

# Implication of membrane localization of target mRNA in the action of a small RNA: mechanism of post-transcriptional regulation of glucose transporter in *Escherichia coli*

Hiroshi Kawamoto, Teppei Morita, Ayumi Shimizu, Toshifumi Inada, and Hiroji Aiba<sup>1</sup>

Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa, Nagoya 464-8602, Japan

Accumulation of phosphosugars such as glucose-6-phosphate causes a rapid degradation of *ptsG* mRNA encoding the major glucose transporter IICB<sup>Glc</sup> in an RNase E/degradosome-dependent manner. The destabilization of *ptsG* mRNA is caused by a small antisense RNA (SgrS) that is induced by phosphosugar stress. In this study, we analyzed a series of *ptsG*–*crp* translational fusions to identify the mRNA region required for the rapid degradation of *ptsG* mRNA. We found that the *ptsG*–*crp* mRNA is destabilized in response to phosphosugar stress when it contains the 5' portion of *ptsG* mRNA corresponding up to the first two transmembrane domains (TM1 and TM2) of IICB<sup>Glc</sup>. The destabilization of *ptsG*–*crp* mRNA was largely eliminated by frameshift mutations in the transmembrane region. The IICB<sup>Glc</sup>–CRP fusion proteins containing more than two transmembrane domains were localized at the membrane. The efficient destabilization of *ptsG*–*crp* mRNA was restored when TM1 and TM2 of IICB<sup>Glc</sup> were replaced by part of the LacY transmembrane region. We conclude that the membrane-targeting property of IICB<sup>Glc</sup> protein rather than the particular nucleotide or amino acid sequence is required for the efficient degradation of *ptsG* mRNA in response to metabolic stress. The stimulation of *ptsG*–*crp* mRNA degradation was completely eliminated when either the *hfq* or *sgrS* gene is inactivated. The efficient mRNA destabilization was observed in the absence of membrane localization when translation was reduced by introducing a mutation in the ribosome-binding site in the cytoplasmic *ptsG*–*crp* mRNA. Taken together, we conclude that mRNA localization to the inner membrane coupled with the membrane insertion of nascent peptide mediates the Hfq/SgrS-dependent *ptsG* mRNA destabilization presumably by reducing second rounds of translation.

[**Keywords:** mRNA localization; mRNA degradation; protein targeting; glucose transporter; small RNA; Hfq]

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External glucose is transported and phosphorylated by the phosphoenolpyruvate (PEP): sugar phosphotransferase system (PTS) in bacteria (Meadow et al. 1990; Postma et al. 1996; Tchieu et al. 2001). The PTS consists of two common cytoplasmic proteins, enzyme I and HPr, and a series of sugar-specific enzyme II complexes (EIIs). The glucose-specific EII (glucose transporter) of *Escherichia coli* consists of a cytoplasmic component IIA<sup>Glc</sup> and a membrane component IICB<sup>Glc</sup>. The phosphoryl group of PEP is sequentially transferred to enzyme I, to HPr, to IIA<sup>Glc</sup>, to IICB<sup>Glc</sup>, and finally to glucose as it is translocated across the membrane. The resulting glucose 6-phosphate (G6P) is metabolized by the Embden-

Meyerhof glycolytic pathway and by the pentose-phosphate pathway to produce numerous intermediary metabolites and energy (Fraenkel 1996).

Recent studies have demonstrated that the *ptsG* gene encoding IICB<sup>Glc</sup> is regulated in quite an intriguing manner at both transcriptional and post-transcriptional levels depending on physiological conditions (Plumbridge 2002). The *ptsG* transcription is regulated simultaneously by two global transcription factors, CRP (cAMP receptor protein)-cAMP and Mlc, in response to glucose availability (Kimata et al. 1997, 1998). Glucose uptake induces *ptsG* transcription by modulating the Mlc-mediated negative regulatory pathway (Kimata et al. 1998; Plumbridge 1998). The glucose transporter itself modulates the localization and activity of Mlc. Dephosphorylated IICB<sup>Glc</sup> generated by glucose uptake binds Mlc, resulting in sequestration of this global transcription factor to the membrane (Lee et al. 2000; Tanaka et al. 2000;

<sup>1</sup>Corresponding author.

E-MAIL i45346a@nucc.cc.nagoya-u.ac.jp; FAX 81-52-789-3001.

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Nam et al. 2001). The membrane localization itself but not binding to IICB<sup>Glc</sup> is directly responsible for the inactivation of Mlc (Seitz et al. 2003; Tanaka et al. 2004).

In addition to the transcriptional control, the expression of *ptsG* is regulated at the post-transcriptional level in response to glycolytic flux. We found previously that the *ptsG* mRNA is dramatically destabilized when the glycolytic pathway is blocked either by mutations at its early stages or by treatment with a nonmetabolizable glucose analog (Kimata et al. 2001). The physiological relevance of this novel post-transcriptional regulation of *ptsG* expression is rather obvious because it prevents glucose uptake to avoid too much accumulation of hexose phosphates that are potentially toxic to cells (El-Kazzaz et al. 2004). On the other hand, the mechanism by which the block of the glycolytic pathway leads to the destabilization of *ptsG* mRNA is apparently complicated and intriguing.

Several important aspects have been uncovered concerning the mechanism and/or pathway of the destabilization of *ptsG* mRNA in response to the metabolic stress. First, the major endoribonuclease RNase E is primarily responsible for the destabilization of *ptsG* mRNA under the stress condition (Kimata et al. 2001). Second, the accumulation of G6P, fructose 6-phosphate, or  $\alpha$ -methylglucoside 6-phosphate somehow triggers the RNase E-mediated-destabilization of *ptsG* mRNA (Morita et al. 2003). Third, the destabilization of *ptsG* mRNA in response to stress induced by the intracellular accumulation of phosphosugars such as G6P is dependent on enolase, a major component of the RNase E-based degradosome (Morita et al. 2004). Fourth, the destabilization of *ptsG* mRNA is dependent on an RNA chaperone Hfq (Morita et al. 2004). Finally, an Hfq-binding small RNA, SgrS, mediates the destabilization of *ptsG* mRNA (Vanderpool and Gottesman 2004). It has been shown that SgrS is induced in response to phosphosugar accumulation resulting in the degradation of *ptsG* mRNA presumably through SgrS-*ptsG* pairing (Vanderpool and Gottesman 2004).

In spite of this progress, several important questions remain unanswered regarding the mechanism by which the *ptsG* mRNA is destabilized in response to phosphosugar stress. In particular, little is known about the features of *ptsG* mRNA that are responsible for the destabilization of mRNA. In this paper, we addressed the question of what features of *ptsG* mRNA are required for the rapid degradation of *ptsG* mRNA. We found that the region from positions +70 to +319 relative to the *ptsG* transcriptional start site is sufficient for the message destabilization. This region includes the 3' one-third of the untranslated leader region corresponding to the predicted target for SgrS and the first 70 codons of *ptsG*. Mutational studies demonstrated that the membrane-targeting property of the translated protein is required for the rapid degradation of *ptsG* mRNA in response to phosphosugar stress. The data presented in this paper suggest that mRNA localization to the inner membrane coupled with the membrane insertion of nascent peptide allowed Hfq/SgrS to act efficiently on the *ptsG* mRNA

by occluding subsequent rounds of translation leading to the RNase E-dependent message degradation.

