested to be involved in cell division (12). However, recently it has been identified as a ribosome-associated peptidyl-prolyl *cis/trans* isomerase (40) involved in protein folding. As a general chaperone, it presumably functions by associating with cytosolic and secretory nascent polypeptide chains (13). It has also been shown to be involved in GroEL-dependent protein degradation (16).

In view of the dedicated involvement of SecB as a pilot chaperone in protein translocation, it is generally expected that the synthesis of SecB (as well as that of other Sec proteins) should correlate with the growth rate and the different growth phases of cells as a function of their need for translocation of essential proteins (33, 41). For example, *secY* is within the *spc* ribosomal protein operon (3, 38). Consequently, its expression is also presumably dependent upon the growth rate. However, the correlation between growth rate and the synthesis of Sec proteins has yet to be established. In this study, by using quantitative immunoblot analysis, we have been able to determine the amounts of SecB and other Sec proteins in cells grown in different media. The results showed that for most of the Sec proteins tested here, the amount produced was indeed growth rate dependent, as expected. Surprisingly, however, the cellular amount of SecB was independent of the growth rate and did not appear to correlate with the levels of other Sec proteins. Furthermore, we have shown that when glucose is used as the sole carbon source, the amount of SecB produced is partially related to catabolic repression.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are derivatives of *E. coli* K-12. MC4100 (F$^-$ *lacU169 araD136 relA rpsL150 flbB5301 deoC7 ptsF25 thi*), ZK4 (MC4100 *recA56*), and the plasmid pHA7E, containing a cloned *crp* gene (15), were kindly provided by J. Beckwith, R. Kolter, and A. Hochschild (Harvard Medical School, Boston, Mass.), respectively. Δ*cya* Δ*crp* mutant strain RH77 (MC4100 Δ*cya851*, Δ*crp-zhd732*::Tn*10* [26]) was a gift from R. Hengge-Aronis (University of Konstanz, Konstanz, Germany). Plasmid pHK205, containing a cloned *secB* gene (18), was a gift from C. A. Kumamoto (Tufts Medical School, Boston, Mass.).

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TABLE 1. Cellular amounts of Sec proteins in various growth media

Medium[a]	Generation time (min)[b]	Amount of Sec protein[c] (ng/mg of total protein ± SD)				
		SecA	SecB	SecE	SecY	Trigger factor
MinA-acetate	123		415 ± 44	82 ± 2.8	87 ± 5.1	2,207 ± 103
MinA-glycerol	95	1,003 ± 109	470 ± 37	86 ± 3.3	137 ± 6.6	2,975 ± 152
MinA-maltose	70		433 ± 15	94 ± 3.9	178 ± 5.6	3,728 ± 75
MinA-glucose	63	1,243 ± 63	224 ± 26	91 ± 1.6	199 ± 6.6	4,133 ± 21
LinA-glycerol	33	1,420 ± 58	338 ± 33	103 ± 5.1	235 ± 4.7	5,216 ± 70
LinA-glucose	28	1,560 ± 170	225 ± 28	107 ± 5.4	266 ± 13.3	5,700 ± 390

[a] In each case the carbon source was present at a concentration of 0.5%.
[b] Generation times were calculated in cells grown to the exponential growth phase.
[c] MC4100 cells were grown to steady state (optical density at 600 nm, ~0.6) in the indicated media. The amounts of protein were determined immunologically. The amounts of SecE and SecY were extrapolated from estimated numbers of copies per cell in minimal glucose medium (26) and calculated from relative ratios in different media. The values are means ± standard deviations of data from 4 to 18 experiments with three independent cultures.

Media. The minimal medium used in this study was the modified minimal medium A (MinA) of Davis and Mingioli (7) plus $CaCl_2$ (5 μg/ml), $FeSO_4 \cdot 7H_2O$ (0.25 μg/ml), and thiamine (10 μg/ml). LinA is buffered L broth (10 g of tryptone, 2 g of yeast extract, and 5 g of NaCl per liter of MinA). All sugar carbon sources were at 0.5% (wt/vol), and antibiotics were used at the following concentrations ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 20 μg/ml.

