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The *Helicobacter pylori* Gene Encoding Phosphatidylserine Synthase: Sequence, Expression, and Insertional Mutagenesis

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The *Helicobacter pylori pss* gene, coding for phosphatidylserine synthase (PSS), was cloned and sequenced in this study. A polypeptide of 237 amino acids was deduced from the PSS sequence. *H. pylori* PSS exhibits significant amino acid sequence identity with the PSS proteins found in the archaebacterium *Methanococcus jannaschii*, the gram-positive bacterium *Bacillus subtilis*, and the yeast *Saccharomyces cerevisiae* but none with its *Escherichia coli* counterpart. Expression of the putative *pss* gene in maxicells gave rise to a product of ~26 kDa, which is in agreement with the predicted molecular mass of 26,617 Da. A manganese-dependent PSS activity was found in the membrane fractions of the *E. coli* cells overexpressing the *H. pylori pss* gene product. This result indicates that this enzyme is a membrane-bound protein, a conclusion which is supported by the fact that the PSS protein contains several local hydrophobic segments which could form transmembrane helices. The *pss* gene was inactivated with a chloramphenicol acetyltransferase cassette on the plasmid. However, an isogenic *pss* gene-disrupted mutant of *H. pylori* UA802 could not be obtained, suggesting that this enzyme plays an essential role in the growth of this organism.

Phospholipids are responsible for membrane integrity, define the membrane permeability barrier of the cell, and serve as the matrix for membrane-associated activities. Phosphatidylethanolamine (PE) is a major phospholipid, accounting for 70 to 80% of the total phospholipids in the membrane of *Escherichia coli*. The first committed step in the biosynthesis of PE is catalyzed by phosphatidylserine synthase (PSS). The PSS enzyme is found in prokaryotes and lower eukaryotes. In *E. coli*, PSS is responsible for the formation of phosphatidylserine from CDP-diacylglycerol and serine, followed by decarboxylation of phosphatidylserine by phosphatidylserine decarboxylase to yield PE (20). Phosphatidylserine, in addition to serving as a precursor for the synthesis of PE, has been suggested to play other cellular roles in prokaryotic and eukaryotic cells. It has been reported that the functional *E. coli pss* gene is required for its motility and chemotaxis (41), whereas the *Saccharomyces cerevisiae* PSS (encoded by the *CHO1* gene) appears to be involved in the control of the net charge of the membrane, solute sequestration, and vacuolar morphology (44).

Genetic and biochemical studies have revealed several differences between known PSSs from the gram-negative and gram-positive bacteria. The *E. coli* PSS contains 452 amino acids and thus is over 275 amino acids longer than that identified in *Bacillus subtilis* (6, 32). In addition, there is little sequence similarity between these two PSS proteins. Rather, the *B. subtilis* PSS exhibits significant sequence homology with the yeast *S. cerevisiae* Pss (28). Furthermore, the *E. coli* PSS is associated with ribosomes and binds to the membrane through electrostatic force (25, 26, 38). This feature is consistent with the presence of extensive hydrophilic stretches in its amino acid sequence (6). In contrast, the *B. subtilis* PSS, like the yeast PSS (28), appears to be a membrane-bound protein (32), as indicated by the fact that the activity of this enzyme in *B. subtilis* and *Bacillus licheniformis* is membrane associated (7, 32).

It is evident that there is genetic and structural divergence between the *E. coli* and *B. subtilis* PSS proteins. However, whether such divergence is a characteristic of gram-positive and gram-negative bacteria remains to be determined. In addition, although it has been suggested that the *E. coli* PSS may not be essential for cell viability, additional evidence is required to confirm this proposal. Identification of additional *pss* genes from other bacteria would provide new insight into evolutionary and functional implications of this enzyme in bacteria. *Helicobacter pylori* is a gram-negative, spiral, microaerophilic bacterium that colonizes the human stomach (27). This pathogen is responsible for gastritis and ulcers (27) and has been implicated in development of gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (34, 35). In this study, we cloned and characterized a *pss* gene from this organism, and functional roles of this gene were characterized by mutagenesis and overexpression in *E. coli* cells.

