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Cloning, Sequencing, and Expression in *Escherichia coli* of the *Bacillus subtilis* Gene for Phosphatidylserine Synthase

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The *Bacillus subtilis pss* gene encoding phosphatidylserine synthase was cloned by its complementation of the temperature sensitivity of an *Escherichia coli pssA1* mutant. Nucleotide sequencing of the clone indicated that the *pss* gene encodes a polypeptide of 177 amino acid residues (deduced molecular weight of 19,613). This value agreed with the molecular weight of approximately 18,000 observed for the maxicell product. The *B. subtilis* phosphatidylserine synthase showed 35% amino acid sequence homology to the yeast *Saccharomyces cerevisiae* phosphatidylserine synthase and had a region with a high degree of local homology to the conserved segments in some phospholipid synthases and amino alcohol phosphotransferases of *E. coli* and *S. cerevisiae*, whereas no homology was found with that of the *E. coli* counterpart. A hydropathy analysis revealed that the *B. subtilis* synthase is very hydrophobic, in contrast to the hydrophilic *E. coli* counterpart, consisting of several strongly hydrophobic segments that would span the membrane. A manganese-dependent phosphatidylserine synthase activity, a characteristic of the *B. subtilis* enzyme, was found exclusively in the membrane fraction of *E. coli* (*pssA1*) cells harboring a *B. subtilis pss* plasmid. Overproduction of the *B. subtilis* synthase in *E. coli* cells by a *lac* promoter system resulted in an unusual increase of phosphatidylethanolamine (up to 93% of the total phospholipids), in contrast to gratuitous overproduction of the *E. coli* counterpart. This finding suggested that the unusual cytoplasmic localization of the *E. coli* phosphatidylserine synthase plays a role in the regulation of the phospholipid polar headgroup composition in this organism.

The CDP-diacylglycerol-dependent phosphatidylserine synthase of *Escherichia coli* catalyzes the committed step in the biosynthesis of a major phospholipid, phosphatidylethanolamine. The synthase is unique in its subcellular localization among phospholipid enzymes: it is associated with ribosomes, not with the membrane, upon disruption of the cell (26, 36). Further studies on its localization have suggested that the synthase is a peripheral membrane protein that interacts with the membrane surface primarily through electrostatic binding with the membrane-associated lipid substrates and acidic phospholipids (25). This feature of the enzyme has been explained by local amino acid sequences of hydrophilic and positive charges (7). These properties of the enzyme appear to be related to the regulated formation of phosphatidylethanolamine in *E. coli* (25, 41).

In contrast, the phosphatidylserine synthases of gram-positive bacilli and the yeast *Saccharomyces cerevisiae* are associated with membranes (5, 9, 23, 24). Among these, *Bacillus licheniformis* phosphatidylserine synthase has been purified to near homogeneity and characterized in some detail: it requires Mn^{2+} and assumes a sequential bi-bi reaction mechanism, in contrast to the ping-pong mechanism exhibited by the *E. coli* counterpart (10). In *B. subtilis*, a membrane-associated phosphatidylserine synthase activity which appears to have characteristics of a sequential bi-bi reaction mechanism has been reported (9), although little is known about its structure and other properties. Introduction of these membrane-associated counterparts of various origins into *E. coli* cells should provide insight into the yet unresolved regulatory mechanism of phosphatidylethanolamine synthesis in *E. coli*. With this view, we have started to clone the structural gene for the phosphatidylserine synthase of *B. subtilis*. This report describes the cloning of the *B. subtilis* phosphatidylserine synthase gene and characterization of its product and the effect of its overproduction on *E. coli* cells.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *B. subtilis* Marburg and *E. coli* K-12 strains and plasmids used in this study are listed in Table 1. Structures of plasmids are shown in Fig. 1.

