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The *secG* deletion mutation of *Escherichia coli* is suppressed by expression of a novel regulatory gene of *Bacillus subtilis*

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Abstract SecG, a membrane component of *E. coli* protein translocase, stimulates the translocation of proteins across the cell membrane through the cycle of topology inversion, which is coupled to the membrane-insertion and deinsertion cycle of SecA [Nishiyama et al. (1996) Cell 85, 71–81]. A gene of *B. subtilis* able to suppress the cold-sensitive phenotype of the *secG* deletion mutant of *E. coli* was cloned and found to encode a novel regulatory protein, ScgR. Similarity search revealed homology with known proteins such as GlnR of *B. subtilis*. Plasmid-encoded ScgR stimulated protein translocation in the deletion mutant. ScgR increased the proportion of cardiolipin at the expense of phosphatidylglycerol, but did not affect the composition of other lipid components of the cell, suggesting that the increased cardiolipin level compensates for the SecG function and thereby stimulates protein translocation.

Key words: Protein translocation; Cardiolipin; Helix-turn-helix motif; SecG; PgsA; ScgR; *Bacillus subtilis*

1. Introduction

The central part of the preprotein translocase in the inner membrane of *E. coli* consists of SecA, SecE, SecG, and SecY proteins [2–5]. SecA, SecE and SecY have been shown to be absolutely essential for protein translocation as well as for cell viability [6,7]. Although SecG is dispensable at 37°C it is especially important for efficient protein translocation [8–10].

Disruption of the *E. coli secG* gene impairs protein translocation and cell growth at low temperature [9]. To explore novel aspects of the SecG function, we have sought genes of *B. subtilis* that are able to suppress the conditional growth defect of the *secG* deletion mutant of *E. coli*. One such gene was recently cloned and found to encode phosphatidylglycerophosphate synthase, PgsA, which is an enzyme required for the synthesis of acidic phospholipids [11]. Overexpression of the *E. coli pgsA* gene in the *secG* deletion mutant stimulated protein translocation at nonpermissive temperature, suggesting that the SecG function can be compensated for by an increased level of cellular acidic phospholipids [11]. Acidic phospholipids are important for the function of SecA, which delivers precursor proteins into the membrane by undergoing the cycle of membrane insertion and deinsertion [12,13]. Furthermore, SecG was also found to undergo the cycle of topology inversion, which seems to be coupled to and thereby stimulates the SecA cycle [1].

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In this report we describe the cloning of another gene from *B. subtilis* that suppresses the growth defect conferred by the *secG* deletion.

2. Materials and methods

2.1. Bacterial strains and media

VK1084 (TG1 *secG::kan*) and KN370 (FS1576 *secG::kan*) strains were grown in L-broth (1% tryptone, 0.5% yeast extract, 1% NaCl) and on L-plates with kanamycin (30 μg/ml). M63 glucose minimal medium [14] supplemented with 20 μg/ml each of all amino acids except methionine was used when the processing kinetics of proOmpA were determined. Ampicillin was added at 100 μg/ml to maintain pGEM3zf(+) or its derivatives in the strains.

2.2. DNA techniques

Chromosomal DNA was prepared from *B. subtilis* as described by Marmur [15]. DNA fragments were purified on a neutral sucrose gradient as described by Palva et al. [16]. Subcloning of the suppressor gene was performed with restriction enzymes and the Takara Kilo-deletion kit. DNA sequences were determined on both strands using the Takara Sequencing System.

2.3. Analysis of lipid components

For the analysis of glycerophospholipids and lipid A 4′-monophosphate derivatives, cells were grown at 30°C in the presence of sodium [^{14}C]acetate (spec. act. 2.11 GBq/mmol, Amersham), as described previously [17]. Glycerophospholipids and lipid A 4′-monophosphate derivatives were sequentially extracted from 30 mg of dry cells as described in [17,18], and analyzed by TLC using the eluents $CHCl_3$/CH_3OH/CH_3COOH (65:25:10, by vol.) for glycerophospholipids and $CHCH_3$/CH_3OH/25% NH_4OH/H_2O (40:25:2:4, by vol.) for lipid A 4′-monophosphate derivatives. Bands were visualized by fluorography and quantitated by optical scanning. Cellular fatty acids were analyzed by GLC and GLC-MS as their methyl esters prepared from 10 mg of freeze-dried cells by saponification and, in parallel, by methanolysis as summarized in [19].