MATERIALS AND METHODS

Bacterial strains and media. Clinical *H. pylori* isolate UA802 was obtained from the University of Alberta Hospital and grown on brain heart infusion-yeast extract (BHI-YE) agar or in BHI-YE broth supplemented with vancomycin and amphotericin B under microaerobic conditions as described previously (9). *E. coli* strains (Table 1) were cultured in LB (39), M9 (39), and supplemented M9 (40) media. Ampicillin (100 μg/ml), chloramphenicol (50 μg/ml), and kanamycin (20 μg/ml) were added to these media when appropriate.

Plasmid constructs. Plasmid pBSHpC10, carrying the *H. pylori ftsH*, *pss*, and partial *copA* genes, was generated previously (10). The *pss* gene was amplified by PCR with two specific primers from pBSHpC10 DNA, F (forward; 5′ATATGAGC TCAAGGAGAATCTCTAATGCCTA3′, corresponding to nucleotides 81 to 94) and R (reverse; 5′ATGAATTCAAGATCATTCCCTCTATG3′, complementary to nucleotides 815 to 831). Restriction endonuclease sites, *Sac*I and *Eco*RI, convenient for cloning were introduced into the 5′ termini of the primers F and R, respectively. In addition, a conserved Shine-Dalgarno (SD) sequence for favorable translation of the *H. pylori pss* gene was also engineered into primer F (underlined nucleotides). The amplified PCR product was digested with *Sac*I and *Eco*RI and ligated with the appropriately cut vector pBluescript KS− (Stratagene, La Jolla, Calif.). A clone, designated pBKHpPss, was selected for further studies. Authenticity of the nucleotide sequence of the PCR-amplified *pss* gene was verified by DNA sequencing. The

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Characteristics	Source or reference
E. coli		
DH10B	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 ara*Δ*139* Δ(*ara leu*)*7697 galU galK* λ$^-$ *rpsL endA1 nupG*	Gibco BRL
CSR603	*rescA1 uvrA6 phr1*	40
CSRDE3	CSR603 with DE3, λ prophage carrying the T7 RNA	52
Plasmids		
pBluescript II SK+	Cloning vector (Ap^r)	Stratagene
pBluescript II KS−	Cloning vector (Ap^r)	Stratagene
pBSHpC10	pBluescript II SK+ with a ~4.6-kb *Xba*I-*Eco*RI fragment	10
pBKHpPss	pBluescript II KS− containing the PCR-amplified *pss* gene under T7 promoter control	This study
pBSHpPss	pBluescript II SK+ containing the PCR-amplified *pss* gene opposite T7 promoter control	This study
pBKHpPssH	pBluescript KS− with a 420-nt *Sac*I-*Hin*dIII fragment containing the partial *pss* gene under T7 promoter control	This study
pBKHpPssHm	pBKHpPss with the insertion of a CAT cassette at a *Hin*dIII site	This study
pBKHpPm/C	pBKHpPssHm containing a ~2-kb *Dra*III-*Eco*RI DNA fragment derived from pBSHpC10 at the 3′ end of the *pss* gene	This study

knockout mutant plasmid pBKHpPssHm was constructed by inserting the chloramphenicol acetyltransferase (CAT) cassette (51) at a *Hin*dIII site which is located in the middle of the *pss* gene (see Fig. 2). To generate a plasmid containing a longer flanking region downstream of the CAT insertion site, primer ZGE24, complementary to nucleotides (nt) 815 to 832 (see Fig. 2) and containing a *Dra*III site, was designed. Subsequently, ligation of the *Sac*I-*Dra*III DNA fragment PCR amplified from pBKHpPssHm with primers F and ZGE24 and the *Dra*III-*Eco*RI DNA fragment from pBSHpC10 into pBluescript II KS− yielded a new recombinant plasmid, pBKHpPm/C. Plasmid constructs used in this study are listed in Table 1.