Growth of bacterial strains and preparation of cell extracts. Cells grown overnight were inoculated into the same fresh media, and cell growth was monitored by determining the optical density at 600 nm. The specific growth rate in a given medium was calculated as $k = \ln 2$/generation time (in hours) (32, 41). During growth, samples were taken at equivalent optical densities, harvested, and washed once with MinA. Harvested cell pellets were kept at −80°C until analyzed.

Frozen cell pellets were resuspended in 2% sodium dodecyl sulfate (SDS) protein buffer (20 mM Tris-Cl [pH 6.8], 2% [wt/vol] SDS, 10% [vol/vol] glycerol) and boiled for 4 min to solubilize the extract, unless otherwise indicated. For the detection of membrane proteins such as SecY and SecE, cell pellets were resuspended in 4% SDS buffer (20 mM Tris-Cl [pH 6.8], 4% [wt/vol] SDS, 10% [vol/vol] glycerol) and incubated at 37°C for 1 h. Extract was passed through a 25-gauge needle to sheer the DNA, and the remaining debris and DNA were removed by centrifugation at 15,000 × *g* for 10 min.

Protein determinations and immunoblotting. Protein concentrations were determined by the Micro-BCA method (Pierce, Rockford, Ill.). Samples were adjusted to the same protein concentration, and 10 to 20 μg of protein was subjected to SDS–12% polyacrylamide gel electrophoresis, unless otherwise indicated. After electrophoresis, proteins were transferred to Immune-lite membranes (Bio-Rad, Hercules, Calif.). For immunoblotting, sera were diluted appropriately and bound antibodies were detected by using alkaline phosphatase-conjugated anti-rabbit immunoglobulin G and a chemiluminescent substrate (Bio-Rad). Immunoblots were exposed to X-ray film, and specific bands were quantified by scanning densitometry (with a Personal Densitometer [Molecular Dynamics, Sunnyvale, Calif.] or a Desktop Densitometer [PDI, Huntington Station, N.Y.]). SecB, SecA, and trigger factor were quantified by using known amounts of the appropriate purified proteins as standards.

DNA manipulations and deletions. Plasmid DNA isolation, digestion, and transformation and other routine DNA manipulations were performed as described by Sambrook et al. (37). All deletions of the *secB* upstream region were derived from plasmid pHK205, which contains about a 1.8-kbp upstream region of the *secB* gene (18). Plasmid pKS101 was constructed by deletion of a *Hin*dIII fragment (−1837 to −389; the numbering is with respect to the translational start site of the *secB* coding sequence) from pHK205 and self-ligation. pKS102 was constructed by deletion of a *Hin*dIII-*Bam*HI fragment (−1837 to −81) followed by filling in the sticky ends with Klenow enzyme (United States Biochemical/Amersham Life Science) and blunt-end ligation. pKS103 was created by deletion of an *Eco*RV fragment (−1837 to +59) and self-ligation of the plasmid, resulting in deletion of the N-terminal coding region of *secB*.

Biochemicals. All chemicals and reagents were reagent grade and were purchased from Sigma Chemical Co. (St. Louis, Mo.), unless otherwise noted. Acrylamide stock solution (Protogel; protein and sequencing grade) was from National Diagnostics (Atlanta, Ga.). Restriction enzymes and T4 ligase were from Boehringer Mannheim (Indianapolis, Ind.). Rabbit antibodies specific for purified SecA, SecB, trigger factor proteins, and synthesized SecY peptides were from our own laboratory stocks, while SecE peptide antibodies were gifts from J. Beckwith and P. Schatz.

