



# An internal hydrophobic helical domain of *Bacillus subtilis* enolase is essential but not sufficient as a non-cleavable signal for its secretion



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## ABSTRACT

Many cytoplasmic proteins without a cleavable signal peptide, including enolase, are secreted during the stationary phase in *Bacillus subtilis* but the molecular mechanism is not yet clear. We previously identified a highly conserved embedded membrane domain in an internal hydrophobic  $\alpha$ -helix of enolase that plays an important role in its secretion. In this study, we examined the role of the helix in more detail for the secretion of enolase. Altering this helix by mutations showed that many mutated forms in this domain were not secreted, some of which were not stable as a soluble form in the cytoplasm. On the other hand, mutations on the flanking regions of the helix or the conserved basic residues showed no deleterious effect. *Bacillus* enolase with the proper hydrophobic helical domain was also exported extracellularly in *Escherichia coli*, indicating that the requirement of the helix for the secretion of enolase is conserved in these species. GFP fusions with enolase regions showed that the hydrophobic helix domain itself was not sufficient to serve as a functional secretion signal; a minimal length of N-terminus 140 amino acids was required to mediate the secretion of the fused reporter GFP. We conclude that the internal hydrophobic helix of enolase is essential but is not sufficient as a signal for secretion; the intact long N-terminus including the hydrophobic helix domain is required to serve as a non-cleavable signal for the secretion of *Bacillus* enolase.

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## 1. Introduction

Many cytoplasmic proteins without any cleavable classical N-terminal signal peptides have been found to be secreted via 'non-classical or non-conventional secretion' [1–4]. Such proteins including enolase have been termed "moonlighting" proteins which display multiple unrelated functions in different sub-cellular locations, [3–6]. It has been proposed that the release outside of cells could be attributed to cell lysis [7,8]. Enolases (EC 4.2.1.11) are essential cytoplasmic enzymes that catalyze the reversible conversion of 2-phosphoglycerate into phosphoenolpyruvate. Although enolases lack a classical signal sequence and typical motifs for membrane or cell wall anchoring, many studies

showed that various enolases can be exported to the cell surface or released to the culture medium in eukaryotic and prokaryotic organisms [5,9,10]. It has been long speculated that there might be an unknown signal for enolase export conserved over a long evolutionary period [11]. Extensive studies from different groups support the presence of an alternative secretion mechanism other than the classical Sec-pathway to drive enolases through membranes to the cell surface or into the extracellular medium [2,11]. *Escherichia coli* enolase was found to be exported into the medium and the export was dependent on covalent binding of its substrate, 2-phosphoglycerate [12]. *Bacillus subtilis* enolase has also been found in the extracellular compartment [8,13], though the mechanism of how the enolase is secreted remains uncertain. We have previously reported that enolase and other cytoplasmic proteins without a cleavable signal sequence can be secreted from *B. subtilis* cells into the medium in the absence of cell lysis [1].

Using the crystal structure of *Enterococcus hirae* enolase [14] as the template, a predicted molecular structure of enolase was modeled by Swiss-Model [15]. *Bacillus* enolase (SI Fig. 1) is composed of one large C-terminal domain (S<sub>139</sub>-K<sub>430</sub>) and one smaller N-terminal domain (P<sub>2</sub>-N<sub>138</sub>). According to Swiss-Plot database (Entry no. **37869**), the C-terminal barrel domain contains four phosphorylation sites, substrate and Mg<sup>2+</sup> binding sites and two catalytic motifs.

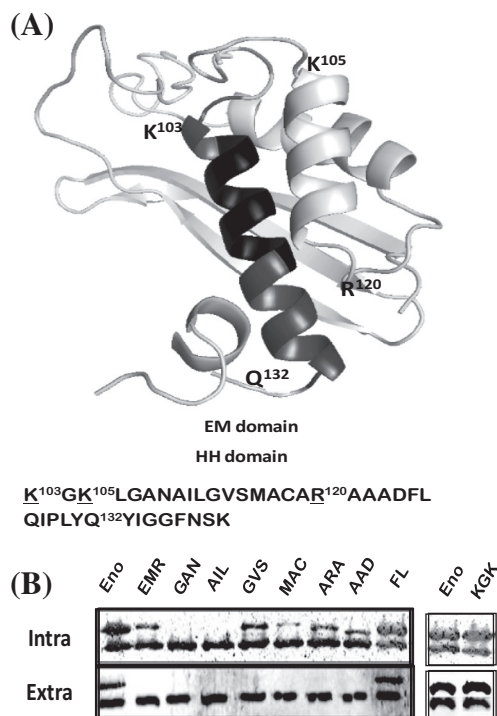
**Abbreviations:** EnoBs, enolase of *Bacillus subtilis*; IPTG, isopropyl  $\beta$ -D-1-thiogalactopyranoside; LB, Luria-Bertani; EM domain, embedded membrane domain; HH domain, hydrophobic  $\alpha$ -helix domain; GFP, Green Fluorescent Protein.