2.4. Other methods

Pulse labeling was performed as described [11], except that [^{35}S]methionine (20 μg/ml) was used instead of Tran^{35}S-label. The suppression of the *secG* deletion mutation was tested as described [11].

3. Results and discussion

3.1. Cloning of a novel transcriptional regulator from B. subtilis *that suppresses the growth defect of a secG deletion mutant of* E. coli

Chromosomal DNA of *B. subtilis* 168 was partially digested with *Sau*3A. Fragments of about 2 kb were separated on a neutral sucrose gradient and ligated with pGEM3zf(+), which had been cut with *Bam*HI and treated with bacterial alkaline phosphatase. The KN370 strain (*secG::kan*) was then transformed with the ligated DNA, followed by the selection of transformants able to grow at 20°C on plates containing ampicillin. One transformant thus isolated was found to harbor a plasmid (pJVK42), which carried a 2.4 kb insert. In order to

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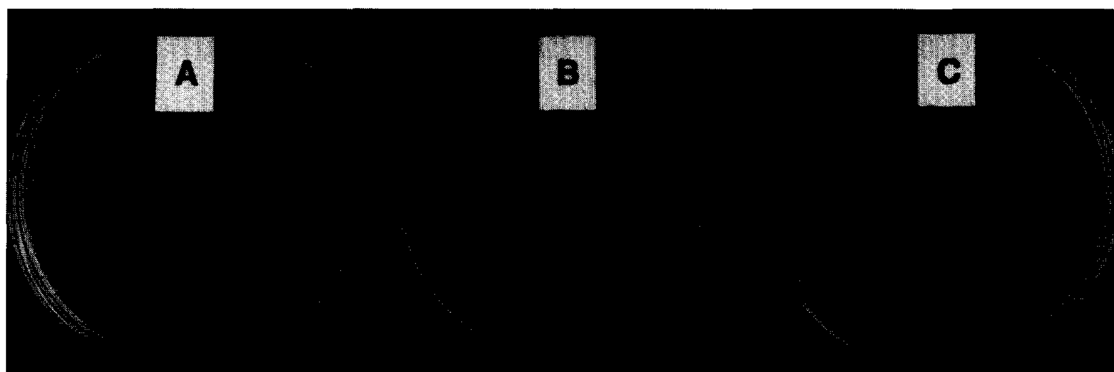

Fig. 1. A 2.4 kb chromosomal fragment from *B. subtilis* carried by pJVK42 restores cell growth of the *E. coli secG* deletion mutant at 20°C. Suppression of the growth defect conferred by the *secG* deletion (*secG::kan*) was examined by transforming KN370 with pJVK42 (A), pGE1 (B), or pGEM3zf(+) (C), and then growing the transformed cells on agar plates at 20°C for 4 days. Colonies were raised by cells harboring a plasmid able to suppress the *secG::kan*. pGE1 carries the *E. coli secG* gene and pGEM3zf(+) is a cloning vector.

confirm that the 2.4 kb insert carried the suppressor gene, we transformed KN370 either with pJVK42, pGEM3zf(+) or pGE1 carrying *E. coli secG* and examined the restoration of growth of the transformants at 20°C [11]. KN370 transformed with the cloning vector, pGEM3zf(+), exhibited no growth at 20°C even after 4 days incubation (Fig. 1C). The secG mutant was, however, able to grow after having been transformed with pJVK42, indicating that a gene in the 2.4 kb insert caused the suppression. Furthermore, the suppression with pJVK42 was as effective as complementation with pGE1 (compare plates in Fig. 1A and B). Comparison of the restriction map of the 2.4 kb insert with that of another suppressor of the secG deletion, *B. subtilis pgsA* gene (pJVK25 in [11]), revealed that the gene in pJVK42 was not *pgsA*.

To subclone the suppressor gene, we first deleted an about 1.4 kb region from the 2.4 kb insert of pJVK42 (Fig. 2). The truncated plasmid (pJVK75) was able to suppress *secG::kan* in KN370. Truncated derivatives were further constructed by deleting parts of the insert of pJVK75 and examined. Deletion of a 0.43 kb *Eco*RV region abolished the suppressor activity (pJVK76). The *Eco*RV fragment alone was insufficient for the suppression (pJVK77). On the other hand, when an insert covers both the *Eco*RV fragment and its outside region, the *secG::kan* mutation was suppressed (pJVK79).