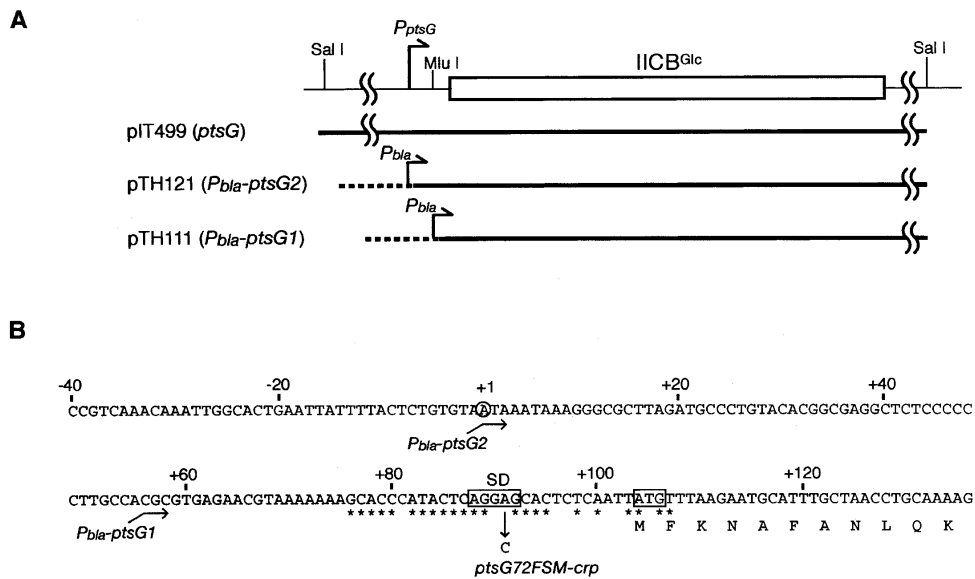
## Results

### *The plasmid-derived ptsG mRNA is destabilized in an RNase E-dependent manner by phosphosugar stress*

We used the cloned *ptsG* gene to analyze the features of *ptsG* mRNA responsible for rapid degradation in response to phosphosugar stress. We first examined the expression of *ptsG* in wild-type cells harboring pIT499 carrying the intact *ptsG* gene (Fig. 1). Total RNAs from cells with and without pIT499 grown in LB medium containing glucose were analyzed by Northern blotting. Semiquantitative analyses revealed that the level of *ptsG* mRNA in wild-type cells harboring pIT499 was more than 10-fold higher than that in cells without the plasmid (Fig. 2A, lanes 1–4). Then, we examined the expression of *ptsG* from pIT499 in  $\Delta$ *pgi* cells lacking phosphoglucose isomerase. The *ptsG* mRNA was destabilized, resulting in a dramatic decrease in the level of the full-length mRNA (Fig. 2A, lane 5). The reduction of *ptsG* mRNA in  $\Delta$ *pgi* cells harboring pIT499 was abolished at 42°C but not at 30°C when the *rne* allele (*ams-1*) encoding a temperature-sensitive RNase E was introduced (Fig. 2B). These results imply that the *ptsG* mRNA generated from pIT499 was destabilized in an RNase E-dependent manner in response to G6P accumulation. The rapid degradation of *ptsG* mRNA was also observed when the wild-type cells harboring pIT499 were grown in LB medium containing  $\alpha$ -methylglucoside ( $\alpha$ MG) (Fig. 2C) as observed previously in cells without the plasmid (Kimata et al. 2001).

### *The first two-thirds of the ptsG leader is dispensable for the mRNA destabilization*

The *ptsG* mRNA possesses a 5'-untranslated leader region (5'-UTR) of 103 nt preceding the structural gene encoding IICB<sup>Glc</sup> (Fig. 1B). It is likely that certain structural features of the *ptsG* promoter and/or leader region would be involved in the destabilization of *ptsG* mRNA. Recently, we demonstrated that the *ptsG* mRNA exhibited the normal response to G6P accumulation when the *ptsG* promoter was replaced with a foreign *bla* promoter on the chromosome (Morita et al. 2004). This *ptsG* allele (*P<sub>bla</sub>-ptsG2*) produces an extended form of *ptsG* mRNA in which several nucleotides derived from the *bla* region are attached to the 5'-end of the wild-type *ptsG* mRNA (see Fig. 1). This implies that the *ptsG* promoter is not required for the destabilization of *ptsG* mRNA and that the modification of the original 5'-end of *ptsG* mRNA does not affect the stability control by phosphosugar stress. We confirmed this by showing that the mRNA derived from the cloned *P<sub>bla</sub>-ptsG2* gene on plasmid pTH121 is normally destabilized in the presence of  $\alpha$ MG (Fig. 3, lanes 1,2). Previously, we constructed a plasmid pTH111 in which the *ptsG* gene lacking the first 56 nt of