Media and bacterial growth. LB broth contained 1% tryptone (Difco, Detroit, Mich.), 0.5% yeast extract (Difco), and 1% NaCl. NBY medium (31) contained 0.8% nutrient broth (Difco), 0.5% polypeptone (Dainihon Seiyaku, Tokyo, Japan), 0.2% yeast extract, and 0.1% NaCl and was adjusted to pH 7.2 with NaOH. SOB medium (19) contained 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 10 mM $MgSO_4$ and was adjusted to pH 7.0 with NaOH. When required, the following supplements were added to the media (per liter): 50 mg of thymine, 5 mg of thiamine hydrochloride, 50 mg of ampicillin, and 25 mg of kanamycin sulfate. Growth of bacteria was monitored by measuring turbidity with a Klett-Summerson photoelectric colorimeter (no. 54 filter). In solid media, 1.5% agar (Difco) was included.

DNA preparation and manipulations. High-molecular-weight chromosomal DNA of *B. subtilis* 160 was prepared by the conventional method of Saito and Miura (38). Plasmid DNAs were prepared from *E. coli* XL1-Blue cells harboring various plasmids by an alkaline lysis method (39). For construction, isolation, and identification of recombinant DNA, the methods described by Sambrook et al. (39) were used. Fragments of DNA were recovered from agarose gels by using a GeneClean II kit from Bio 101 (La Jolla, Calif.). Restriction endonuclease digestion, filling of cohesive ends, and ligation were performed with enzymes from Takara Shuzo (Kyoto,

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
B. subtilis 160	*trpB160*	H. Saito
E. coli		
OS2101	*pssA1* (formerly *pss-1*) *pyrD34 thyA33 galK35 str*	32
OS2124	*pyrD34 thyA33 galK35 str*	32
CSR603	*recA1 uvrA6 phr-1*	40
XL1-Blue	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac* (F′ *proAB*$^+$ *lacI*^{q} *lacZΔM15*::Tn*10*)	4
Plasmids		
pWSK29	pSC101, multicloning site with T7ϕ10 and *lac* promoters, Amp^r	44
pWSK129	pWSK29, Km^r instead of Amp^r	44
pOZ2	pWSK129 carrying a 4.6-kb fragment of *B. subtilis*	This work
pBM201	pWSK29 carrying the 4.6-kb insert of pOZ2	This work
pBM2	pWSK29 carrying a 2,035-base *Pst*I fragment of pBM201	This work
pBM22	pWSK29 carrying a 1,224-base fragment of pBM201	This work
pBM102	pBM201 lacking 346 bases from the *Pst*I fragment of the 4.6-kb insert	This work
pBM213	pWSK29 carrying *Eco*RI-*Ava*II fragment of pBM201	This work
pBM61	pBM201 lacking 730 bases from 5′ end of the 4.6-kb insert	This work
pPS3155	pBR322, *E. coli pssA*$^+$	33
pBR322	Tet^r Amp^r	3
pMS1	pBR322, lacking Amp^r	This work
pMS8	pMS1 carrying *Eco*RI-*Ava*II fragment of pBM201	This work

