Overexpression of phosphatidylglycerophosphate synthase restores protein translocation in a *secG* deletion mutant of *Escherichia coli* at low temperature

Vesa P. Kontinen, Hajime Tokuda

Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan

Received 10 March 1995; revised version received 29 March 1995

Abstract **The *E. coli secG* deletion mutant is unable to grow and is defective in protein translocation at low temperature. A gene of *Bacillus subtilis*, which is able to restore the growth of the deletion mutant at low temperature, was found as a multi-copy suppressor. Sequencing of this gene revealed significant homology to *E. coli pgsA*, which encodes phosphatidylglycerophosphate synthase, an enzyme involved in acidic phospholipid synthesis. A plasmid carrying *E. coli pgsA* also restored the growth of the deletion mutant. Furthermore, protein translocation in the deletion mutant was stimulated when it harbored a plasmid carrying *pgsA*. A possible mechanism underlying the *pgsA*-dependent suppression of the *secG* deletion mutation is discussed.**

Key words: Protein translocation; Acidic phospholipid; SecA; SecG; PgsA

1. Introduction

Genetic and biochemical studies have revealed several components which comprise the preprotein translocation apparatus in the inner membrane of *E. coli* [1–4]. Reconstitution of the protein translocation apparatus into proteoliposomes has clarified that SecA, SecE and SecY are absolutely required for translocation [5,6]. SecG has been shown to be required for efficient protein translocation [7–9]. Furthermore, SecD has been demonstrated to facilitate the release of translocated proteins from the spheroplast membrane [10].

Many mutations in these components are known to impair protein translocation at low temperature, suggesting that some step in protein export is cold-sensitive [11]. A low temperature may inhibit insertion of a signal peptide or components of the translocation apparatus into the phospholipid membrane, leading to impaired translocation. SecA, which requires acidic phospholipids for its function [12], has been shown to be inserted deep into the membrane upon protein translocation [13].

SecG is needed for protein translocation and cell growth, especially at low temperature [7,8]. In this study, we searched for genes of Bacillus subtilis which are able to restore the growth of the *secG* deletion mutant at low temperature. Such genes may contain a *secG* homologue or another one capable of taking over the SecG function. Characterization of such suppressors may expose novel aspects of the SecG function and, more generally, protein translocation. One of the suppressors was found to be homologous to *E. coli pgsA* [14,15], which encodes phosphatidylglycerophosphate synthase (PgsA), an important enzyme in the synthetic pathway for acidic phospholipids.

2. Materials and methods

2.1. Bacterial strains and growth conditions

VK1084 (TG1 *secG::kan*), KN370 (FS1576 *secG::kan*), and KN425 (W3110 M25 *secG::kan*) [8] were grown in L-broth or M63 medium [16] supplemented with 30 μg/ml of kanamycin. The M63 also contained 20 μg/ml each of all amino acids other than Cys and Met. Strains harboring pGEM3zf(+), pBR322 or their derivatives were grown on media containing 100 μg/ml of ampicillin.

2.2. Testing of suppression of the secG deletion

The KN370 strain was transformed with a saturating amount of plasmid DNA. The transformed cells were appropriately diluted, and then plated on L-broth containing 1.5% agar and 100 μg/ml of ampicillin. The plates were then incubated at either 20°C or 37°C for 5 days or 1 day, respectively. After the incubation, the plates were examined for the growth of transformants.

2.3. DNA manipulations

Chromosomal DNA was isolated from *B. subtilis* as described [17]. DNA fragments were purified by means of electro-elution from the agarose gel. Restriction enzymes and a Takara Kilodeletion kit were used for subcloning of the suppressor gene. DNA sequencing was performed with a Toyobo Sequencing kit, as recommended by the manufacturer. The sequence was determined on both strands.

2.4. Pulse labeling

Cells were grown to the stationary phase on M63 and then used to inoculate 5.5 ml of fresh M63 to give a turbidity of 0.1 at 660 nm. Cultures were grown to the exponential phase (turbidity, 0.6) at 37°C, followed by incubation at 20° for 2 h. These cells were labeled with Tran^{35}S-label (20 μCi/ml) for 1 min at 20°C, chased, immunoprecipitated with an anti-OmpA antibody, and then analyzed by fluorography as described [8].