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**Fig. 1.** Predicted ribbon structures of N-terminal domain of *Bacillus* enolase and the importance of HH domain on enolase secretion. (A). Top panel: predicted structures of the 140 aa N-terminal domains *Bacillus* enolase using the Swiss-Plot [15]. The HH  $\alpha$ -helix (#106–126) and EM (#110–118) domains are highlighted. Three basic residues (K<sup>103</sup>, K<sup>105</sup>, R<sup>120</sup>), and a small loop replacement (Q<sup>132</sup>) around HH domain are indicated. (B). Mutations in the HH domain affected the secretion of enolase. Different glycine-substituted mutants were expressed in *B. subtilis* WB600BHM. Equal volumes of soluble whole-cell lysate (Intra) and supernatant (Sup) fractions were examined by immunoblots. EnoBs antibodies detected both chromosomal wild-type (which were used as internal controls) and plasmid FLAG-tagged enolase (higher band, confirmed with FLAG antibodies). Eno: wild-type enolase; EMR: EM domain replaced enolase [1]; all other mutations are glycine replacements as described in Section 2.

In the N-terminal motif, a long unbent hydrophobic  $\alpha$ -helix (HH) domain (A<sub>108</sub>–L<sub>126</sub>) of enolase resides (Fig. 1A). Within this HH domain, a core region (A<sub>110</sub>–C<sub>118</sub>) is predicted as a membrane-embedded (EM) domain [1] by the PSSM\_SVM scheme [16] that may be involved in interacting with membranes. Mutation analyses by deletion of EM domain or replacement (EMR) of the enolase showed that this EM domain indeed is important for its secretion [1].

The EM domain is part of the larger HH domain that has been predicted as a signal peptide or a transmembrane domain (signalP 3.0 server; [17]). In this work we further examined the nature of this EM domain and the surrounding HH domain for the secretion of enolase in *B. subtilis* (EnoBs). We constructed a series of mutations in the domain of the cloned EnoBs to determine their effects on its secretion in *B. subtilis*. We found that the EnoBs with native hydrophobic helix, but not mutated, domain can also be exported in *E. coli* as in *B. subtilis*, indicating that the importance of the HH domain in the secretion of EnoBs. Moreover, we have identified an N-terminal region including the HH domain of EnoBs that facilitates the secretion of the reporter protein GFP. Taken all data together, we conclude that this highly conserved HH domain is important, but not sufficient in its secretion.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids, culture conditions and growth

Bacterial strains and plasmids are listed in SI Table 1. *B. subtilis* WB600BHM and *E. coli* strain DH5 $\alpha$  were grown in Luria–Bertani

(LB) broth or agar plates containing 0.2% glucose at 37 °C. The following antibiotics were used as required: ampicillin (100  $\mu$ g/mL), chloramphenicol (25  $\mu$ g/mL for *E. coli* and 5  $\mu$ g/mL for *Bacillus*), and kanamycin (10  $\mu$ g/mL).

### 2.2. Cloning and expression of *B. subtilis* enolase mutants

Constructions of the wild-type *B. subtilis* *eno* gene and its mutations on shuttle vector pDG148 using overlapping PCR were described previously [1]. To construct FLAG-tagged enolase and mutants, the wild-type and mutated *eno* genes were PCR amplified and cloned into the *SacI*/*PstI* sites of pGTN-FLAG [1].