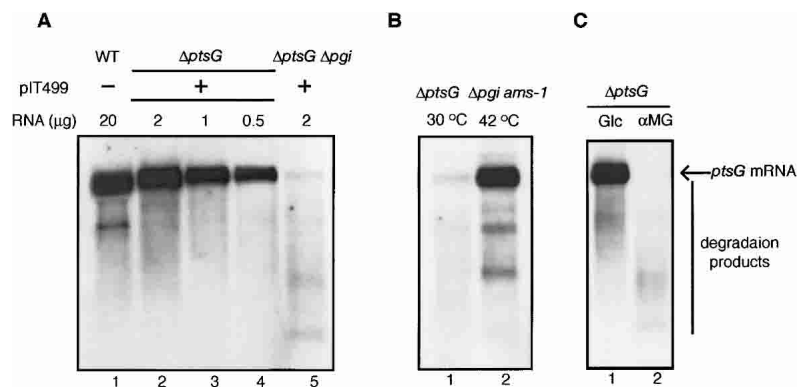


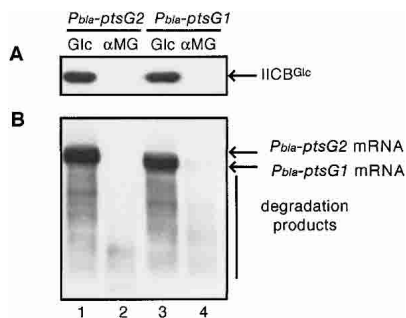
**Figure 1.** (A) The *ptsG* gene and its variants. The map of the *ptsG* region is shown at the top along with relevant restriction sites. The open box represents the coding region of IICB<sup>Glc</sup>. The thick lines below the map indicate the cloned regions on plasmids. The arrows represent the start and direction of transcription from two promoters ( $P_{ptsG}$  and  $P_{bla}$ ). Plasmid pIT499 carries the 3.2-kb fragment containing the intact *ptsG* gene with the native promoter ( $P_{ptsG}$ ). Plasmid pTH121 carries the  $P_{bla}$ -*ptsG2* gene in which the *ptsG* gene with its entire leader region is under the control of  $P_{bla}$ , while pTH111 carries the  $P_{bla}$ -*ptsG1* gene in which the *ptsG* gene lacks the 5' portion up to +56 of the leader region. (B) Nucleotide sequence around the *ptsG* leader region. The nucleotide corresponding to the transcription start site (+1) of  $p_{ptsG}$  is encircled. Numbering is relative to the transcription start site. Arrows show the 5'-ends of  $P_{bla}$ -*ptsG2* and  $P_{bla}$ -*ptsG1*. The Shine-Dalgarno sequence (SD) and the translation initiation codon are boxed. The amino acid sequence of the N terminus of IICB<sup>Glc</sup> is shown below the nucleotide sequence. The nucleotides complementary to SgrS RNA proposed by Vanderpool and Gottesman (2004) are shown by asterisks. The vertical arrow denotes the nucleotide substitution in the *ptsG-crp* fusion gene (see Fig. 6).

5'-UTR is under the control of the *bla* promoter (Takahashi et al. 1998). The *bla* promoter to drive the *ptsG* in pTH111 is the same as that used in *ptsG2*. The *ptsG* allele in pTH111 was designated as  $P_{bla}$ -*ptsG1*. By using this construct, we examined whether the 5' portion of the *ptsG* 5'-UTR is required for the destabilization of *ptsG* mRNA. The 5'-truncated *ptsG* mRNA was stably expressed when cells harboring pTH111 were grown in

the presence of glucose while it was rapidly degraded when cells were grown in the presence of  $\alpha$ MG (Fig. 3, lanes 3,4). Further truncation of the 5'-UTR at least up to +70 affected neither the expression of *ptsG* mRNA nor its response to phosphosugar stress (H. Kawamoto, T. Morita, and H. Aiba, unpubl.). We conclude that the first 70 nt of the *ptsG* UTR is not required for the stability control of *ptsG* mRNA by phosphosugar stress.

**Figure 2.** Expression and response to phosphosugar stress of plasmid-borne *ptsG*. (A) IT1568 (wild-type) (lane 1), AS16 ( $\Delta ptsG$ ) harboring the *ptsG* plasmid pIT499 (lanes 2-4), and AS18 ( $\Delta ptsG \Delta pgi$ ) harboring pIT499 (lane 5) were grown to  $A_{600} = 0.5$ . Glucose was added at a final concentration of 1% and incubation was continued for 20 min. Total RNAs were prepared, and indicated amounts of RNA samples were subjected to Northern blot analysis using the *ptsG* probe. (B) AS20 ( $\Delta ptsG \Delta pgi \Delta ms-1$ ) harboring pIT499 was grown at 30°C to  $A_{600} = 0.5$ . Glucose was added at a final concentration of 1%, and incubation was continued at the indicated temperature for 20 min. Total RNAs were prepared and 2  $\mu$ g of RNA samples was subjected to Northern blot analysis using the *ptsG* probe. (C) AS16 harboring pIT499 was grown to  $A_{600} = 0.5$ . Glucose (Glc) or  $\alpha$ -methylglucoside ( $\alpha$ MG) was added at a final concentration of 1%, and incubation was continued for 20 min. Total RNAs were prepared and 2  $\mu$ g of RNA samples was subjected to Northern blot analysis using the *ptsG* probe.





**Figure 3.** Expression and response to phosphosugar stress of the variant *ptsG* mRNAs. (A) AS16 harboring the *P*<sub>bla</sub>-*ptsG2* plasmid pTH121 (lanes 1,2) or the *P*<sub>bla</sub>-*ptsG1* plasmid pTH111 (lanes 3,4) were grown with 1% Glc (lanes 1,3) or αMG (lanes 2,4) to  $A_{600} = 0.6$ . Total proteins were prepared and samples equivalent to 0.0075  $A_{600}$  units were subjected to Western blot analysis using anti-IIB antibody. (B) Cells were grown to  $A_{600} = 0.5$ . Glc (lanes 1,3) or αMG (lanes 2,4) was added at a final concentration of 1%, and incubation was continued for 20 min. Total RNAs were prepared and 3 μg of RNA samples was subjected to Northern blot analysis using the *ptsG* probe.

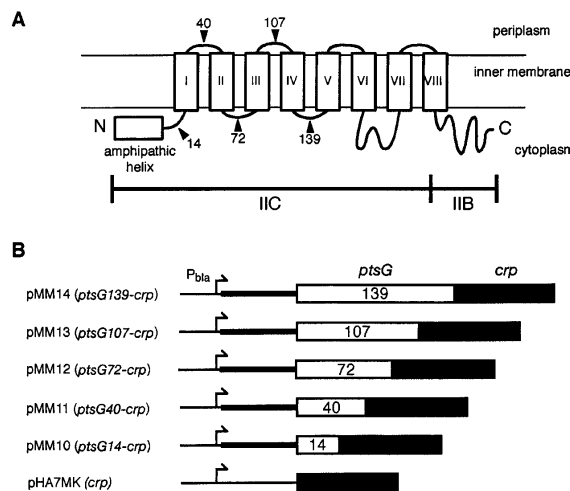
#### The region up to the transmembrane segment II is sufficient for the response to phosphosugar stress

To define further the features of *ptsG* mRNA responsible for the rapid degradation in response to phosphosugar stress, a series of *ptsG*-*crp* translational fusions encoding hybrid proteins with N-terminal regions of IICB<sup>Glc</sup> and the entire CRP were constructed (Fig. 4). IICB<sup>Glc</sup> consists of a transmembrane IIC domain of ~400 residues and a cytoplasmic IIB domain of ~90 residues (Buhr and Erni 1993). The IIC domain contains eight transmembrane segments (TM) along with an N-terminal amphipathic sequence (Buhr and Erni 1993). The fusions were designed to produce IIC-CRP proteins in which the N-terminal 14, 40, 72, 107, and 139 residues of IICB<sup>Glc</sup> were joined to the CRP ORF. These positions correspond to those just after the N-terminal amphipathic sequence, TM-I, TM-II, TM-III, and TM-IV, respectively. Cells harboring a control plasmid pHA7MK expressing the *crp* mRNA or each of five different plasmids carrying *ptsG*-*crp* fusions were grown in the presence of either glucose or αMG. Total RNAs and proteins were prepared and analyzed by Northern and Western blotting, respectively (Fig. 5). When cells were grown in the presence of glucose, all fusion genes produced significant amounts of hybrid proteins and mRNAs (Fig. 5A,B, lanes 3,5,7,9,11). On the other hand, the levels of hybrid proteins and *ptsG*-*crp* mRNAs were dramatically reduced when cells harboring pMM12, pMM13, or pMM14 were grown in the presence of αMG (Fig. 5A,B, lanes 4,6,8,10,12). The reduction of hybrid proteins and *ptsG*-*crp* mRNAs by αMG were no longer observed when RNase E was inactivated (data not shown). These results clearly indicate that the *ptsG*-*crp* chimeric mRNAs were rapidly degraded in response to phosphosugar stress as in the case of the native *ptsG* mRNA. The presence of αMG weakly reduced the expression of the *ptsG*-*crp* mRNAs when