3. Results and discussion

3.1. Cloning of a gene from B. subtilis that suppresses the defective growth of a secG deletion mutant at low temperature

Chromosomal DNA of *B. subtilis* Marburg 168 was restricted with *Hin*dIII or *Pst*I. After size-selection (2–4 kb), the fragments were ligated with pGEM3zf(+), which had been cut with the same enzymes. The VK1084 strain (*secG::kan*) was then transformed with the ligated DNA, followed by selection of transformants resistant to ampicillin and able to grow at 20°C. Two transformants carrying *Hin*dIII fragments were able to grow in the cold. Characterization of the plasmids in the transformants revealed that they carry the same insert of about 2.4 kb.

We transformed KN370 (*secG::kan*) with one of these plas-

*Corresponding author. Fax: (81) (3) 3818 9435.

SSDI 0014-5793(95)00378-9

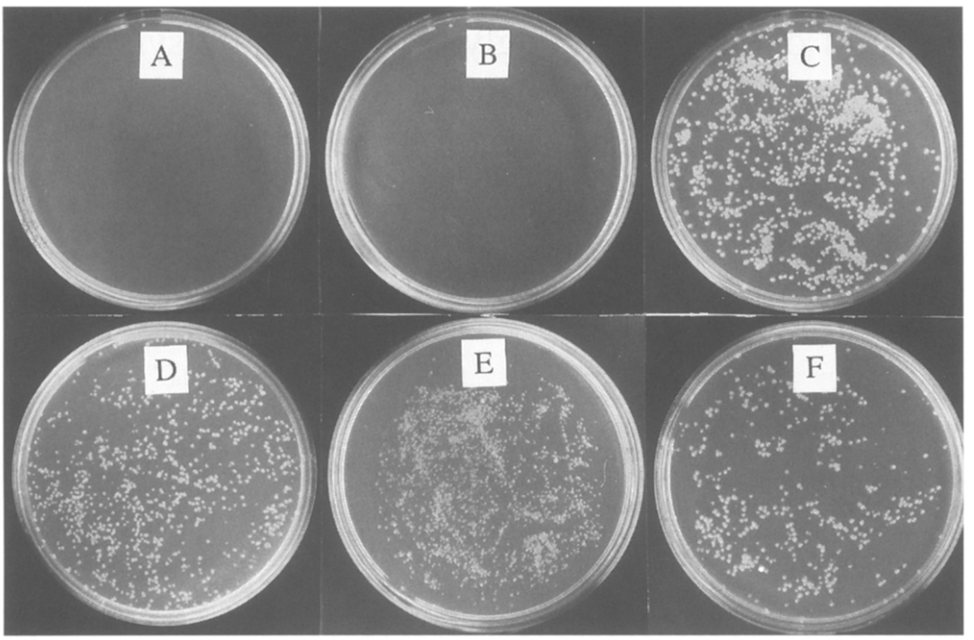

Fig. 1. Ability of *pgsA* to restore the growth of the *E. coli secG* deletion mutant at 20°C. KN370 was transformed with pGEM3zf(+) (A), pBR322 (B), pGE1 (C), pJVK25 (D), pJVK43 (E), or pJVK63 (F), and then examined for the suppression of *secG::kan*. pGE1 carries *secG*. pJVK25 was constructed by cloning the 2.4 kb *B. subtilis* DNA fragment carrying the *pgsA* homologue into pGEM3zf(+). pJVK43 and pJVK63 carry the *E. coli pgsA* gene on pGEM3zf(+) and pBR322, respectively.

mids (pJVK25) to confirm the suppression of the cold-sensitive growth of this strain [8]. As a control, KN370 was also transformed with the cloning vector, pGEM3zf(+), and pGE1, which carries the gene for *E. coli* SecG [8]. The transformants harboring pGEM3zf(+) did not exhibit growth even after five days incubation in the cold (Fig. 1A). On the other hand, transformation with pJVK25 restored the growth of KN370 (D) as effectively as that with pGE1 (C).