The insert of pJVK75 was then sequenced from both strands. The nucleotide sequence revealed one major open reading frame of 330 bp that could encode a protein of 110 amino acid residues (Fig. 3A). The calculated molecular mass of the protein is 13.1 kDa. A ribosome binding site, putative −10 and −35 regions were found immediately upstream from the translation initiation codon. The translation termination codon was followed by a possible stem loop structure with similarity to transcription terminators.

Comparison of the deduced amino acid sequence of the suppressor gene, referred to here as scgR (suppressors of the cold-sensitive *secG* deletion mutation), with sequences in databases, revealed similarities with proteins of known function. GlnR of *B. subtilis* [20], MerR of various eubacteria [21] and $TipA_L$ of *Streptomyces lividans* [22], all transcriptional regulators of gene expression exhibit significant homology in the N-terminal region. The segment of ScgR from residues 16 to 79 had 52, 35 and 37% identity with a corresponding segment of GlnR, MerR (*Thiobacillus ferrooxidans*) and $TipA_L$, respectively (Fig. 3B), suggesting that ScgR is a transcriptional regulator of *B. subtilis* and somehow functions in *E. coli*. Indeed, *scgR* was found to be identical to *tnrR* of *B. subtilis*, that has been cloned as an activator of expression of various nitrogen regulated genes (S. Fisher, personal communication). The conserved N-terminal region contains a helix-turn-helix motif, which is the DNA binding domain of transcriptional regulators and essential for their function. The nonconserved C-terminal region of ScgR may also be functionally important, since the C-terminal domain of MerR has been shown to be involved in binding of a ligand, Hg(II), which is an activator of the expression of a MerR regulated gene, *mer* [21].

3.2. *scgR restores the growth of a* secG *deletion mutant at low temperature by stimulating protein translocation*

Overexpression of the *E. coli* and *B. subtilis pgsA* genes stimulates protein translocation in the *secG* deletion mutant

Table 1
Phospholipid composition of the *secG* deletion mutants expressing *scgR* of *B. subtilis*

Phospholipid	Percentage of total phospholipids[a]			
	KN370		VK1084	
	pGEM3zf(+)	pJVK75[b]	pGEM3zf(+)	pJVK75[b]
CL+PG	35.8 (35–36.6)	35.2 (34.9–35.5)	37.3 (34.9–39.6)	37.6 (35.7–39.4)
PE	64.2 (63.4–65)	64.8 (64.6–65)	62.8 (60.4–65.1)	62,5 (60.6–64.3)
CL	7.7 (7–8.5)	10 (9.8–10.3)	6.7 (6.3–7)	10.7 (10.1–11.3)
PG	28.1 (28–28.1)	25.2 (25.1–25.2)	30.6 (28.6–32.6)	26.9 (25.6–28.1)

[a]The percentages of phospholipids are means from two determinations. The range of the values is shown in parentheses.
[b]The proportion of specified glycerophospholipid in two *secG* deletion mutants harboring either pGEM3zf(+), or pJVK75. pJVK75 has been constructed by cloning the *scgR* gene of *B. subtilis* into pGEM3zf(+).

and thereby allows the mutant to grow at 20°C [11]. To examine whether the same mechanism underlies the suppression of the *secG* deletion with *scgR*, we determined the kinetics of processing of proOmpA to OmpA in VK1084 harboring either pJVK75 or pGEM3zf(+) by pulse-chase and immunoprecipitation experiments (Fig. 4A). The pulse-chase analysis revealed that *scgR* gene carried by the multi-copy plasmid indeed stimulated processing of proOmpA in the deletion mutant. In the presence of pJVK75, about 50% of the precursor was processed to mature OmpA at 1 min after the chase (Fig. 4B). A longer time, about 2 min, was required with VK1084 cells harboring pGEM3zf(+) to obtain the same level of conversion of the precursor.