cells harboring pMM10 or pMM11, while it did not affect the expression of *crp* mRNA. Taken together, we conclude that the information responsible for the rapid degradation in response to phosphosugar stress is contained within the region from positions +70 to +349 of the *ptsG* mRNA. This region consists of the 3' one-third of the leader region (+70 to +103) including the Shine-Dalgarno sequence and the region corresponding to the N-terminal portion of IICB<sup>Glc</sup> containing the first two transmembrane segments along with the amphipathic sequence (+103 to +349). The nucleotide position +349 corresponds to amino acid position 72.

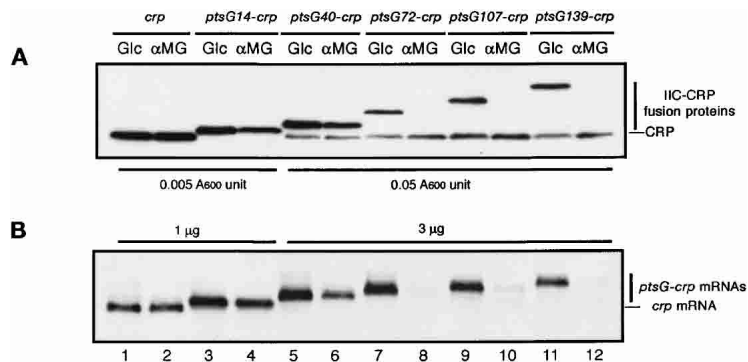
#### The membrane-targeting property of the transmembrane segments is important for the rapid degradation of *ptsG*-*crp* mRNA

It has been proposed that a 32-nt-long region from positions +77 to +107 of *ptsG* mRNA is the direct target of SgrS RNA because it is partially complementary to that of SgrS at nucleotide positions 157-187 (Vanderpool and Gottesman 2004). Our observation that the *ptsG14*-*crp* mRNA containing +70 to +142 region is moderately reduced in response to the phosphosugar stress is consistent with the proposed base-pairing model by Vanderpool and Gottesman (2004). However, it is also clear that this region alone is not sufficient for the efficient destabilization of *ptsG* mRNA. To examine the role of the



**Figure 4.** (A) Membrane topology of IICB<sup>Glc</sup>. The deduced membrane topology of IICB<sup>Glc</sup> is taken from Buhr and Erni (1993). The portions corresponding to IIC and IIB domain are labeled. The N-terminal amphipathic helix and eight transmembrane (TM) domains are represented as rectangles. Arrowheads represent the sites (amino acid residue number of IICB<sup>Glc</sup>) to which the *crp* gene is fused. (B) Schematic representation of *ptsG*-*crp* translational fusion genes. Open and filled rectangles represent *ptsG* and *crp* coding sequences, respectively. The numbers in open rectangles represent amino acid positions of IICB<sup>Glc</sup>. The thick lines represent the part of the *ptsG* leader region. Arrows represent the direction of transcription driven by *P*<sub>bla</sub>.

**Figure 5.** Expression and response to phosphosugar stress of *ptsG*-*crp* translational fusions. (A) IT1568 harboring pHA7MK (lanes 1,2), pMM10 (lanes 3,4), pMM11 (lanes 5,6), pMM12 (lanes 7,8), pMM13 (lanes 9,10), or pMM14 (lanes 11,12) was grown with 1% Glc or  $\alpha$ MG to  $A_{600} = 0.6$ . Total proteins were prepared, and indicated amounts of samples were subjected to Western blot analysis using anti-CRP antibody. (B) Cells were grown to  $A_{600} = 0.5$ . Glc or  $\alpha$ MG was added at a final concentration of 1%, and incubation was continued for 20 min. Total RNAs were prepared and indicated amounts of RNA samples were subjected to Northern blot analysis using the *crp* probe.



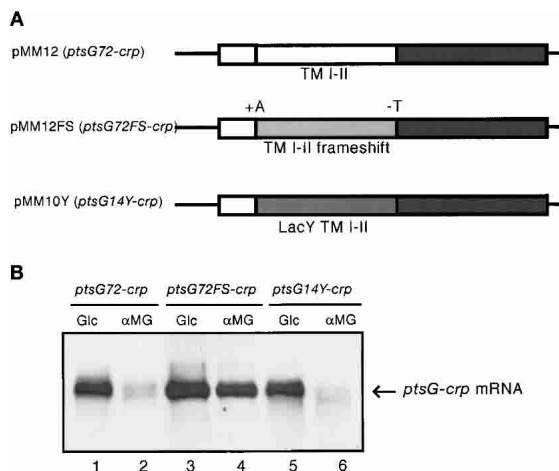
transmembrane region in the mRNA destabilization, we introduced one nucleotide insertion at position +145 and one nucleotide deletion at position +349 in the *ptsG72*-*crp* fusion gene to generate the *ptsG72FS*-*crp* gene (Fig. 6A). These frameshift mutations, which create no intervening nonsense codons, cause only a slight change in the nucleotide sequence but a dramatic change in the amino acid sequence of TM-I and TM-II. Northern blot analysis revealed that the level of mRNA produced from the *ptsG72FS*-*crp* gene was only moderately reduced by  $\alpha$ MG (Fig. 6B, lanes 4,5). This suggests that the amino acid sequence but not nucleotide sequence of the TM-I/TM-II region is required for the efficient destabilization of *ptsG* mRNA.

The next question is how the amino acid sequence of the TM-I/TM-II region participates in the marked destabilization of *ptsG* mRNA. One possibility is that a particular amino acid sequence within this region somehow plays a role in the regulation of mRNA stability. Alternatively, the membrane-targeting property of the transmembrane segments may be needed for the destabilization of *ptsG* mRNA. To examine these possibilities, the region corresponding to TM-I and TM-II in the *ptsG72*-*crp* was replaced with that corresponding to two transmembrane segments derived from LacY (LacY TM-I–TM-II) to construct the *ptsG14Y*-*crp* allele (Fig. 6A). Northern blot analysis indicated that the mRNA produced from the *ptsG14Y*-*crp* allele was stably expressed in the presence of glucose, while it was markedly destabilized in the presence of  $\alpha$ MG (Fig. 6B, lanes 5,6). This strongly suggests that the membrane-targeting property rather than a particular amino acid sequence itself within TM-I and TM-II of IICB<sup>Glc</sup> is crucial for the rapid degradation of *ptsG* mRNA.