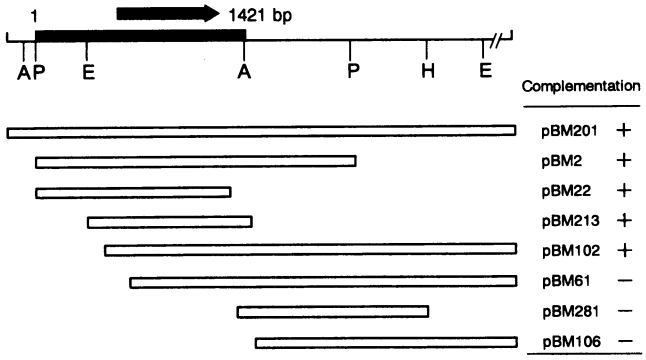

FIG. 1. Physical map of the *B. subtilis pss* locus and complementation of *E. coli pssA1* mutation. The physical map of the 4.6-kbp fragment of the *B. subtilis* chromosome cloned on pWSK29 (44), designated pBM201, is presented. The thick solid line indicates the sequenced region presented in Fig. 2. The thick filled arrow shows the direction and extent of the reading frame of the *pss* gene. Horizontal bars below indicate the regions subcloned on the respective plasmids (see also Table 1 for details) listed on the right. pBM106 and pBM281 could not complement the *pssA1* mutation but did complement the *psd-2* mutation (15, 34). In all cases except for pBM2 and pBM22, the T7ϕ10 promoter of pWSK29 is located on the left end of the subcloned fragment. pBM2 and pBM22 have the *lac* promoter on the left end in place of the T7ϕ10 promoter. Complementation of temperature sensitivity of OS2101 (*pssA1*) was examined on NBY plates at 42°C. Abbreviations: A, *Ava*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I.

Japan), Nippon Gene (Tokyo, Japan), and New England Biolabs (Beverly, Mass.) under conditions recommended by the suppliers. Competent cells of strains OS2101 and XL1-Blue were prepared by the SOB medium method described by Inoue et al. (19) and the standard $CaCl_2$ method (39), respectively.

DNA sequencing. Subclones for DNA sequencing were obtained by cloning the defined restriction fragments and overlapping deletion fragments made by using exonuclease III and S1 nuclease into pWSK29 vector (44). Single-stranded DNA templates were prepared from phage M13KO7-infected XL1-Blue cells harboring each of the subclone plasmids (39). The chain termination reaction was performed as described in a manual supplied by United States Biochemical Corporation (Cleveland, Ohio), using Sequenase version 2.0 DNA polymerase and a fluorescein-labeled 21-mer M13 universal primer (Yuki Gosei Kogyo, Tokyo, Japan). Nucleotide mixtures containing 7-deaza-dGTP and 7-deaza-dATP (Pharmacia, Uppsala, Sweden) were used to prevent formation of intrastrand secondary structures. Reaction products were analyzed with a model DSQ-1 automated laser fluorescent DNA sequencer (Shimadzu, Kyoto, Japan). To verify the sequence data, both strands were sequenced. The DNA sequence and the deduced amino acid sequence were examined with sequence analysis programs of GENETYX software (Software Development Co., Tokyo, Japan).

Analysis of plasmid-encoded proteins. Detection and molecular weight determination of the phosphatidylserine synthase were performed by the maxicell method as described previously (31, 40). Strain CSR603 harboring a plasmid was grown in M9 medium (5 ml) supplemented with 1% Casamino Acids and 1 μg of thiamine per ml to 50 Klett units (about 2×10^8 cells per ml) and irradiated with a 15-W germicidal lamp for 12 s from a distance of 50 cm. Cells were then incubated at 37°C with shaking overnight in the presence of 100 μg of D-cycloserine (Sigma, St. Louis, Mo.) per ml. Then, the cells were washed with M9 buffer and suspended in 2 ml of Hershey medium (31). After 1 h of incubation for starvation, the cells were incubated with 25 μCi of L-[^{35}S]methionine (1,152 Ci/mmol; American Radiolabeled Chemicals, Inc., St. Louis, Mo.) per ml for 1 h. Then, the cells were collected and solubilized in a sample buffer (3% sodium dodecyl sulfate, 5% mercaptoethanol) at 100°C for 3 min. Labeled protein was subjected to sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis and visualized with a BAS 1,000 Mac bioimaging analyzer (Fuji Photo Film, Tokyo, Japan).

Phospholipid analysis. Lipids were extracted by the method of Bligh and Dyer as modified by Ames (1) and separated by Silica Gel 60 (Merck, Darmstadt, Germany) thin-layer chromatography with chloroform-methanol-acetic acid (65:25:10, vol/vol/vol). Spots of phospholipids were visualized by uniformly spraying a Dittmer-Lester reagent (8) and analyzed with a high-speed thin-layer chromatography scanner (model CS-920; Shimadzu, Kyoto, Japan). The molar percentage of each component was calculated.