RESULTS AND DISCUSSION

The amount of SecB synthesized is dependent on the carbon source, not the growth rate. SecB synthesis has previously been assumed to be correlated with the growth rate and growth conditions as related to the need for protein export. To verify this, we determined the amount of intracellular SecB protein in an *E. coli* K-12 strain, MC4100, grown in various media. The total amount of SecB in samples taken during the exponential phase of cell growth was measured by quantitative immunoblotting. Interestingly, these results showed that the amount of SecB in cells grown in glucose minimal medium is relatively small (~220 ng of SecB/mg of total protein) (Table 1) compared to the amounts of trigger factor (~4.1 μg/mg of total protein) (Table 1) and two other chaperones, DnaK and GroEL (9.8 and 13 μg/mg of total protein, respectively [41]). The concentration of SecB was estimated to be around 1,000 copies per cell in glucose minimal medium and thus was present at only 10 to 20% of the levels of other chaperone-like proteins (16,000, 10,000, and 5,000 copies per cell for GroEL, DnaK, and trigger factor, respectively [41]). As a molecular chaperone, SecB is not inducible by environmental stress, in contrast to other molecular chaperones which have been shown to be dependent upon growth rate (41). In addition, the levels of SecB were relatively constant in different growth phases (data not shown). As such, the low levels of SecB and its growth rate-independent synthesis set SecB apart from other molecular chaperones.

Surprisingly, the levels of SecB were higher when cells were grown in glycerol media than when they were grown in glucose media. The levels of SecB synthesized were similar when cells were grown on the same carbon source, despite differences in medium composition, which resulted in a greater than twofold difference in growth rates (Fig. 1). Although Randall (34) demonstrated that SecB binds to many nonnative, denatured proteins in vitro, Kumamoto and Francetić (21) showed that the binding of SecB in vivo is very specific for a small number of protein precursors. In addition, the binding of SecB to these precursors has been shown to be transient (17). The selectivity for a subset of precursor proteins and the transient binding characteristics suggest that SecB is capable of supporting protein translocation even in relatively low concentrations. If this is the case, the translocation of large amounts of SecB-dependent precursors, such as pro-OmpA (30,000 copies per cell), pre-maltose binding protein (pre-MBP) (10,000 to 20,000 copies per cell), and pre-LamB (100,000 copies per cell) (9, 31, 41), can conceivably be guided by a much smaller amount of SecB chaperone (250 copies of functional tetramers per cell). Interestingly, synthesis of pre-MBP and pre-LamB is repressed in the presence of glucose (30), conditions under which SecB synthesis is also reduced.

The *gpsA* gene, encoding L-glycerol-3-phosphate dehydrogenase, which is involved in glycerol-mediated membrane bio-

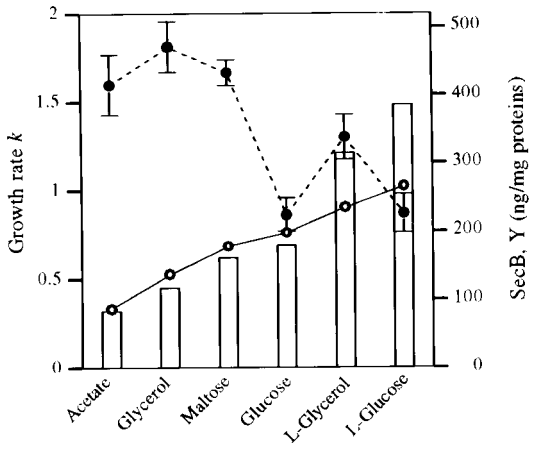

FIG. 1. Growth rate-independent synthesis of SecB. The amounts of SecB and SecY in MC4100 cells grown in different growth media under steady-state conditions were measured by quantitative immunoblotting. The amounts of SecB (●) and SecY (○) were taken from Table 1. Columns denote specific growth rates k, where k is ln2/generation time (in hours). Error bars indicate the standard deviations.

genesis (20), is located downstream of the *secB* gene. Indeed, the start codon of the *gpsA* structural gene is overlapped by the stop codon of *secB* (39). To determine whether the high levels of SecB that were observed in the glycerol medium might reflect a glycerol-specific expression of the coupled dehydrogenase, the levels of SecB were examined in media with other carbon sources, such as maltose or acetate. The results showed that the amount of SecB in cells grown on these carbon sources was similar to that observed in cells grown on glycerol (Fig. 1; Table 1). These data would indicate that while there may still be translational coupling of the two genes, synthesis of SecB is not subject to glycerol-specific regulation. These results also showed that the cellular levels of SecB are rather specifically repressed in glucose medium.