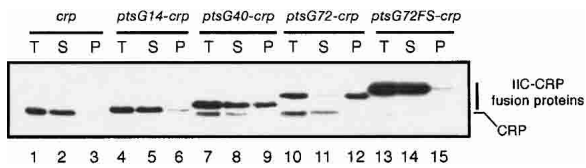
#### Proteins generated from mRNAs sensitive to phosphosugar stress are membrane-associated

To confirm the role of the membrane-targeting property of the transmembrane segments in the rapid degradation of *ptsG* mRNA, we performed a cell fractionation assay by Western blotting using anti-CRP antibody. As expected, the wild-type CRP was found exclusively in the soluble cytoplasmic fraction (Fig. 7, lanes 1–3). Under the same condition, IIC72–CRP possessing TM-I and TM-II generated from pMM12 appeared predominantly

in the membrane fraction (Fig. 7, lanes 10–12), while IIC14–CRP lacking the transmembrane segments generated from pMM10 appeared predominantly in the soluble fraction (Fig. 7, lanes 4–6). IIC40–CRP possessing only TM-I was found in both soluble and membrane fractions (Fig. 7, lanes 7–9). As expected, IIC72FS–CRP generated from pMM12FS appeared almost exclusively in the cytoplasmic fraction (Fig. 7, lanes 13–15). These results again support our claim that the rapid degradation of *ptsG* mRNA in response to phosphosugar stress is tightly coupled with the membrane-targeting property of translated proteins. In addition, it should be noted that the expression levels of fusion proteins attached to membrane are greatly reduced compared to those of cytoplasmic



**Figure 6.** Expression and response to phosphosugar stress of *ptsG*-*crp* translational fusion variants. (A) Schematic representation of altered *ptsG*-*crp* translational fusions. The rectangles in white and black represent the *ptsG* and *crp* coding regions, respectively. The rectangles in light and dark gray represent TM I–II of IICB<sup>Glc</sup> with frameshift mutations and LacY TM I–II, respectively. The frameshift mutations just before and after TM I–II are shown as +A (insertion) and –T (deletion) in the *ptsG72FS*-*crp* gene. (B) Northern blot analysis of altered *ptsG*-*crp* mRNAs. IT1568 harboring pMM12 (lanes 1,2), pMM12FS (lanes 3,4), or pMM10Y (lanes 5,6) was grown to  $A_{600} = 0.5$ . Glc or  $\alpha$ MG was added at a final concentration of 1%, and incubation was continued for 20 min. Total RNAs were prepared and 3  $\mu$ g (lanes 1,2,5,6) or 1  $\mu$ g (lanes 3,4) of RNA samples was subjected to Northern blot analysis using the *crp* probe.



**Figure 7.** Subcellular localization of proteins. IT1568 harboring pHA7MK (lanes 1–3), pMM10 (lanes 4–6), pMM11 (lanes 7–9), pMM12 (lanes 10–12), or pMM12FS (lanes 13–15) was grown with 1% Glc to  $A_{600} = 0.6$ . Total proteins (T) were prepared and fractionated by centrifugation to obtain supernatant (S) and pellet (P). The fractionated protein samples were subjected to Western blot analysis using anti-CRP antibody. The proteins samples used are 0.005  $A_{600}$  unit (lanes 1–6), 0.075  $A_{600}$  unit (lanes 7–12), and 0.01  $A_{600}$  unit (lanes 13–15).

mic proteins (e.g., cf. lanes 10 and 13 in Fig. 7). The reduced expression of membrane proteins could be due to either inefficient translation or reduced stability of proteins. However, we observed no significant difference in the protein stability among these proteins (data not shown). This suggests that the translation of membrane proteins is less efficient.

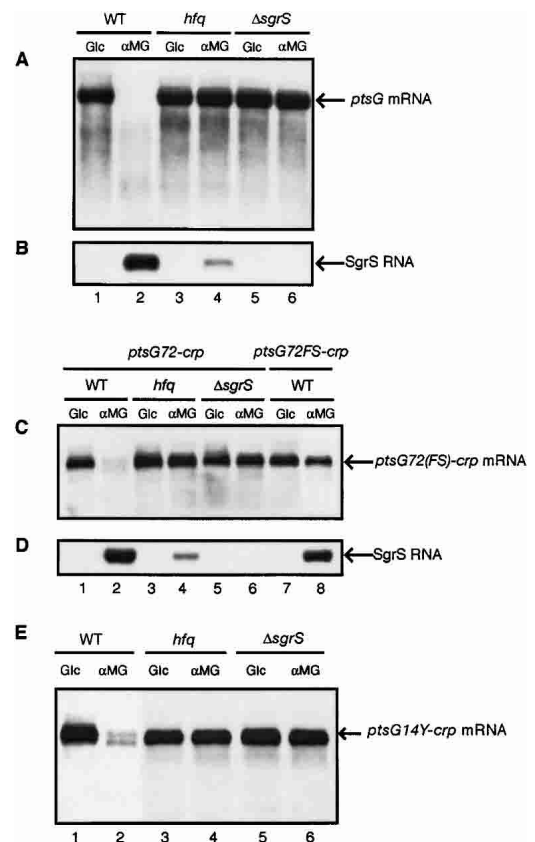
#### The destabilization of *ptsG* mRNA in response to phosphosugar stress is dependent on Hfq and a small RNA (*SgrS*)

It is highly possible that Hfq is involved in the destabilization of *ptsG* mRNA in response to phosphosugar stress because this RNA chaperon has been shown to participate in post-transcriptional regulation by affecting stability and/or translation of several mRNAs often with small RNAs (Gottesman 2002, 2004; Storz et al. 2004; Valentin-Hansen et al. 2004). To test this possibility, we examined the effect of the *hfq* mutation on the expression of *ptsG* and *ptsG-crp* mRNAs in response to phosphosugar stress. The rapid degradation of *ptsG* mRNA in response to phosphosugar stress was abolished in cells lacking Hfq, while the *hfq* mutation did not affect the expression of *ptsG* mRNA in the presence of glucose (Fig. 8A,C, lanes 1–4). This implies that the destabilization of *ptsG* mRNA is dependent on Hfq. Recently, Vanderpool and Gottesman (2004) have discovered that the synthesis of SgrS, an Hfq-binding small RNA, is induced by phosphosugar stress and acts as an antisense RNA to mediate the destabilization of *ptsG* mRNA. We extended their results by using strains expressing *P<sub>bla</sub>-ptsG* and *ptsG-crp* fusion genes. SgrS was highly induced in cells harboring *ptsG* plasmids in the presence of  $\alpha$ MG where the *P<sub>bla</sub>-ptsG* and *ptsG72-crp* but not *ptsG72-crp* mRNAs are destabilized (Fig. 8B,D). The destabilization of *P<sub>bla</sub>-ptsG* and *ptsG72-crp* mRNAs no longer occurs in  $\Delta$ *sgrS* cells (Fig. 8A,C, lanes 5,6). We also observed that the destabilization of *ptsG14Y-crp* mRNA in the presence of  $\alpha$ MG was abolished in *hfq* or  $\Delta$ *sgrS* cells (Fig. 8E). The level of SgrS RNA under the condition of phosphosugar stress is dramatically reduced in the *hfq* cells, suggesting that Hfq is also involved in the stabilization of

*SgrS* RNA (Fig. 8B,D, lane 4). The reduction in the level of the *SgrS* (*RyaA*) RNA in an *hfq* mutant was previously observed (Zhang et al. 2003). The stabilization of RNA by Hfq has been shown for several other Hfq-binding small RNAs (Sledjeski et al. 2001; Moller et al. 2002; Masse et al. 2003; Moll et al. 2003).