To subclone the suppressor gene, we deleted parts of the 2.4 kb insert in pJVK25 with restriction enzymes and exonuclease III. Among the pJVK25 derivatives thus constructed, pJVK40 was found to carry the shortest insert (about 0.85 kb) having the activity to suppress *secG::kan* (Fig. 2). We sequenced the insert in pJVK40. The nucleotide sequence contained an open-reading frame that encodes a protein of 193 amino acid residues (molecular, weight 21,300) (Fig. 3A). A good ribosome binding

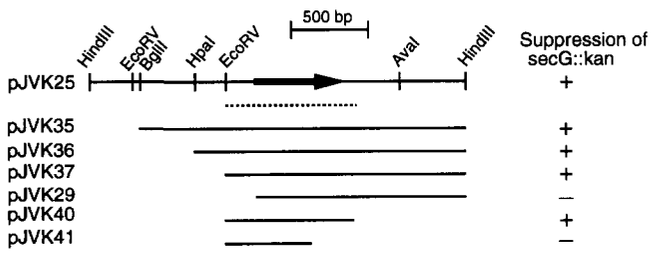

Fig. 2. Suppressor activity of pJVK25 and its derivatives. A restriction map of the 2.4 kb fragment of the *B. subtilis* chromosome containing the *pgsA* gene is shown. Parts of the insert in pJVK25 were deleted with appropriate restriction enzymes to construct pJVK35, pJVK36 and pJVK37. The insert in pJVK25 was deleted with exonuclease III to construct pJVK29. The insert in pJVK37 was further deleted with exonuclease III to construct pJVK40 and pJVK41. The ability of these derivatives to suppress *secG::kan* is indicated. The arrow indicates the coding region of *pgsA*. The broken line indicates the sequenced region.

site, and possible −10 and −35 regions were found in the upstream region. The translation stop codon was followed by another putative ribosome binding site and a new start codon, suggesting that the 0.85 kb insert also carries a part of another cistron. Since pJVK40 carries only the first 26 codons of the putative second cistron, the first cistron is most likely responsible for the suppressor activity.

Comparison of the deduced amino acid sequence of the 21.3 kDa protein with those deposited in the NBRF (PIR) database revealed three significantly homologous sequences. Those are phosphatidylglycerophosphate synthase (PgsA) from *E. coli, Mycobacterium leprae* and *Pseudomonas fluorescens*. On alignment, the sequences of the four PgsA proteins exhibit high conservation throughout (Fig. 3B). Pairwise comparison of the PgsA sequences performed with the Homogapp program (Genetyx) revealed that *B. subtilis* PgsA exhibits highest identity with *M. leprae* PgsA (36.2%), followed by with *P. fluorescens PgsA* (35%) and *E. coli* PgsA (33.2%).

On the *E. coli* chromosome, the *uvrC* gene is located in the upstream region of *pgsA*. The two genes are only about 60 bps apart [15]. Since the 0.85 kb fragment of the *B. subtilis* chromosome contains only 181 bps of the upstream region of *pgsA*, we do not know whether or not the gene organization of this region is conserved between *E. coli* and *B. subtilis*. We also sought homologues for the deduced amino-terminal sequence of the putative second cistron. No homologous sequences were found, however.

3.2. *Restoration of cell growth and protein translocation in the* secG *deletion mutant by overexpression of* E. coli pgsA

To determine whether or not a plasmid carrying *E. coli pgsA* also restores the growth of KN370 at low temperature, the pMA1 plasmid, which carries the genes for *E. coli* PgsA and