To express the EnoBs in *B. subtilis* WB600BHM, the transformants containing a plasmid with cloned *eno* gene were grown in LB medium with 0.2% glucose at 37 °C. When the cell cultures reached O.D. 0.2, IPTG (1 mM) was added to induce the cloned enolase expression as described [1]. After 10 h of cultivation for *B. subtilis*, 10 mL of cells were collected by centrifugation, whole cell lysate and medium supernatant were prepared as described [1]. The cells were re-suspended in 5 mL of 10 mM Tris–HCl (pH 8.0) buffer containing 1 mM EDTA (TE buffer), followed by French Press to break the cells. The whole cell lysates were centrifuged at 12,000 $\times$ g for 10 min to remove cell debris and insoluble materials, and the supernatants were collected as soluble whole cell lysates. The samples were adjusted for protein amounts where necessary, using chromosomal enolase as internal control, and were mixed with the equal amount of 2 $\times$  sample buffer for immunoblot analysis [1]. *B. subtilis* enolase antibodies were used under condition in which *E. coli* enolase was not detectable.

### 2.3. Construction of Green Fluorescent Protein fusions with N-terminus of enolase

Green Fluorescent Protein (GFP) was amplified from pBAD-gfpuv (Clontech, Mountain View, CA) and the restriction sites *XbaI* and *SphI* were introduced at 5' and 3' sites of GFP, respectively. The PCR product was digested by *XbaI*/*SphI* and subcloned onto the corresponding sites of *E. coli*/*B. subtilis* shuttle vector pDG148 in which the cloned gene was placed under the control of P<sub>spac</sub> promoter, resulting in the plasmid pDGGFP. To construct the different lengths of N-terminus of enolase fusion with GFP, PCR-amplified N-terminus fragments were inserted into pDGGFP and resulted in nine different plasmids (SI Table 1). The sequences of the entire inserts in the pDGGFP-derived plasmids were confirmed by DNA sequencing.

### 2.4. Secretion of EnoBs and isolation of outer membrane vesicles in *E. coli*

The overnight cultures of *E. coli* DH5 $\alpha$  containing cloned EnoBs gene were inoculated into fresh LB medium with 0.2% glucose at 37 °C with shaking. When cell cultures reached O.D. 0.2, IPTG (1 mM) was added to induce the cloned enolase expression as described [1]. After 8 h cells were collected by centrifugation and the supernatant was treated as with *B. subtilis* above. The outer membrane vesicles (OMV) were pelleted by ultracentrifugation (100,000 rpm, 30 min, 4 °C) in a TLA-100.3 rotor (Beckman Max-XP), and washed with 0.5 M NaCl to remove loosely bound enolase with membranes. The extracellular proteins in the spent medium were precipitated using DOC–TCA method [18].

### 2.5. Immunoblots

Protein samples were analyzed by immunoblots as described [1]. The rabbit primary antibodies for *Bacillus* enolase, *E. coli* OmpA, SecA and SecY were from the laboratory stock and for GFP and

FLAG (F7425) were purchased from Sigma–Aldrich. Goat anti-rabbit alkaline-phosphatase-linked antibodies (Bio-Rad) were used as secondary antibodies.

## 2.6. Reagents and chemicals

All reagents and chemicals are reagent grades, purchased from commercial companies.

## 3. Results and discussion

### 3.1. The hydrophobic $\alpha$ -helix domain is important for enolase secretion in *Bacillus*

We previously showed that a hydrophobic EM domain (aa#110–118) (Fig. 1A) is important in the secretion of enolase: the deletion or EM replacement (EMR) of recombinant enolase was expressed as soluble forms but failed to secrete [1]. The EM domain is within a longer hydrophobic  $\alpha$ -helix (HH; aa #106–123). In this study we further examined the effects of the mutations in this domain and the surrounding residues on its secretion.

We constructed substitutions by glycine in the EM domain that provide the conformational flexibility and was less hydrophobic including  $^{110}\text{AIL} \rightarrow ^{110}\text{GGG}$ ,  $^{114}\text{VSG} \rightarrow ^{114}\text{GGG}$ ,  $^{116}\text{MAC} \rightarrow ^{116}\text{GGG}$  in pGTN-FLAG plasmids. Both the wild-type and the cloned FLAG-tagged enolases were detected by enolase antibodies; the former served as internal controls for amounts of chromosomal enolase secretion, and the later was larger due to FLAG-tag (which was confirmed with FLAG antibodies: data not shown, but see Fig. 2B). The expression/stability levels of some mutated soluble enolases were significantly affected by glycine-substitution mutations as compared to wild-type. Most were not detectable even in the whole cell fractions indicating that the mutated proteins were not stable, especially the  $^{110}\text{AIL} \rightarrow ^{110}\text{GGG}$  (Fig. 1B). There was a minor band detected from the  $^{116}\text{MAC}$  mutant, while  $^{114}\text{VSG}$  was expressed 2-fold lower than WT enolase (Fig. 2A), showing the differential levels of stability of glycine-mutated soluble enolases in the EM domain. However, none of these mutant enolases could