#### The *SgrS*-mediated degradation of *ptsG-crp* fusion mRNA lacking TM domains is enhanced when its translation is reduced

As shown above, the action of *SgrS*/Hfq on the target *ptsG-crp* mRNAs is weak in the absence of membrane



**Figure 8.** Effects of *hfq* or *sgrS* mutation on the degradation of *ptsG* and *ptsG-crp* mRNAs. (A,B) AS16, AS21 ( $\Delta$ *ptsG hfq::cat*), and AS23 ( $\Delta$ *ptsG ΔsgrS*) harboring pTH111 were grown to  $A_{600} = 0.5$ . Glc, or  $\alpha$ MG was added at a final concentration of 1%, and incubation was continued for 20 min. Total RNAs were prepared and 3  $\mu$ g of RNA samples was subjected to Northern blot analysis using *ptsG* or *sgrS* probes. (C,D) IT1568, TM122 (*hfq::cat*), and TM542 ( $\Delta$ *sgrS*) harboring pMM12, and IT1568 harboring pMM12FS were grown to  $A_{600} = 0.5$ . Glc, or  $\alpha$ MG was added at a final concentration of 1%, and incubation was continued for 20 min. Total RNAs were prepared and 3  $\mu$ g of RNA samples was subjected to Northern blot analysis using *crp* or *sgrS* probes. (E) IT1568, TM122, and TM542 harboring pMM10Y were grown to  $A_{600} = 0.5$ . Glc or  $\alpha$ MG was added at a final concentration of 1%, and incubation was continued for 20 min. Total RNAs were prepared and 3  $\mu$ g of RNA samples was subjected to Northern blot analysis using the *crp* probe.

localization presumably due to an efficient translation. To investigate a possible link between translation efficiency and SgrS/Hfq action, we introduced a single nucleotide substitution at position +90 in the Shine-Dalgarno sequence of *ptsG72FS-crp* (Fig. 1B). We examined the expression of the mutant fusion gene (*ptsG72FSM-crp*) in the presence of either glucose or  $\alpha$ MG by Western and Northern blotting. As expected, the expression of the fusion protein was dramatically decreased even in the normal condition (Fig. 9A, lane 3). In addition, the mRNA level was also markedly reduced in the presence of glucose presumably due to a reduced stability caused by inefficient translation (Fig. 9B, lane 3). Interestingly, however, the mutation markedly enhanced the degradation of mRNA in the presence of  $\alpha$ MG (Fig. 9B, lane 4), therefore in the presence of SgrS, resulting in no detectable fusion protein (Fig. 9A, lane 4). Thus, the SgrS-mediated degradation of the cytoplasmic fusion mRNA is apparently stimulated when its translation is slowed down. In other words, SgrS/Hfq retains the ability to act efficiently in the absence of membrane localization depending on the translation status of the target mRNA.

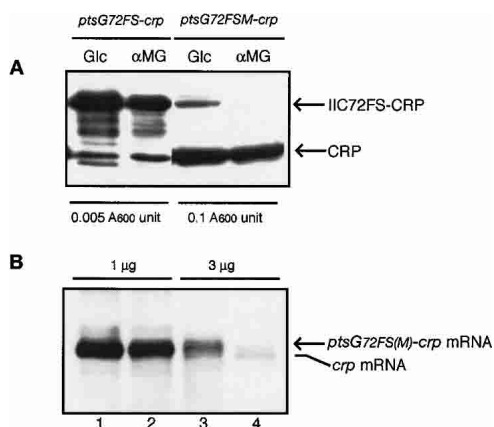
## Discussion

A class of small noncoding RNAs is involved in the regulation of gene expression at post-transcriptional levels by altering translation or stability of target mRNAs in bacteria (Gottesman 2002, 2004; Storz et al. 2004). These RNAs are believed to act through base-pairing with target mRNAs in an Hfq-dependent manner. For example,

OxyS RNA inhibits the translation of target mRNAs by occluding the ribosome-binding sites (Altuvia et al. 1998; Zhang et al. 1998). In contrast, the DsrA and RprA RNAs stimulate *rpoS* translation by preventing formation of a translation-inhibitory hairpin structure in the *rpoS* 5'-untranslated region (Lease et al. 1998; Majdalani et al. 1998, 2002). Recent studies have shown further that small RNAs could alter the stability of target mRNAs. Typically, RyhB RNA induces a rapid degradation of its target mRNAs in an Hfq- and RNase E-dependent manner (Masse and Gottesman 2002; Masse et al. 2003). SgrS (RyaA) RNA is one of a group of newly identified small noncoding RNAs that bind to Hfq (Zhang et al. 2003). The work by Vanderpool and Gottesman (2004) demonstrated that SgrS mediates the destabilization of *ptsG* mRNA presumably through base-pairing with *ptsG* mRNA. They have shown that SgrS is expressed in response to phosphosugar accumulation and that SgrS expression is correlated with greatly reduced levels of *ptsG* mRNA. They have also found that the expression of *sgrS* is regulated by a novel transcriptional activator SgrR that may activate *sgrS* transcription in response to high intracellular levels of phosphosugars.

The major question addressed in the present study is what features of *ptsG* mRNA are responsible for the rapid decay by RNase E under conditions of phosphosugar accumulation. We first showed that the 5' portion up to +70 in the *ptsG* mRNA is dispensable for the destabilization of message. Then, we used a series of *ptsG-crp* translational fusions to specify the downstream boundary in the *ptsG* mRNA required for the response to phosphosugar stress. We found that the region up to the second transmembrane segment of IICB<sup>Glc</sup> is sufficient for the efficient message destabilization. The region required for the efficient destabilization of message covers the region complementary to SgrS RNA (see Fig. 1B). Thus, our data are consistent with the SgrS-*ptsG* mRNA base-pairing model by Vanderpool and Gottesman (2004).