Phosphatidylserine synthase activity assay. Cells grown to mid-log phase in LB medium were suspended in a potassium phosphate buffer (100 mM, pH 7.4), disrupted by sonication with a Branson Sonifier, and fractionated into soluble and crude membrane fractions by centrifugation for 1 h at 40,000 × *g* after removal of cell debris (by centrifugation at 5,000 × *g* for 10 min). The tightly packed pellet was suspended in 100 mM potassium phosphate buffer (pH 7.4) and used as the membrane fraction. The *B. subtilis*-type phosphatidylserine synthase activities in these membrane and soluble fractions were as-

sayed essentially by the method of Dutt and Dowhan (9). The 0.1-ml reaction mixture contained Tris-maleate (125 mM, pH 7.5), 1% Triton X-100 (wt/vol), 10 mM $MnCl_2$, 10 mM L-[3-^{3}H]serine (360 dpm/nmol; Amersham), 0.125 mM CDP-diacylglycerol (Serdary Research Laboratories, London, Ontario, Canada), 0.5 mM phosphatidylserine (Serdary Research Laboratories), and an enzyme fraction (50 μg of protein). After incubation at 30°C for 10 or 30 min, the reaction was terminated by the addition of methanol containing 0.1 N HCl. Chloroform-soluble products were separated from water-soluble components by phase partitioning, and CDP-diacylglycerol-dependent incorporation of L-[3-^{3}H]serine into the chloroform-soluble fraction was measured.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide databases with accession number D38022.

RESULTS AND DISCUSSION

Cloning and sequencing of the structural gene for phosphatidylserine synthase of *B. subtilis*. Our strategy to clone the *B. subtilis* structural gene for phosphatidylserine synthase was to isolate chromosomal fragments that complemented a temperature-sensitive *pssA* mutation of *E. coli*. To do this, strain OS2101 (*pssA1*), which cannot form colonies at 42°C on an NBY agar plate (31), was used. Fragments of the *B. subtilis* chromosomal DNA (2 to 5 kbp) obtained after partial digestion with *Sau*3AI were inserted into the *Bam*HI site in the multiple cloning sites of pWSK129 DNA and then used to transform *E. coli* OS2101 (*pssA1*). Individual kanamycin-resistant transformants appearing on NBY agar plates at 30°C were suspended in liquid NBY medium and then spread on NBY plates for selection at 42°C. Three clones that grew at 42°C were obtained from ca. 18,000 independent transformants. Restriction endonuclease digestion of the three recombinant plasmids showed that 4.6-, 4.8-, and 6.8-kbp fragments were cloned and that they had a common 3-kbp region. To identify the functional region that complemented the *pssA1* mutation, we subcloned the 4.6-kbp insert of plasmid pOZ2 into pWSK29, designated pBM201, and produced a series of deletion derivatives by using internal restriction sites (Fig. 1). An *Eco*RI-*Ava*II fragment (the plasmid carrying it was designated as pBM213) was capable of complementation.

We sequenced the DNA region responsible for complementation of *pssA1* and its flanking regions (Fig. 2). An open reading frame, starting at a GTG codon at nucleotide 582 and extending to a TAG codon at nucleotide 1,113, encodes a polypeptide of 177 amino acid residues which has homology with the *S. cerevisiae* phosphatidylserine synthase (see below). No significant coding frame was detected on the opposite strand. Further sequencing of the downstream region revealed a second open reading frame which encoded a 263-amino-acid-residue polypeptide showing homology with phosphatidylserine decarboxylases of various origins (34). The biological significance of this novel arrangement of the genes will be described elsewhere. In front of the possible initiation codon of the first open reading frame, a putative ribosome binding site, 5′-GGAGGT-3′, was found. Promoter consensus sequences of gram-positive organisms (13), designated P1 and P2, starting at nucleotides 357 and 417, respectively, were present upstream of the putative ribosome binding site.