3.3. Analysis of cellular lipid components of the secG *deletion mutants expressing scgR of* B. subtilis

We proposed previously that the increased level of acidic phospholipids owing to the overexpression of *pgsA* most probably causes the stimulation of protein translocation in the *secG* deletion mutant [11]. Indeed, recent analysis of phospholipid composition revealed that the level of acidic phospholipids (cardiolipin plus phosphatidylglycerol) in VK1084 increased from 36 to 45% at the expense of phosphatidylethanolamine upon the overexpression of *B. subtilis pgsA*. The altered phospholipid composition could possibly facilitate the insertion of SecA into the inner membrane and thereby stimulate protein translocation. To examine whether ScgR also alters the phospholipid composition, we determined the level of four glycerophospholipids of KN370 and VK1084 harboring either pGEM3zf(+) or pJVK75. A change in the fatty acid composition could probably also stimulate protein translocation in the deletion mutant by altering membrane fluidity. Therefore, we analyzed the cellular fatty acid composition of the strains expressing *scgR*. Furthermore, characterization of the lipid A moiety of lipopolysaccharide was carried out after acid hydrolysis.

The proportions of four glycerophospholipids are listed in Table 1. Although the acidic phospholipids (CL and PG) accounted for approx. 35% of the total amount of phospholipids in either strain regardless of the expression of *scgR*, the proportion of CL increased at the expense of PG when the strain harbored pJVK75 carrying *scgR*. The level of PE was independent of whether or not the strains expressed *scgR*. The

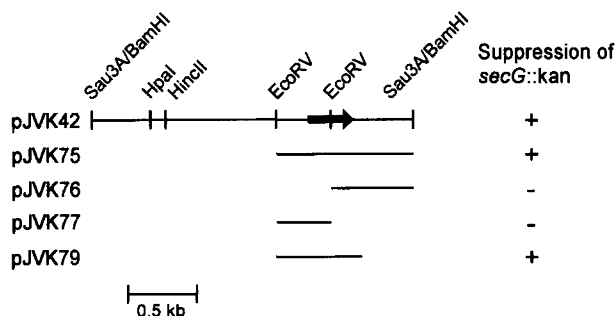

Fig. 2. Restriction map of the 2.4 kb fragment in pJVK42 and location of the suppressor gene, *scgR*. Truncated derivatives of pJVK42 were constructed by deleting parts of the 2.4 kb fragment. Four plasmids thus obtained were tested for the ability to restore the growth of KN370 at 20°C as described in the legend for Fig. 1. The panel on right shows the suppression of *secG::kan*. The arrow indicates the *scgR*-ORF.

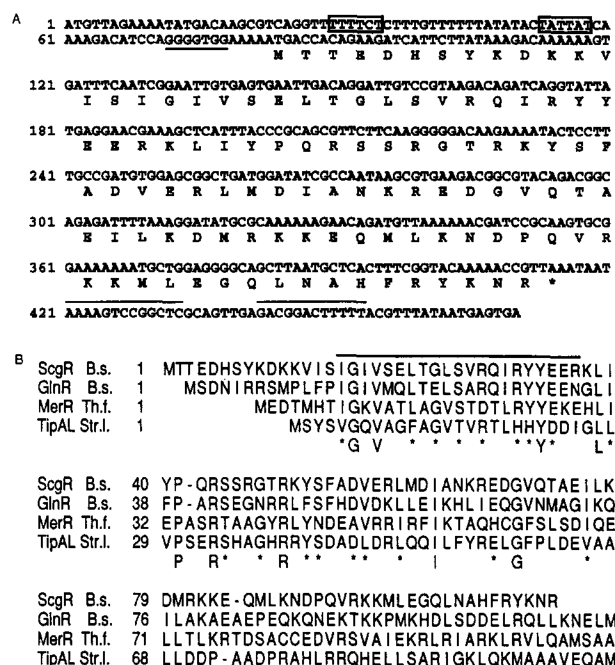

Fig. 3. Nucleotide and deduced amino acid sequences of the *scgR* gene of *B. subtilis* (A) and comparison of the amino acid sequence with the sequences of three prokaryotic transcriptional regulators (B). (A) Potential ribosome-binding site is underlined, putative −10 and −35 regions are boxed and transcriptional terminator is overlined. (B) The sequence of ScgR was aligned with GlnR of *B. subtilis*, MerR of *Thiobacillus ferrooxidans* and TipA$_L$ of *Streptomyces lividans* by using the Malinp program (Genetyx). The line above the sequences indicates the helix-turn-helix motif of the regulatory proteins. Highly conserved amino acid residues are shown below the alignment: upper-case letters and star indicate the residues conserved in four and three of the sequences, respectively. Part of the C-terminal region of GlnR, MerR and TipA$_L$ is not shown.