However, our results indicate that the *ptsG* region complementary to SgrS RNA alone is not sufficient for the strong destabilization of the message because the *ptsG-crp* fusion mRNAs missing the transmembrane region are only moderately decreased in the stress conditions where SgrS is highly expressed. Our data clearly demonstrate that the *ptsG* region up to the second transmembrane segment is necessary for the efficient destabilization of *ptsG* mRNA. One simple explanation for this result is that the mRNA region corresponding to two transmembrane segments somehow are involved in the interaction with SgrS and/or components of RNase E degradosome. Another possibility is that the translated nascent peptide of the transmembrane segments of IICB<sup>Glc</sup> plays some roles in the SgrS-*ptsG* interaction and/or the action of RNase E degradosome. However, our experiments clearly exclude these possibilities because the transmembrane segments of IICB<sup>Glc</sup> can be replaced with those derived from an unrelated protein LacY. Thus, the simplest interpretation of our data is that membrane targeting ability of translated protein is cru-



**Figure 9.** Effect of down-mutations in the ribosome-binding site on the response to phosphosugar stress of the cytoplasmic *ptsG-crp* mRNA. (A) IT1568 harboring pMM12FS (lanes 1,2) or pMM12FSM (lanes 3,4) was grown with 1% Glc or  $\alpha$ MG to  $A_{600} = 0.6$ . Total proteins were prepared, and the indicated amounts of samples were subjected to Western blot analysis using anti-CRP antibody. (B) Cells were grown to  $A_{600} = 0.5$ . Glc or  $\alpha$ MG was added at a final concentration of 1%, and incubation was continued for 20 min. Total RNAs were prepared, and the indicated amounts of RNA samples were subjected to Northern blot analysis using the *crp* probe.

cial for the destabilization of *ptsG* mRNA in response to phosphosugar stress.

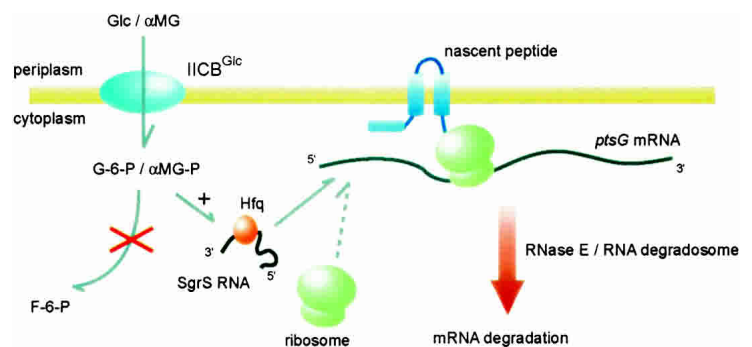
The sugar-specific permeases (EIIs) of PTS generally contain N-terminal amphipathic sequences of 13–20 residues that are believed to function in membrane insertion possibly cooperatively with the first hydrophobic transmembrane segment (Saier 1989; Saier et al. 1989; Yamada et al. 1991). Recently, it has been shown that mannitol permease (MtlA) is recognized by the bacterial signal recognition particle resulting in cotranslational targeting of the growing MtlA polypeptide to the inner membrane (Beck et al. 2000). IICB<sup>Glc</sup> has an N-terminal amphipathic sequence of 13 residues preceding eight transmembrane segments (Buhr and Erni 1993). Therefore, it is very likely that IICB<sup>Glc</sup> is also inserted into membranes in a cotranslational manner. If this is the case, the *ptsG* mRNA under translation is expected to localize near the inner membrane because translation is also coupled with transcription in bacteria. Taken together, we propose that mRNA localization to the inner membrane coupled with the membrane insertion of nascent peptide is a critical event for the Hfq/SgrS- and RNase E-mediated destabilization of the *ptsG* mRNA.

There are several possible models to explain the requirement of mRNA localization to the inner membrane for the rapid degradation of *ptsG* mRNA in response to phosphosugar stress. Firstly, the preferential localization of RNase E-based degradosome near the membrane could explain this. In this regard, there is a report that RNase E seems to be predominantly located near the cytoplasmic membrane (Liou et al. 2001). We showed recently that enolase within the degradosome is required for the destabilization of *ptsG* mRNA (Morita et al. 2004). Thus, one possible mechanism by which enolase stimulates *ptsG* mRNA degradation would be to modulate the membrane localization of RNase E. Further experiments are certainly needed to examine whether RNase E is indeed located near the membrane and whether enolase affects subcellular localization of RNase E. Secondly, the requirement of the mRNA localization to the inner membrane for the rapid degradation of message can be explained if SgrS or SgrS/Hfq complex were preferentially localized near the inner membrane by some mechanism. It is unlikely that SgrS or SgrS/Hfq itself is

localized near membrane. However, additional factors including enolase could mediate the localization of SgrS/Hfq to the inner membrane. The third possibility would be that the action of either SgrS/Hfq or RNase E is somehow inhibited in the particular *ptsG*–*crp* fusion mRNA when it is in the cytoplasm by an unknown factor, while this inhibition is relieved when the mRNA is localized near the membrane. Thus, it is plausible that additional factor(s) other than RNase E, enolase, Hfq, SgrS, and SgrR are also involved in the regulation of stability of *ptsG* mRNA in response to phosphosugar stress. A simple attractive idea is that the ribosome itself is acting as an additional player for the regulation of stability of *ptsG* mRNA. Consistent with this idea, we have shown that the reduced translation of the cytoplasmic target mRNA by introducing single base substitutions in the ribosome-binding site allowed SgrS/Hfq to act efficiently in the absence of membrane localization.

Taken together, we would like to propose the following scenario concerning the regulation of translation and stability of *ptsG* mRNA (Fig. 10). When Mlc is inactivated in the presence of glucose, *ptsG* transcription is induced. As shown by Vanderpool and Gottesman (2004), and confirmed here, no SgrS RNA is made under nonstress conditions. Translation of *ptsG* mRNA occurs normally and the newly synthesized nascent polypeptide of IICB<sup>Glc</sup> would be cotranslationally targeted into the inner membrane. Under stress conditions, SgrS RNA is efficiently transcribed. We hypothesize that new rounds of translation of *ptsG* mRNA already being translated would be less effective compared to the first round of translation because the membrane localization of message may somehow affect the entry of new ribosomes. The fact that the expression level of IIC72–CRP retaining the membrane-targeting ability is markedly lower compared to that of IIC72FS–CRP, the nonmembrane counterpart of IIC72–CRP, supports this assumption. The inhibition of the ribosome entry may allow SgrS along with Hfq to act on the *ptsG* mRNA, resulting in SgrS–*ptsG* pairing, and therefore the RNase E-dependent degradation of the *ptsG* mRNA. When the target mRNA lacks the membrane-localization ability, the entry of new ribosomes occurs effectively, which, in turn, prevents the action of SgrS/Hfq on the *ptsG* mRNA. It re-

**Figure 10.** A model for *ptsG* mRNA degradation in response to the phosphosugar stress. In nonstress conditions, *ptsG* mRNA is translated and IICB<sup>Glc</sup> protein is targeted to cytoplasmic membrane in a cotranslational manner, resulting in localization of mRNA near the membrane. New rounds of ribosome entry into the *ptsG* mRNA being translated would be inhibited due to the membrane localization of message. Under stress conditions, SgrS RNA is highly transcribed by the action of SgrR. The inhibition of the ribosome entry may allow SgrS/Hfq to act on the *ptsG* mRNA, resulting in SgrS–*ptsG* pairing and therefore the RNase E-dependent degradation of the *ptsG* mRNA. When the target mRNA lacks the membrane-localization ability, the entry of new ribosomes occurs effectively, which, in turn, prevents the action of SgrS/Hfq on the *ptsG* mRNA.





mains to be studied whether the destabilization of *ptsG* mRNA is caused directly by SgrS–mRNA base-pairing or is an indirect effect of the inhibition of translation of *ptsG* by SgrS–mRNA base-pairing.