To determine the functional region, various deletion derivatives were constructed. A 346-bp deletion from the 5′ end (the *Pst*I site), which yielded pBM102, did not affect the efficiency of complementation (Fig. 1). However, when deletion was extended up to nucleotide 730, the resultant plasmid, pBM61, did not complement the *pssA1* mutation. A deletion from the 3′ end up to nucleotide 1245 did not affect the complementation by the resultant plasmid, pBM22, supporting the assignment shown in Fig. 2. The homology with the phosphatidylserine synthase of *S. cerevisiae* also suggests that this open reading frame is the coding frame of the structural gene for the phosphatidylserine synthase of *B. subtilis*. Since this reading frame was verified to code for the *B. subtilis* phosphatidylserine synthase by the enzyme assay described below, we designate the gene *pss*.

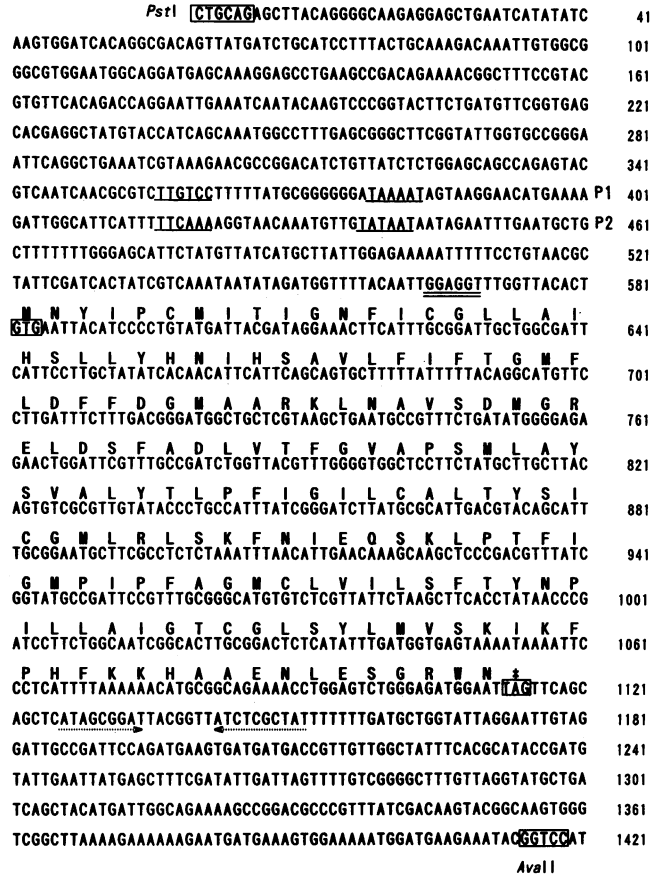

FIG. 2. Nucleotide sequence of the *B. subtilis pss* gene locus. The nucleotide sequence of the 1,421-bp region from the *Pst*I site to the *Ava*II site is presented. Numbering begins at the first letter of the *Pst*I site. Possible promoter regions, P1 and P2, are underlined. A putative ribosome binding site is double underlined, and putative initiation and termination codons are boxed. The dotted arrows indicate inverted repeats of a possible rho-independent terminator. The deduced amino acid sequence of the phosphatidylserine synthase is presented in single-letter code.

Characterization of the product of the *B. subtilis pss* gene. The nucleotide sequence of the *pss* gene predicted an extremely hydrophobic protein; the deduced primary sequence was composed of 62% hydrophobic residues, and the remainder contained 18% hydrophilic and 20% neutral residues. Comparison of the codon usage with the codon preference statistics of weakly and strongly expressed *E. coli* genes (14) suggests that *B. subtilis pss* has the characteristics of a low-level expression gene as in the case of the *E. coli pssA* (27).