A

```
ATCCGCACCGGATATGCACCTAATCTGAAAATAAAAATCAACGGTAAGGTTCTTTCATAT   60

GAACTTGATCCGAAAAAAGTGATGGCACAAACCATAAAGATTGTAAATAAAAAGGAAGAA  120

                -35                -10
AAGTCATCTTAATTACCAGATGACTTTTCTTCACGTCCGAGTATGAAATTGGAGGGGCTT  180

TATGTTTAACTTACCAAATAAAATCACACTAGCTAGAATCGCATTAATCCCAATCTTCAT  240
 M  F  N  L  P  N  K  I  T  L  A  R  I  A  L  I  P  I  F  M

GATTATCATGCTGGCGCCGTTTGACTGGGGCAGGCTCGAAGTTGGAGACGAATCCATCCC  300
 I  I  M  L  A  P  F  D  W  G  R  L  E  V  G  D  E  S  I  P

GGTCGCACATTTGGCGGGGGCCATTCTATTTATTATTGCATCTACAACAGACTGGGTGGA  360
 V  A  H  L  A  G  A  I  L  F  I  I  A  S  T  T  D  W  V  D

CGGGTATTATGCCCGAAAATTAAATCTCGTAACAAACTTCGGGAAATTTCTTGATCCGCT  420
 G  Y  Y  A  R  K  L  N  L  V  T  N  F  G  K  F  L  D  P  L

TGCGGACAAACTGCTCGTATCCGCAGCGTTAATTATCCTTGTTCAATTTGATCTCGCTCC  480
 A  D  K  L  L  V  S  A  A  L  I  I  L  V  Q  F  D  L  A  P

AGCTTGGATGGTCATTGTGATTATCAGCAGGGAGTTTGCCGTGACAGGTTTAAGGCTTGT  540
 A  W  M  V  I  V  I  I  S  R  E  F  A  V  T  G  L  R  L  V

ATTAGCCGGAACAGGAGAAGTCGTAGCTGCTAACATGCTTGGTAAAATCAAAACCTGGGC  600
 L  A  G  T  G  E  V  V  A  A  N  M  L  G  K  I  K  T  W  A

ACAGATTATTGCGGTATCTGCATTGCTTCTTCATAATCTTCCATTTGAACTTGTGTCATT  660
 Q  I  I  A  V  S  A  L  L  L  H  N  L  P  F  E  L  V  S  F

CCCGTTTGCAGACTTGGCGCTGTGGGTAGCCGTCTTCTTTACTGTCGTCTCAGGCTGGGA  720
 P  F  A  D  L  A  L  W  V  A  V  F  F  T  V  V  S  G  W  E

ATATTTCTCTAAAAATTGGGAAGCGTTAAAAACATCTAACTAAGAAAGGAGGACTTCGTC  780
 Y  F  S  K  N  W  E  A  L  K  T  S  N  *

ATGGAATTTCCAAAAAAAGCAGAAATAATTGCAGTCGGTTCAGAGTTGCTGCTTGGTCAA  840
 M  E  F  P  K  K  A  E  I  I  A  V  G  S  E  L  L  L  G  Q

ATCGCCAATACAAATGCC                                             858
 I  A  N  T  N  A
```

B

```
BsPgsA   1:  MFNLPNKITLARIALIPIFMIIMLAPFDWGRLEVGDESIPVAHLAGAIL
EcPgsA   1:  MQFNIPTLLTLFRVILIPFFVLVFYLPVTW-----------SPFAAALI
PfPgsA   1:   MNIPNLITVLRVLLIPIFILLFYLPYNWSYM-----------AASSV
MlPgsA   1:    MVDCVRIANLANILTGLRLMLVPVFLVALFAGNGHEIVGRIAAFVI

BsPgsA  50: FIIASTTDWVDGYYARKLNLVTNFGKFLDPLADKLLVSAALIILVQFDLA
EcPgsA  51: FCVAAVTDWFDGFLARRWNQSTRFGAFLDPVADKVLVAIAMVLVTE-HYH
PfPgsA  49: FAFAAATDWLDGYLARRLEQSTPFGAFLDPVADKLMVAVALVLLVQ-EHG
MlPgsA  47: FTVACITDRLDGLLARRYGMATEFGAFVDPIADKTLIGAALIGLSMLGDL

BsPgsA 100: PAWMVI---VIISREFAVTGLR--LVLAGTGEVVA-ANMLGKIKTWAQII
EcPgsA 101: SWWVTLPAATMIAREIIISALREWMAELGKRSSVA-VSWIGKVKTTAQMV
PfPgsA  99: NLWLTLPAAVIIGREIVVSALREWMAELGRTRPGGRCPTWANRKTAAQML
MlPgsA  97: PWWVTV---LIMTRELGVTLLR----LAVIHRGVIPASWGGKLKTVVQAV

BsPgsA 150: AVSALLLHNLPFELVSFPFADLALWVAVFFTVVSGWEYFSKNWEALKTSN
EcPgsA 151: ALAWLLWRPNIW--VEYAGIAL-FFVAAVLTLWSMLQYLSAARADLLDQ
PfPgsA 149: ALVILLANPPAFTFWVLLGYAF-LLIAGGLTLWSMLQYLRAAWPHLKTDG
MlPgsA 147: AIGLFLLPLTQLSVPFHVGAAVVMAVAVVLTVVTGVDYVAGTIRAVRGVH

PfPgsA 199: RKEIKLFESRG
MlPgsA 197: QTAS
```