Preparation of membrane and soluble fractions of bacterial cells. *E. coli* cells were grown for 3.5 h and induced by 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 1 h. Cells were then washed three times with a potassium phosphate buffer (100 mM [pH 7.4]) and resuspended in the same buffer. Cells were cracked with a French press at 8,000 lb/in^2. The cell extracts were centrifuged at 12,900 × *g* at 4°C for 10 min. The supernatants were collected and recentrifuged in a Beckman TL 100 ultracentrifuge (100.2 rotor) at 80,000 rpm at 4°C for 30 min. The supernatant was collected as the cytoplasmic fraction. The membrane fraction was washed once with the above-described buffer and recentrifuged in the same ultracentrifuge at 80,000 rpm at 4°C for 30 min. The resulting membrane pellets were homogenized in 200 μl of the same buffer.

Assay of PSS activity. PSS activity was assayed with or without the presence of 10 mM $MnCl_2$ as previously described (32). L-[3-^{3}H]serine was purchased from Amersham/Canada (Oakville, Ontario, Canada), whereas phosphatidylserine and CDP-diacylglycerol were obtained from Sigma-Aldrich Canada (Mississauga, Ontario, Canada). The reactions were conducted at 30°C for 30 min and then terminated by addition of 1 ml of methanol containing 1 N HCl. Chloroform-soluble ^{3}H-labeled products were partitioned from the aqueous phase, dried at 60°C, and measured by the method of Larson et al. (23).

Other techniques. Plasmid DNA was prepared by the method of Birnboim and Doly (2). Chromosomal DNA from *H. pylori* was extracted as described by Ezaki et al. (8). Sequential deletions of recombinant plasmid DNA were then made by using the Erase-a-Base system as specified by the supplier (Promega, Madison, Wis.). Nucleotide sequence was determined with thermocycling Sequenase purchased from Amersham Life Science, Inc. (Cleveland, Ohio), following the procedure specified in the supplier's manual. The *pss* gene was expressed in modified maxicell strain *E. coli* CSRDE3 cells (52) and analyzed by sodium dodecyl sulfate–polyacrylamide (15%) gel electrophoresis as described previously (10). Plasmid mutants were introduced into *H. pylori* UA802 by a natural transformation procedure (50).

Analysis of the nucleotide sequence. The nucleotide sequence reported in this study was analyzed by using the software package (version 8) of the Genetics Computer Group (Madison, Wis.).

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited with GenBank under accession no. AF005718.

RESULTS

Analysis of the nucleotide sequence of the *pss* gene. The *pss* gene from *H. pylori* UA802 is located in the middle region of the recombinant plasmid pBSHpC10 (Fig. 1). The genes flanking the upstream and downstream sequences of *H. pylori pss* are *ftsH* and *copA*, respectively, as reported previously (9–11). Nested deletions of both DNA strands of pBSHpC10 were made and sequenced with Sequenase. The open reading frame of the *pss* gene starts at nt 88 and ends at nt 798, specifying 237 amino acids with a predicted molecular weight of 26,617 (Fig. 2). A less conserved SD sequence (43) was predicted in the region upstream from the start codon (Fig. 2). At least three −10 regions (Pribnow box) reside within the region upstream of the *pss* gene. The putative transcription elements −10 and −35 boxes, which are most similar to those recognized by the major *E. coli* RNA polymerase Eσ^{70}, are as indicated in Fig. 2. The 3′ end of the *pss* gene overlaps the 5′ end of the *copA* gene (11). However, the genes *copA* and *copP* are transcribed as a single operon (11).

A hydropathy profile of the deduced amino acid sequence of the *pss* gene was predicted by the method of Kyte and Doolittle (21). The overall sequence of this protein is highly hydrophobic (Fig. 3A), and eight hydrophobic stretches were identified. In Fig. 3B, a model of the membrane topology of the *H. pylori* PSS protein was generated by using the methods developed by Turner and Weiner (47) as well as von Heijne (49). Eight transmembrane helices connected by three small cytoplasmic loops containing charged residues were consistently predicted. Such a feature suggests that this PSS is a membrane-bound protein.