Comparison of the amounts of other Sec proteins to that of SecB. The absence of growth rate-dependent synthesis of SecB led us to examine the production of other Sec or Sec-related proteins under the same growth conditions. Our data confirmed a previous report (41) that trigger factor is indeed growth rate dependent (Table 1). In addition, the cellular amounts of SecA and SecY were also found to be related to growth rate, as were the level of SecE, although the change in the latter was relatively small (Table 1; Fig. 1). Additionally, our estimation of the relative amounts of these proteins was comparable to values given in previous reports (25, 31). Thus, as expected, the synthesis of these Sec proteins was found to be growth rate dependent, in marked contrast to that of SecB. It has also been determined that changes in SecB synthesis, either in overproducing strains or in a null mutant, have no effect on the cellular amounts of either SecA or trigger factor. Conversely, overproduction of trigger factor had no significant effect on the level of SecB (data not shown). These observations are consistent with the previous findings of Rollo and Oliver (36) in that mutations within other Sec components result in hyperexpression of SecA but a mutation in SecB does not lead to a similar increase.

Our observations raise several interesting issues. First, it has been generally assumed that the synthesis of components involved in the Sec pathway is dependent on the growth rate and the growth phase of cells. This growth rate dependency presumably was necessary to support the increased need for protein export in faster-growing cells. Indeed, one of the prevalent explanations for the differences in viability of *secB* null mutant strains in rich and poor media (20) was that the *secB* null mutants were not viable in rich media because the absence of SecB led to an increased accumulation of defective precursor proteins in the cytoplasm. This adverse effect on the physiology of the cell, it was thought, would be greater in media that supported faster cell growth, as this would lead to a heavier demand for SecB. However, our data show that the synthesis of SecB is independent of growth rate, and thus a growth rate-dependent need for SecB does not appear to be the basis for the nonviability of *secB* null strains in L broth. Second, although functional interactions between SecB and other Sec components have been shown to exist (2, 14, 27), as has competitive binding of precursors between trigger factor and SecB (24, 33), there is no apparent correlation between SecB and other Sec proteins in either their cellular amounts or their growth dependency. Third, the estimated number of export sites on the membrane of each cell ranges from 200 to 500 (29), and each of these sites consists primarily of membrane components like SecY and SecE, which have been shown to be growth rate dependent (Table 1). The other Sec component, SecA, is present in 800 to 900 copies per cell (as estimated from SecA amounts in glucose minimal medium). This determination is consistent with that made by Oliver and Beckwith (500 to 1,000 copies per cell [31]) but differs significantly from that of Mizushima et al., who estimated that SecA is present in 2,500 to 5,000 copies per cell (29). Since SecA is equally divided between the cytosol and the membrane (31), SecA is presumably also present in about 400 to 450 copies (or 200 to 225 functional dimers) per cell, embedded in the membrane export sites (4). These estimations suggest that there is an approximately stoichiometric relationship between the number of SecB chaperone molecules (~250 functional tetramers per cell) and the number of export sites in cells grown in the presence of glucose. Conversely, the number of SecB copies appears to be in excess of the number of export sites (as defined by the amount of SecY) in poorer media with acetate or glycerol as the sole carbon source (Fig. 1). Indeed, using the same estimation as made previously, the ratio of SecB molecules to export sites could be as high as 4:1. The physiological significance of this finding is not clear. However, it is tempting to speculate that in slower-growing cells, more SecB is required to keep the precursors in the competent form so that they will be translocated through the limited number of export sites; i.e., more SecB is required to keep the precursors competent for prolonged periods of time.