Fig. 3. Nucleotide sequence of the *pgsA* gene of *B. subtilis* and alignment of the amino acid sequences of the four PgsA proteins. (A) Ribosome binding sites are underlined, and putative −10 and −35 regions are boxed. The deduced amino acid sequences are also shown. (B) The PgsA proteins are from *B. subtilis* (BsPgsA), *E. coli (EcPgsA)*, *P. fluorescens* (PfPgsA), and *M. leprae* (MlPgsA). Residues conserved in at least two of the PgsA proteins are shaded. The alignment was performed with the Malinp program (Genetyx).

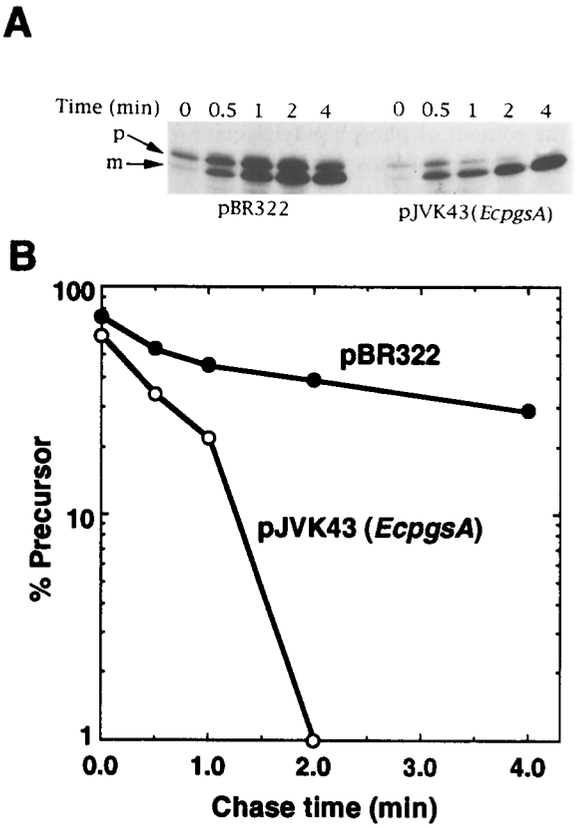

Fig. 4. Expression of the plasmid-encoded *E. coli* PgsA stimulates the processing of proOmpA in the *secG* deletion mutant at 20°C. (A) Cells of KN425 (*secG::kan*) harboring pJVK43 or pBR322, the cloning vector, were pulse-labeled and then chased for the specified times. OmpA (m) and proOmpA (p) were immunoprecipitated, and then subjected to SDS-PAGE and fluorography. (B) The amounts of proOmpA and OmpA on the fluorogram in A were determined by densitometric scanning. The percentage of proOmpA over the total amount of OmpA materials was calculated. The numbers of methionine and cysteine residues in proOmpA (8) and OmpA (7) were used in the calculation.

UvrC, was digested with *Bgl*II to delete the UvrC-encoding region [15]. The deleted plasmid, pJVK43, is about 2 kb shorter than pMA1. KN370 was transformed with pJVK43 and then examined for growth at 20°C. The transformed cells exhibited growth (Fig. 1E), indicating that the expression of the *pgsA* gene carried by the multi-copy plasmid restores the growth of the *secG* deletion mutant. Expression of the *E. coli pgsA* gene from a high copy plasmid, pJVK63, also restored the growth of KN370 (Fig. 1F), whereas the strain harboring a vector, pBR322, did not grow at all (Fig. 1B). Taken together, these results indicate that overexpression of *pgsA*, whether it is derived from *B. subtilis* or *E. coli*, causes suppression of the cold-sensitive growth of the *secG* deletion mutant.