To determine whether this *pss* gene actually codes for *B. subtilis* phosphatidylserine synthase, OS2101 (*pssA1*) cells har-

TABLE 2. Membrane localization of the phosphatidylserine synthase of *B. subtilis*[a]

Strain	Fraction	Sp act	
		10 mM $MnCl_2$	Without $MnCl_2$
OS2101/pBM213	Membrane	8.2	0.3
	Soluble	<0.2	<0.2
OS2101/pWSK29	Membrane	<0.2	<0.2
	Soluble	<0.2	<0.2
OS2124	Membrane	<0.2	<0.2
	Soluble	<0.2	<0.2

[a] Cells were grown at 42°C in LB medium supplemented with 50 μg of ampicillin per ml and harvested at 200 Klett units. Fractionation of the cells and the enzyme assay were performed as described in Materials and Methods. Specific activities (nanomoles of labeled serine incorporated per minute per milligram of protein) were averages of duplicate samples which did not differ more than 5%.

boring plasmid pBM213, which contained the reading frame with minimal flanking regions, were examined. By the assay for *B. subtilis* phosphatidylserine synthase, reported by Dutt and Dowhan (9), Mn^{2+}-dependent production of chloroform-soluble materials was observed solely in the membrane fraction from the mutant cells harboring pBM213, not in the soluble fraction (Table 2). No activity was detected in either the membrane or soluble fraction from *E. coli* OS2124 (*pssA*$^+$) under these assay conditions specific for the *B. subtilis* enzyme. The reading frame was therefore concluded to code for the phosphatidylserine synthase of *B. subtilis*.

The protein product of the *pss* gene was then examined by the maxicell method of Sancar et al. (40). Figure 3 shows an autoradiogram of ^{35}S-labeled proteins separated by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis. Strain CSR603 harboring pMS8, a pMS1 derivative containing the *pss* gene, gave protein bands with approximate molecular weights of 37,000 (37K) and 18K. The 37K band was observed in both pMS8 and pMS1 (a pBR322 derivative lacking the *amp* gene), and the size corresponded to that of the product of the *tet* gene (40). The band of an 18K protein was thus concluded to be the product of a *B. subtilis pss* gene. This observed molecular weight of the product agreed well with the molecular weight of 19,613 deduced from the nucleotide sequence.

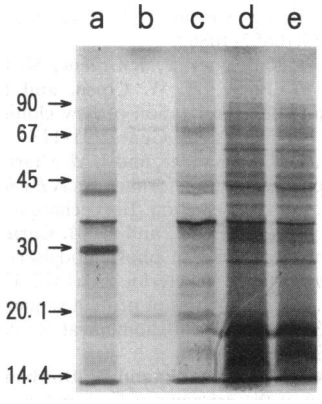

FIG. 3. Labeling and detection of *B. subtilis* phosphatidylserine synthase protein by the maxicell method. An autoradiogram of sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis that separated ^{35}S-labeled proteins is shown. Bands of 28 and 31K were assigned to β-lactamase, and the band at 37K was assigned to the *tet* gene product (40). Molecular weight standards are α-lactalbumin (14.4K), soybean trypsin inhibitor (20.1K), carbonic anhydrase (30K), ovalbumin (43K), bovine serum albumin (67K), and phosphorylase *b* (94K). Lanes: a, pBR322; b, no plasmid; c, pMS1; d and e, independent clones of pMS8.

Sequence comparison of *B. subtilis* phosphatidylserine synthase. The sequence of *B. subtilis* phosphatidylserine synthase was examined for homology with known protein sequences by using GENETYX software. Although the synthase showed no sequence homology with the *E. coli* counterpart (452 residues) (7), it has homology with the product (276 residues) coded for by the *CHO1* (*PSS*) gene of *S. cerevisiae* (21, 29) (data not shown). The two products had 35% common residues, and when the related amino acid substitutes were included, the homology value increased to 56% (within the *B. subtilis* synthase sequence of residues 17 to 163). Interestingly, its molecular weight is quite different from that of the *B. licheniformis* counterpart, which is 53K (10).