level of lyso-PE remained low (less than 0.7%) in all the strains, indicating that the expression of *scgR* did not affect the hydrolysis of phosphatidylethanolamine by phospholipase A2 [17]. The ratio of saturated (12:0, 14:0, 16:0 and 18:0) to unsaturated (16:1 and 18:1) fatty acids of phospholipids was not altered by the expression of *scgR*. TLC patterns of [^{14}C]acetate-labeled lipid A 4′-monophosphate species were identical for all the strains studied, indicating that *scgR* had no effect on the structure of this lipid. Taken together, these results indicate that increase in the level of acidic phospholipids, especially cardiolipin, brought about by the overexpression of *pgsA* or *scgR*, causes the suppression of the cold-sensitive phenotype of the *secG* deletion mutant.

In *E. coli*, the composition of acidic phospholipids is dependent on the growth phase. Acidic phospholipids, which are dominant at exponential and stationary growth phases, are phosphatidylglycerol and cardiolipin, respectively [23]. Although ScgR is a putative transcriptional regulator of *B. subtilis* and its exact function in *E. coli* is unknown, the protein seems to interfere with the regulation of acidic phospholipid composition through either increased synthesis or decreased degradation of cardiolipin.

ScgR is identical to TnrA, which activates the expression of nitrogen regulated genes of *B. subtilis*, and may function as a stress regulator under nitrogen-limited condition or at low

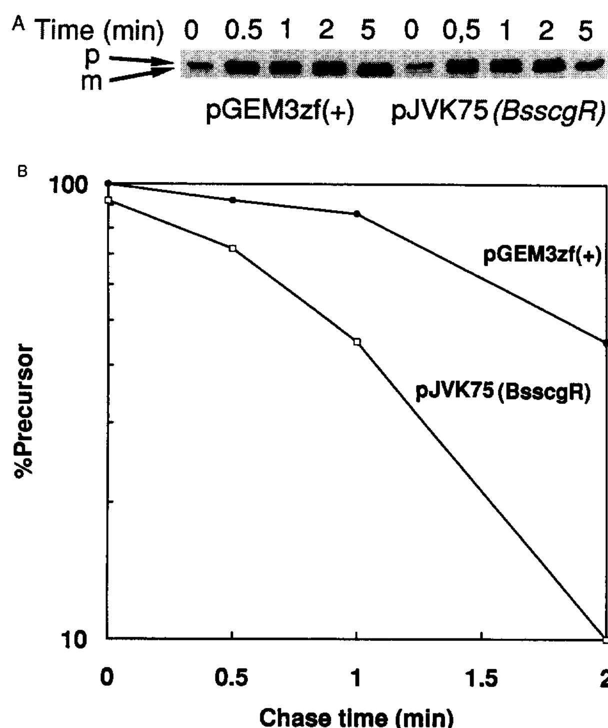

Fig. 4. Processing of proOmpA is stimulated by ScgR in the *secG* deletion mutant. (A) Cultures of VK1084 harboring pJVK75 or pGEM3zf(+) were grown to the exponential phase at 37°C, followed by incubation at 20°C for 2 h. The cells were then labeled with a 1 min pulse of [^{35}S]methionine (20 μCi/ml) that was chased with nonradioactive methionine. At the indicated times samples were taken and subjected to immunoprecipitation with anti-OmpA antibodies. Immunoprecipitated OmpA (m) and proOmpA (p) were separated by SDS-PAGE and visualized by fluorography. (B) Bands on the fluorogram shown in (A) were optically scanned and percentage of proOmpA over the total amount of OmpA materials was calculated. The graph shows the percentages up to two minutes of chase. The number of methionine residues in proOmpA (6) and OmpA (5) was used in the calculation.

temperature. Identification and characterization of genes that are affected by *B. subtilis* ScgR are important for clarification of the roles of SecG and acidic phospholipids in protein translocation.

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