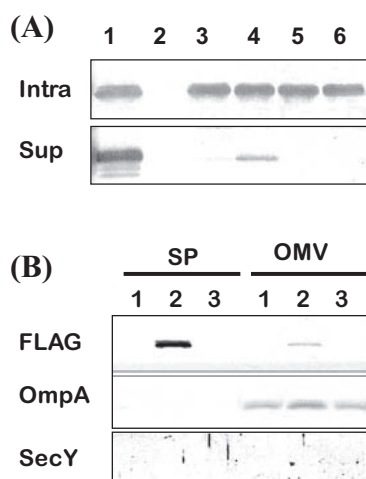
be detected extracellularly (Fig. 1B), indicating that the hydrophobicity or sequence specificity of the EM domain in enolase plays a critical role on the protein stability and/or its secretion in *B. subtilis*.

The EM domain is within a longer HH domain (Fig. 1A; SI Fig. 1). To further analyze how this helix affects the secretion of enolase, Gly-substituted mutants in the plasmids were constructed around the HH domain including  $^{103}\text{KKG}$ ,  $^{108}\text{GAN}$ ,  $^{119}\text{ARA}$ ,  $^{122}\text{AAD}$ , and  $^{125}\text{FLG}$ . We found that while wild-type chromosomal enolase secreted normally (which again was used as internal controls for the expression and secretion), mutations on  $^{108}\text{GAN}$ ,  $^{119}\text{ARA}$  and  $^{122}\text{AAD}$  affected secretion of mutated enolases (Fig. 1B). Similar to the EM  $^{110}\text{AIL}$  domain mutation, the mutated  $^{108}\text{GAN}$  protein was undetectable in either intra- or extracellular portions. Intracellular soluble mutated  $^{119}\text{ARA}$  and  $^{122}\text{AAD}$  proteins showed 3–4 folds lower than WT. On the other hand,  $^{103}\text{KKG}$  and the  $^{125}\text{FLG}$  mutation on both ends of the helix showed similar expression and secretion levels as the wild-type (Fig. 1B). These data indicates that the mutations on  $^{103}\text{KKG}$  and  $^{125}\text{FLG}$  outside the HH domain do not affect the export of enolase, unlike those in the HH domain. The mutations within, but not outside, the HH domain demonstrated the importance of this helix on enolase secretion.

We further examine some other regions around this domain in enolase secretion. We constructed another enolase mutant following the HH domain on pGTN-FLAG. The small helix was disrupted by substituting a proline residue (Q132P). The mutated enolase was expressed and secreted normally as chromosomal enolase (Data not shown). Thus, the hydrophobic helix plays an important role on enolase secretion. It has been reported that positive-charged amino acid residues could provide additional interactions between membrane proteins and negatively charged phospholipids [19–21]. There are two positively charged residues (K103 and K105) around and one (R120) in the EM domain and these residues are highly conserved in enolases of different species (not shown). We constructed three mutations (K103G, K105G & R120G) to determine the importance of these EM flanking charged residues in its secretion. The results showed that glycine-substituted single mutations had no effect on exporting enolase (data not shown). We further constructed three double mutations of enolase that two of the three positively charged residues had been changed to glycine, and resulted in three double mutants including K103G + K105G, K103G + R120G, and K105G + R120G. None of the mutants affected secretion (Fig. 1B, right panel  $^{103}\text{KKG}$ , and data not shown). Taken together, the positive-charge residues within this region did not affect enolase secretion in *B. subtilis*. Previously, Chen et al. [22] reported that positively charged residues in *Bacillus* may not be essential for export as longer hydrophobic sequences may compensate the lack of positively charged residues on *Bacillus* signal peptides.