The effect of cAMP and CRP on SecB synthesis. The lower levels of SecB in glucose medium could result from changes in either the stability or the synthesis of SecB. In vivo labeling and pulse-chase experiments showed that there was no significant change in the stability of SecB, whereas a decrease in the synthesis of new SecB was observed when cells were shifted from a glycerol to a glucose medium (as determined by using the ratio of SecB to EF-G as an indicator [data not shown]). This specific reduction suggested that SecB synthesis might be regulated by catabolite repression. To substantiate this possibility, the effect of externally added cyclic AMP (cAMP) on the levels of SecB was examined. The presence of cAMP caused SecB levels in wild-type cells that were grown in glucose medium to increase by about 50% (Table 2). As expected, there was no change in SecB when cAMP was added in the *cya crp* double-mutant RH77, which lacks the ability to synthesize endogenous cAMP and its functional mediator, cAMP receptor protein (CRP) (Table 2). When the wild-type *crp* gene was restored in RH77 cells through transformation with the plas-

TABLE 2. Effects of exogenous cAMP and mutations in *cya* and *crp* on synthesis of SecB

Strain	Relevant genotype	Plasmid	Relative amount of SecB (mean ± SD)[a]	
			Without cAMP	With cAMP
MC4100	Wild type, *cya*$^+$ *crp*$^+$	None	1.00 ± 0.062	1.57 ± 0.137
RH77	Δ*cya* Δ*crp*	None	0.93 ± 0.051	0.94 ± 0.103
		pHA7E (*crp*$^+$)	1.66 ± 0.147	6.66 ± 0.503
		pBR322 (*cya crp*)	1.01 ± 0.071	1.07 ± 0.042

[a] Cells were grown in MinA with glucose to steady state, reinoculated into fresh medium in the presence or absence of cAMP (5 mM final concentration), and then allowed to grow for 3.5 generations. The relative amounts of SecB were compared by immunoblotting, setting the level of SecB in MC4100 cells grown in glucose at 1.00. The values are averages of data from four to eight experiments with two independent cultures.

TABLE 3. Effects of *secB* gene upstream deletions on SecB synthesis

Strain	Relevant genotype	Plasmid[b]	Relative amount of SecB (mean ± SD)[a]	
			Without cAMP	With cAMP
ZK4	MC4100 *recA56*	None	1.06 ± 0.06	1.48 ± 0.04
		pHK205	39.6 ± 2.23	55.5 ± 1.94
		pKS101	11.4 ± 0.74	25.1 ± 3.18
RH77	MC4100 Δ*cya* Δ*crp*	None	0.96 ± 0.08	0.97 ± 0.11
		pHK205	36.5 ± 1.56	36.3 ± 2.99
		pKS101	7.87 ± 0.87	7.12 ± 1.03

[a] The relative amounts of SecB were determined by immunoblotting as described in the footnote in Table 2, and the level of SecB in wild-type MC4100 cells grown on glucose was set at 1.00. The values are averages of data from four to eight experiments with two to three independent cultures.

[b] The relevant physical map of pHK205 and deletion constructions are shown in Fig. 2.

mid pHA7E, the addition of exogenous cAMP caused SecB levels to increase fourfold (Table 2). These data demonstrate that the addition of exogenous cAMP affects the cellular level of SecB by partially relieving the catabolite repressive effect of glucose. Additionally, this relief requires the presence of both the *cya* and *crp* gene products. The cAMP-CRP complex has been shown to be a global regulator of numerous cellular mechanisms and gene expressions, either as an activator or as a repressor (1). In the case of SecB, the cAMP-CRP complex appears to function as an activator since its presence leads to an increase in SecB levels in glucose medium. The decreased synthesis of SecB in the presence of glucose and its recovery through the addition of cAMP are typical responses observed with glucose-elicited catabolic repression (11, 28). With the current data, it is not clear, however, whether the cAMP-CRP complex acts directly or indirectly at the level of *secB* transcription. It is possible that SecB synthesis is related to the synthesis of exported proteins which are themselves under catabolic repression. In this regard, it is worth noting that maltose binding protein and LamB, which are known to be dependent on SecB for their export (10), have also been shown to be positively regulated by the cAMP-CRP complex (30). Even so, there was no parallel increase in the level of SecB observed when maltose binding protein was induced to a high level in maltose medium (Table 1).