Overexpression of *pgsA* may stimulate protein translocation and thereby allow the *secG* deletion mutant to grow at 20°C. We therefore examined the processing of proOmpA to OmpA in KN370 cells harboring either pJVK43 or pBR322 by means of pulse chase experiments (Fig. 4). The processing rate in KN370 was indeed stimulated by the presence of pJVK43. The mature OmpA was already predominant over proOmpA at 0.5 min after the chase, and proOmpA was hardly detectable at 2 min in KN370 cells harboring pJVK43. On the other hand,

KN370 cells harboring pBR322 contained a significant amount (about 29%) of proOmpA even at 4 min after the chase.

The *pgsA* gene carried by a multi-copy plasmid has been shown to alter cellular phospholipid compositions; i.e. increase in the content of phosphatidylglycerol from 22% to 35% and decrease in that of phosphatidylethanolamine from 76% to 64% have been reported [18]. The results presented in this paper, therefore, indicate that the SecG function can be compensated for by an increase in the level of acidic phospholipids. Acidic phospholipids are known to be important for the SecA function [12]. Furthermore, it has recently been reported that externally added SecA is inserted deep into the membrane upon protein translocation [13]. The smooth insertion of SecA is therefore critically important for efficient protein translocation. The results presented in this paper suggest that both SecG and acidic phospholipids stimulate protein translocation by facilitating the SecA insertion, especially when the membrane fluidity is low in the cold. Detailed molecular mechanism underlying the compensation for the SecG function by the increase in the level of acidic phospholipids is currently under investigation with reconstituted proteoliposomes. Furthermore, it seems important to examine whether or not the overexpression of *pgsA* also suppresses other cold sensitive mutations of *secD*, *secE* and *secY* [11].

Acknowledgements: We wish to thank Drs. Isao Shibuya and Hiroshi Matsuzaki of Saitama University for the pMA1 plasmid and valuable suggestions, Dr. Fujio Kawamura of this institute for *B. subtilis* Marburg 168, and Miss Saori Nishikawa for her secretarial support. This work was supported by Grants to H.T. from the Ministry of Education, Science and Culture of Japan (Nos. 05454620 and 06658096), and by a JSPS Postdoctoral Fellowship for Foreign Researchers in Japan to V.P.K.

References

[1] Schatz, P.J. and Beckwith, J. (1990) Annu. Rev. Genet. 24, 215–248.
[2] Bieker, K.L., Phillips, G.J. and Silhavy, T.J. (1990) J. Bioenerg. Biomembr. 22, 291–310.
[3] Wickner, W., Driessen, A.J.M. and Hartl, F.-U. (1991) Annu. Rev. Biochem. 60, 101–124.
[4] Tokuda, H. (1994) FEBS Lett. 346, 65–68.
[5] Brundage, L., Hendrick, J.P., Schiebel, E., Driessen, A.J.M. and Wickner, W. (1990) Cell 62, 649–657.
[6] Akimura, J., Matsuyama, S., Tokuda, H. and Mizushima, S. (1991) Proc. Natl. Acad. Sci. USA 88, 6545–6549.
[7] Nishiyama, K., Mizushima, S. and Tokuda, H. (1993) EMBO J. 12, 3409–3415.
[8] Nishiyama, K., Hanada, M. and Tokuda, H. (1994) EMBO J. 13, 3272–3277.
[9] Hanada, M., Nishiyama, K., Mizushima, S. and Tokuda, H. (1994) J. Biol. Chem. 269, 23625–23631.
[10] Matsuyama, S., Fujita, Y. and Mizushima, S. (1993) EMBO J. 12, 265–270.
[11] Pogliano, K.J. and Beckwith, J. (1993) Genetics 133, 763–773.
[12] Lill, R., Dowhan, W. and Wickner, W. (1990) Cell 60, 271–280.
[13] Economou, A. and Wickner, W. (1994) Cell 78, 835–843.
[14] Gopalakrishnan, A.S., Chen, Y.-C., Temkin, M. and Dowhan, W. (1986) J. Biol. Chem. 261, 1329–1338.
[15] Usui, M., Sembongi, H., Matsuzaki, H., Matsumoto, K. and Shibuya, I. (1994) J. Bacteriol. 176, 3389–3392.
[16] Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
[17] Marmur, J. (1961) J. Mol. Biol. 3, 208–218.
[18] Ohta, A., Waggoner, K., Radominska-Pyrek, A. and Dowhan, W. (1981) J. Bacteriol. 147, 552–562.