In conclusion, the present study has shed a light on a unique feature, implication of mRNA localization to membrane, of small RNA-mediated mRNA degradation. It is certainly interesting to know whether this is true for other membrane-associated messages that may be regulated by small RNAs in bacteria. Finally, this novel mechanism could be relevant to the action of small regulatory RNAs in eukaryotes too.

## Materials and methods

### Media and growth conditions

Cells were grown aerobically at 37°C unless specified otherwise in LB medium. Antibiotics were used at the following concentrations when needed: ampicillin (50 µg/mL); chloramphenicol (15 µg/mL), and tetracycline (15 µg/mL). Bacterial growth was monitored by determining the optical density at 600 nm.

### Bacterial strains and plasmids

The *E. coli* K12 strains and plasmids used in this study are listed in Table 1. IT1568 (W3110 *mle*) was used as a parent wild-type strain. Other strains were constructed by one-step gene inactivation protocol (Datsenko and Wanner 2000). When necessary, the *cat* marker was eliminated by using an FLP expression plasmid pCP20. Plasmid pHA7MK was constructed from pHA7M

by introducing a KpnI site just after the initiation codon of CRP. To construct pMM10, pMM12, pMM13, pMM14, and pMM15, DNA regions between a BamHI site upstream of P<sub>bla</sub> and various sites corresponding to amino acid positions 14, 40, 72, 103, and 140 of IICB<sup>Glc</sup> in pTH111 were amplified by PCR and cloned between BamHI and KpnI sites of pHA7MK.

### Northern blotting

Total RNAs were isolated from cells grown to mid-log phase as described (Aiba et al. 1981). For Northern blot analysis, indicated amounts of total RNAs were resolved by 1.2% agarose gel electrophoresis in the presence of formaldehyde and blotted on to Hybond-N<sup>+</sup> membrane (Amersham Biosciences). The mRNAs were visualized using digoxigenin (DIG) reagents and kits for nonradioactive nucleic acid labeling and detection system (Roche Molecular Biochemicals) according to the procedure specified by the manufacturer. The following DIG-labeled DNA probes were prepared by PCR using DIG-dUTP: a 305-bp fragment corresponding to the 5' region of *ptsG*; a 621-bp fragment corresponding to the *crp*; and a 150-bp fragment corresponding to *sgrS*.

### Western blotting

Bacterial cells grown in LB medium containing appropriate antibiotics(s) or carbon(s) were harvested and suspended in 100 µL of SDS-PAGE loading buffer (62.5 mM Tris-HCl at pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.1% bromophenol blue). The sample was heated at 100°C for 5 min. Indicated amounts of protein samples were subjected to a 12% or 15% polyacrylamide–0.1% SDS gel electrophoresis and transferred to Immobilon membrane (Millipore). The membranes were

**Table 1.** Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant genotype and property	Source
Strain		
IT1568	W3110 <i>mle</i>	Wild type
AS13	Δ <i>ptsG</i> :: <i>cat</i>	This study
AS16	Δ <i>ptsG</i>	This study
TM224	Δ <i>pgi</i>	Morita et al. 2004
AS17	Δ <i>pgi</i> Δ <i>ptsG</i> :: <i>cat</i>	This study
AS18	Δ <i>ptsG</i> Δ <i>pgi</i>	This study
AS20	Δ <i>ptsG</i> Δ <i>pgi</i> <i>ams-1 zce726</i> :: <i>Tn10</i>	This study
TM122	<i>hfq</i> :: <i>cat</i>	This study
AS21	Δ <i>ptsG</i> <i>hfq</i> :: <i>cat</i>	This study
TM540	Δ( <i>sgrR</i> – <i>sgrS</i> ):: <i>cat</i>	This study
TM542	Δ( <i>sgrR</i> – <i>sgrS</i> )	This study
AS22	Δ <i>ptsG</i> Δ( <i>sgrR</i> – <i>sgrS</i> ):: <i>cat</i>	This study
AS23	Δ <i>ptsG</i> Δ( <i>sgrR</i> – <i>sgrS</i> )	This study
Plasmid		
pIT499	Derivative of pSTV28 carrying the intact <i>ptsG</i>	Kimata et al. 1997
pTH111	Derivative of pBR322 carrying the P <sub>bla</sub> - <i>ptsG1</i>	Takahashi et al. 1998
pTH121	Derivative of pBR322 carrying the P <sub>bla</sub> - <i>ptsG2</i>	This study
pHA7MK	Derivative of pBR322 carrying the P <sub>bla</sub> - <i>crp</i>	This study
pMM10	Derivative of pBR322 carrying the <i>ptsG14</i> - <i>crp</i>	This study
pMM11	Derivative of pBR322 carrying the <i>ptsG40</i> - <i>crp</i>	This study
pMM12	Derivative of pBR322 carrying the <i>ptsG72</i> - <i>crp</i>	This study
pMM13	Derivative of pBR322 carrying the <i>ptsG107</i> - <i>crp</i>	This study
pMM14	Derivative of pBR322 carrying the <i>ptsG139</i> - <i>crp</i>	This study
pMM12FS	Derivative of pBR322 carrying the <i>ptsG72FS</i> - <i>crp</i>	This study
pMM10Y	Derivative of pBR322 carrying the <i>ptsG14Y</i> - <i>crp</i>	This study
pMM12FSM	Derivative of pBR322 carrying the <i>ptsG72FSM</i> - <i>crp</i>	This study

treated with anti-IIB (Tanaka et al. 2000) or anti-CRP (Ishizuka et al. 1993) antibodies. Signals were visualized by the ECL system (Amersham).

#### Protein localization assay

Cells harboring indicated plasmids were grown in 20 mL of LB medium to  $A_{600}$  of 0.6. A 0.5-mL aliquot of cell cultures was directly sonicated for 5 sec twice at 0°C, with a 10-sec interval in between. The sonicated samples were centrifuged at 15,000g (4°C) for 3 min. The pellets were dissolved in 30  $\mu$ L of SDS sample buffer and used as the membrane fraction. The supernatant fraction was precipitated with cold trichloroacetic acid (5% final concentration). The precipitates were collected by centrifugation, washed with 70% acetone, dissolved in 30  $\mu$ L of SDS sample buffer, and used as the cytoplasmic fraction.

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