### 3.2. Secretion of *Bacillus* enolase in *E. coli*

We have shown that deletion or replacement of this entire helix reduces the secretion of enolase in *B. subtilis*. Since it has been reported previously that *E. coli* enolase can be exported out of the cells into the medium [12], so we also examined EnoBs secretion and the importance of the highly conserved HH domain on enolase secretion in *E. coli*. We found that the cloned EnoBs could also be secreted in *E. coli* (Fig. 2A, lane 1). As shown in Fig. 2A comparing to wild-type EnoBs, the EM replacement (lane 3) and mutations on  $^{114}\text{VS}$  and  $^{116}\text{MAC}$  with glycine (lanes 5, 6) totally abolished the secretion of mutated EnoBs while AIL mutant was found to be secreted at a very low level (lane 4). These data demonstrate the importance of the HH domain on EnoBs secretion in both *B. subtilis* and *E. coli*. Taken together, these results support the hypothesis that the  $\alpha$ -helix region plays an important role in the export process.



**Fig. 2.** HH domain is important for *B. subtilis* enolase secretion in *E. coli*. (A) Samples of soluble cell lysates (Intra) and supernatant (Sup) were analyzed by immunoblots with EnoBs antibodies under conditions *E. coli* enolase was not detected. Lane 1, wild-type EnoBs; lane 2, DH5 $\alpha$  (pDG148) as a control for non-cross reaction with *E. coli* intrinsic enolase; lane 3, EMR [1]; lane 4, AIL  $\rightarrow$  GGG; lane 5, GVS  $\rightarrow$  GGG; lane 6, MAC  $\rightarrow$  GGG. (B) Immunoblot analyses of extracellular supernatant (SP) and spontaneously released outer membrane vesicles (OMV) using the specific antisera as indicated: FLAG is the marker for EnoBs; OmpA is a marker for outer membranes; and SecY is a marker for cytoplasmic membranes. Lane 1, DH5 $\alpha$ ; lane 2, EnoFLAG (pEnoFLAG); lane 3, VS  $\rightarrow$  GG (pVSFLAG).



In Gram-negative bacteria, the spontaneously released outer membrane vesicles (OMV) have been found to carry some 'secreted' proteins without typical cleavable signal peptides. Balsalobre et al. reported that a major portion of the secreted *E. coli*  $\alpha$ -hemolysin was located inside the released OMVs [23]. Ferrari et al. showed that enolase of group B *Neisseria meningitidis* could be identified from both detergent-derived OMVs and the spontaneously released OMVs [24], though the amounts of the enolase in the released OMVs were not quantified. We determined how much the exported FLAG-tagged EnoBs was associated with OMVs released in *E. coli*. To verify the nature of the isolated *E. coli* OMV, the specific inner (SecY) and outer membrane (OmpA) protein marker were monitored. SecY could not be detected in either the supernatant or the isolated OMVs; the quality of the OMV was indicated with the OmpA as expected (Fig. 2B). Under such conditions, only 2% of EnoBs was detected in the OMV fraction. Washing the OMV with 0.5 M NaCl further removed loosely bound enolase to a negligible amount (0.7%). Thus, it is not likely that EnoBs is exported via OMVs. Rather, the export is via an unknown mechanism for EnoBs to cross the *E. coli* membranes into the medium. We have previously shown that the secretion of EnoBs in *B. subtilis* is also unlikely via membrane vesicles [1].

### 3.3. The signal necessary for enolase secretion

The sequence alignment of *E. coli* and *B. subtilis* enolases revealed that the HH domain described above is highly conserved [1]. Based on the notion that a hydrophobic  $\alpha$ -helix is important for interactions between membrane proteins and the membrane [25,26], we hypothesized that the enolase internal HH domain may play an important role for targeting itself to the membrane. To test this possibility, a plasmid pDG148 harboring the reporter *gfp* gene was constructed, resulting in the plasmid pDGGFP. Various regions of the 140 residues N-terminus of EnoBs (Fig. 1A) was fused onto pDGGFP to determine which domain can be used as a signal to direct GFP across membranes and to secrete into the growth medium. We further dissected the N-terminus of enolase to determine the minimal length for carrying GFP to export. The HH domain and 8 other hybrid proteins were constructed with different lengths of EnoBs N-terminus and fused to pDGGFP: p $\Delta$ N16GFP ( $\Delta$ 1–16), p $\Delta$ N40GFP ( $\Delta$ 1–40), p $\Delta$ N54GFP ( $\Delta$ 1–54), p $\Delta$ N81GFP ( $\Delta$ 1–81), p $\Delta$ N99GFP ( $\Delta$ 1–99), pN126GFP, pN118-GFP, pN109-GFP, and pSp-GFP (106–123, Sp; putative signal).