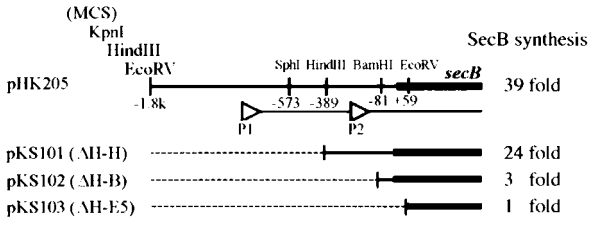

FIG. 2. Physical maps of the upstream regions of *secB* gene constructions, and synthesis of SecB in glycerol medium. Plasmid pHK205, containing the cloned chromosomal *secB* gene, includes ~1.8 kbp upstream of the *secB* coding region. Deletion constructions were carried out as described in Materials and Methods. Solid lines indicate *secB* gene upstream regions, dotted lines indicate deleted segments, and boxes indicate the *secB* coding region. Also shown (triangles) are the putative distal promoter P1 and proximal promoter P2 as determined by genome sequencing (35). Restriction sites are as follows: MCS, multiple cloning sites; E5, *Eco*RV; H, *Hin*dIII; and B, *Bam*HI. The numbering is in reference to the *secB* translation coding region. The levels of SecB synthesized in cells containing pHK205 or various upstream deletion plasmids grown in glycerol minimal medium were compared immunologically. The values are presented with respect to the level of SecB from wild-type cells without any plasmid (set at 1.00).

DNA sequence analysis of the *secB* region from the *E. coli* genome (39) suggested that there are two possible promoters upstream of the *secB* gene. To obtain preliminary information on whether there is a relationship between these two putative promoters and the effect of glucose on the synthesis of SecB, we examined deletions within the upstream putative promoters in the pHK205 plasmid (Fig. 2). The overall levels of SecB were increased approximately 39-fold in cells harboring the pHK205 plasmid compared to levels in wild-type cells without the plasmid in glycerol medium (Fig. 2). The increase in the amount of SecB was about 24-fold in pKS101 transformants, in which the distal putative promoter, P1, had been deleted (Fig. 2). Indeed, the pattern of changes in SecB levels in cells grown in glucose medium and the recovery with cAMP of SecB amounts with the multicopy plasmid pHK205 were similar to those observed for synthesis of SecB derived from chromosomal *secB* (Table 3). However, the reduction in glucose and the recovery of SecB with cAMP were more pronounced in cells containing only the proximal putative promoter, P2 (in pKS101) (Fig. 2). The reduction of SecB in glucose medium was about 5-fold and the recovery with cAMP was 2-fold with pKS101, compared to a 2-fold reduction and 1.5-fold recovery with pHK205 (Table 3). The levels of SecB from these plasmids in the Δ*cya* Δ*crp* double-mutant strain, RH77, were further examined. When pHK205 was expressed in RH77 cells, the levels of SecB were similar to those of pHK205 in wild-type cells and did not increase upon addition of cAMP. On the other hand, in RH77 cells containing pKS101, the levels of SecB were slightly decreased and did not respond to the addition of cAMP (Table 3). These results further substantiate the possible involvement of cAMP and CRP in the synthesis of SecB. Our data suggest that the region 5′ proximal to *secB* within pKS101 is likely to be responsible for the effect of glucose and the cAMP-CRP-mediated effect upon SecB synthesis, although additional, more extensive studies are needed to confirm this. Such studies provide the focus of our ongoing research in this area.

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