The *B. subtilis* synthase had a region showing a high degree of local homology to the conserved domains in phospholipid synthases and amino alcohol phosphotransferases of *E. coli*, *S. cerevisiae*, and CHO cells (18, 21, 28) (Fig. 4). In this region, the phosphatidylserine synthases of *B. subtilis* and *S. cerevisiae* had 53% common residues. The consensus sequence, $DGx_2ARx_8Gx_3Dx_3D$, in the four enzymes of *S. cerevisiae* (18) and *E. coli* phosphatidylglycerophosphate synthase (12, 43) was found in the *B. subtilis* synthase (indicated by asterisks in Fig. 4). In addition, in the flanking regions of this consensus sequence, significant sequence homologies between the *B. subtilis* and yeast phosphatidylserine synthases and *E. coli* phosphatidylglycerophosphate synthase were observed (indicated with plus signs). These conserved regions are likely to correspond to the reaction mechanism, rather than mere affinity to substrates, of the enzyme, since the substrates for these synthases are different from those for phosphotransferases. Further, no significant sequence homology was found with the *E. coli* phosphatidylserine synthase, which adopts a ping-pong reaction mechanism (35), in contrast to the ordered sequential bi-bi reaction mechanisms assumed for these synthases (2, 10, 11, 16).

The hydropathy profile of the phosphatidylserine synthase of *B. subtilis* was then examined by the moving-segment analysis of Kyte and Doolittle (22). The result revealed six highly hydrophobic segments with sufficient length of traversing membrane and three weakly hydrophilic segments (data not shown). This prediction was consistent with its actual membrane localization, whereas it showed a sharp contrast to the cytoplasmic *E. coli* counterpart, which is primarily hydrophilic (7). The conserved domain (Fig. 4) of the *B. subtilis* enzyme contained a relatively hydrophilic segment. A thorough inspection of the segment indicated that it could present an amphiphilic α-helix structure (data not shown), suggesting a mode of interaction with membrane at one side and the opposite side facing with the cytoplasm (20), as previously shown for the yeast cholinephosphotransferase (17). Many of the conserved amino acids in the segment were charged and constituted the hydrophilic face. The *E. coli pgsA3* allele, encoding a severely defective phosphatidylglycerophosphate synthase, has a replacement of Thr at codon 60 with Pro in the conserved region (43), indicating an essential role of this segment in enzymatic function.

Effect of overproduced *B. subtilis* phosphatidylserine synthase on *E. coli* cells. Plasmid pBM22, containing the *pss* gene under the control of the *lac* promoter, was introduced into OS2101 (*pssA1*) cells, and the effect of overproduction of *B. subtilis* phosphatidylserine synthase was examined (Table 3). Cells were cultivated at 42°C in the presence or absence of

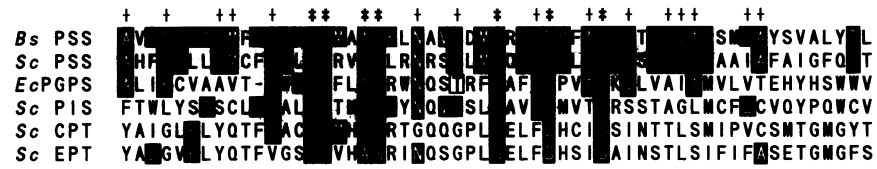

FIG. 4. Alignment of the amino acid sequences of the homologous segments within enzymes of phospholipid synthesis. The *B. subtilis* phosphatidylserine synthase sequence (*Bs* PSS; residues 31 to 87) is aligned with the sequences from the region of local homology (28) of *E. coli* phosphatidylglycerophosphate synthase (*Ec* PGPS; residues 36 to 91) (12, 43) and *S. cerevisiae* phosphatidylserine synthase (*Sc* PSS; residues 116 to 172) (21, 29), phosphatidylinositol synthase (*Sc* PIS; residues 45 to 101) (28), diacylglycerol cholinephosphotransferase (*Sc* CPT; residues 99 to 155) (17), and ethanolamine phosphotransferase (*Sc* EPT; residues 85 to 141) (18). The residues identical with *Bs* PSS are shaded in black. Among these residues, those conserved in all of the enzymes are indicated by asterisks at the top. A plus sign indicates a residue that is homologous among *Ec* PGPS, *Bs* PSS, and *Sc* PSS. The homologous groups of amino acids were assigned according to the mutation data matrix (6). PGPS of the *E. coli pgsA3* mutant has a replacement of residue 60 from wild-type Thr (boxed) with Pro (43).