We found that while GFP itself cannot be secreted into the medium in *B. subtilis* (Fig. 3, lane 1), the whole N-terminus fusion (N140-GFP) was capable of directing GFP fusion protein across the *B. subtilis* membranes (Fig. 3, lane 2). Previously, Gan et al. [27] have predicted a transmembrane domain on *Echinococcus*

*granulosus* enolase from aa<sub>104–124</sub> as a possible signal on how enolase exports to cell surface. However, we found that such putative HH signal (#106–123) itself was insufficient to direct GFP secretion (Fig. 3, lane 6). Moreover, fusions with GFP of various N-terminus deletions up to the HH domains of EnoBs showed that none of the constructs could direct GFP secretion, and many in fact were unstable in the cytoplasm as revealed by immunoblot analysis (data not shown). In these assays, the amounts of SecA in the cytosol were used as an internal control (Fig. 3, upper panel), and the EnoBs were used as controls for expression and secretion (Fig. 3, lower panel). There are three  $\beta$ -sheets were predicted at N-terminus; it appears that modifications of these  $\beta$ -sheets may destabilize the structure. As a result only very few recombinant GFPs can be recovered as stable proteins in the intracellular compartment.

In contrast, truncation from the C-terminal end of the 140 residues of N-terminus showed a normal expression, but no secretion was observed. The constructs of GFP fusions with increasing N-terminus of EnoBs were stable in the cells (Fig. 3; Intracellular, lanes 2–6). Surprisingly, even the construct with N-terminal 126 residues (N126-GFP) that includes the HH domain could not serve as the signal for directing the secretion of the reporter-GFP (Fig. 3, extracellular, lane 3); only with the additional 14 residues N140aa fusion (N140-GFP) could be secreted (lane 2). These results showed that the whole 140-residues N-terminus domain of EnoBs (Fig. 1A) is needed as a signal to direct fused GFP to the medium. Thus even though the HH domain is necessary, it is not sufficient to direct the EnoBs secretion (Fig. 1A), even with the N-terminus up to HH domain; it requires the additional  $\beta$ -sheet residues beyond the HH domain (Fig. 1A) to function as a secretion signal. It appears that the long N-terminus 140 aa of enolase is structurally unique and must be maintained to function as a signal for secretion (Fig. 1A). Similarly to *B. subtilis* enolase, a yeast enolase N-terminal fragment of 169 amino acids can target a cytoplasmic invertase to the cell surface [5].

Taken together, the HH domain of enolase is required for its secretion in *B. subtilis* and *E. coli*, suggesting that this is a general phenomenon. To export protein across the membrane, the N-terminus 140 residues of enolase is needed to promote the secretion of a reporter such as GFP fusion. These results showed that the HH domain is necessary, but not sufficient, to serve as a non-cleavable signal for the secretion. Although the extracellular function of enolase is not yet identified, it is possible that *B. subtilis* enolase has some unknown extracellular functions which may be required during the stationary phase or for bacterial survival.

### Acknowledgments

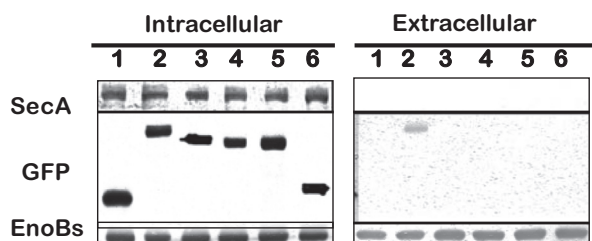
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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2014.03.032>.

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**Fig. 3.** Whole N-terminus domain of EnoBs is necessary as a signal for the secretion of recombinant GFP. Series deletion of N-terminus from its C-terminal were prepared and fused with GFP. Recombinant GFPs were expressed in *B. subtilis*. Soluble intracellular and extracellular samples were analyzed for GFP (middle panel) were detected using immunoblots with antibodies. SecA (upper panel) was used as a marker for no secretion, and chromosomal enolase (lower panel) as a marker for expression and secretion. Lane 1, GFP; lane 2, N140-GFP; lane 3, N126-GFP; lane 4, N118-GFP; lane 5, N109-GFP; lane 6, #106–123Eno-GFP.

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