isopropyl-β-D-thiogalactoside (IPTG) and collected at 200 Klett units, and the enzyme activities in membrane and soluble fractions, as well as phospholipid compositions, were determined. OS2101 harboring pWSK29 (vector) showed no synthase activity in either fraction and contained a low level of phosphatidylethanolamine as previously described (32). On the other hand, OS2101 harboring pBM22 showed a Mn^{2+}-dependent phosphatidylserine synthase activity in the membrane fraction, and its phosphatidylethanolamine content increased to 74% of the total phospholipids. After the addition of IPTG, the enzyme activity increased exclusively in the membrane fraction, accompanying a significant increase in phosphatidylethanolamine content (up to 90% of the total phospholipids). A similar increase (up to 93%) was observed in the wild-type strain OS2124 harboring pBM22. This was in a remarkable contrast to the case of the *E. coli* counterpart; amplification of the *E. coli pssA* gene resulted in an increase in the enzyme activity but not in the phosphatidylethanolamine content (33, 37). We confirmed this with *E. coli* strain OS2101 (*pssA1*) harboring a multicopy recombinant plasmid pPS3155 (*pssA*$^+$ [33]) (data not shown).

By carefully examining the available experimental data concerning the characteristics of *E. coli* phospholipid enzymes and properties of genetically manipulated *E. coli* cells, Shibuya proposed the dynamic adjustment model for the regulated synthesis and turnover of phospholipids to achieve the optimal polar headgroup composition in *E. coli* (41). In this model, phosphatidylserine synthase is present in two forms: only the molecules bound to acidic phospholipids on the membrane by electrostatic interaction participate in phosphatidylserine synthesis, whereas others are functionally latent. Thus, phosphatidylethanolamine synthesis is positively regulated by the total amount of acidic phospholipids present in membrane. Genetic manipulations of phosphatidylglycerophosphate synthase (30) and the membrane level of phosphatidic acid (42) eventually resulted in altered rates of phosphatidylserine synthesis. The straightforward increase in phosphatidylethanolamine content in response to the amplification of the membrane-bound *B. subtilis* phosphatidylserine synthase (Table 3) strongly supports the properties and behaviors of the *E. coli* phosphatidylserine synthase formulated in the model referred to above.

TABLE 3. Amplification of the phosphatidylserine synthase of *B. subtilis* and the effect on the phospholipid composition of *E. coli* cells[a]

Plasmid harbored by strain OS2101	Sp act		Phospholipid composition (%)		
	Membrane	Soluble	PE	PG	CL
pWSK29	<0.6	<0.6	59	22	19
pBM22	18	<0.6	74	21	6
pBM22 + 10 μM IPTG	58	<0.6	88	8	4
pBM22 + 1 mM IPTG	246	<0.6	90	6	5

[a] Cells of OS2101/pBM22 were grown at 42°C in LB medium with 50 μg of ampicillin per ml supplemented with the indicated concentrations of IPTG or 0.2% glucose and harvested at 200 Klett units. Cells of OS2101/pWSK29 were grown in LB medium with 50 μg of ampicillin per ml. Fractionation of the cells, enzyme assay, and phospholipid analysis were performed as described in Materials and Methods. Specific activities (nanomoles of labeled serine incorporated per minute per milligram of protein) were averages of duplicate samples which did not differ more than 5%. Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin.

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