Sequence comparison of the *H. pylori* PSS protein with known PSSs. The sequence of the *H. pylori* PSS was compared with known protein sequences in the nucleotide/protein sequence databases by using the Blastp program included in the Genetics Computer Group package. A protein sequence from another *H. pylori* strain, A68, which is identical to our PSS sequence was found in GenBank (nucleotide sequence accession no. U59625). However, it appears that no further analysis on this sequence was carried out. The *H. pylori* PSS exhibits an extensive degree of overall sequence homology with known

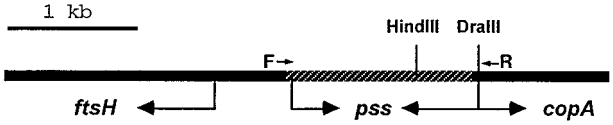

FIG. 1. Schematic representation of the *H. pylori pss* gene location in plasmid pBSHpC10 (10). The *H. pylori copA* and *ftsH* genes were previously characterized (10, 11). Two restriction sites used for insertional mutagenesis with the CAT cassette, *Hin*dIII and *Dra*III, are marked. Locations of the two PCR primers F (forward) and R (reverse) used for amplification of the *pss* gene are indicated by arrows.

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        10                  30                  50
TTAGCGCTTGTAAAATTTTTATTCCCATAATTGATTTTAAAATCGTTTTTATTCCTATTC
        70                  90 -35              110      -10
TAAAAGCCAAGTTACAAAGAATCTCTAATGCCTATTAACCCTCTCTATCTTTTCCCCAAT
                SD          M  P  I  N  P  L  Y  L  F  P  N
       130                 150                 170
CTTTTCACCGCTAGCAGTATTTTTTTAGGCATGATGAGTATTTTTTACGCTTCCAGTTAC
L  F  T  A  S  S  I  F  L  G  M  M  S  I  F  Y  A  S  S  Y
       190                 210                 230
CAATTTGTCATGGCGTGTTGGTTAGTGGTAGCGAGCCTTATTTTAGACGGGCTTGATGGG
Q  F  V  M  A  C  W  L  V  V  A  S  L  I  L  D  G  L  D  G
       250                 270                 290
CGTGTCGCAAGGCTTACCAACACCACCAGCAAGTTTGGTATTGAATTTGACTCCCTAGCT
R  V  A  R  L  T  N  T  T  S  K  F  G  I  E  F  D  S  L  A
       310                 330                 350
GATGTAATCGCTTTTGGAGTAGCCCCAAGTCTTATTACTTACTTTTATGTGGGGTATAAC
D  V  I  A  F  G  V  A  P  S  L  I  T  Y  F  Y  V  G  Y  N
       370                 390                 410
TTTGGGCGCATAGGCATGGCGGTGAGCGCGTTGTTTGTGATTTTTGGAGCGATACGATTA
F  G  R  I  G  M  A  V  S  A  L  F  V  I  F  G  A  I  R  L
       430                 450                 470
GCGCGATTCAATATCAGCACCAACACAAGCGACCCCTATTCTTTTATCGGTATCCCCATC
A  R  F  N  I  S  T  N  T  S  D  P  Y  S  F  I  G  I  P  I
       490                 510                 530
CCTGCGGCGGCGGTATTGGTGGTGCTTTGCGTGTTATTGGATAACAAATACCATTTTTTA
P  A  A  A  V  L  V  V  L  C  V  L  L  D  N  K  Y  H  F  L
       550                 570                 590
                        HindIII
GAAGGAAATACCGAAAAATTATTTTTAAGCTTCATTGTTTTACTAGGGGTGCTTATGGTG
E  G  N  T  E  K  L  F  L  S  F  I  V  L  L  G  V  L  M  V
       610                 630                 650
AGCAATATCCGCTACCCTAATTTTAAAAAAGTCAAATGGAATCTCAAGCTTTTCATCTTA
S  N  I  R  Y  P  N  F  K  K  V  K  W  N  L  K  L  F  I  L
       670                 690                 710
GTGTTGATTTTTTTATCGTTAGTGTTTGTGCGCCCTTTAGAGGCTTTAAGCGTGTTTATG
V  L  I  F  L  S  L  V  F  V  R  P  L  E  A  L  S  V  F  M
       730                 750                 770
GGGTTGTATTTGATTTATGGCATCATTCGGTGGCTCTTTTTAATGGTAAAAATTATTTTT
G  L  Y  L  I  Y  G  I  I  R  W  L  F  L  M  V  K  I  I  F
       790                 810                 830   DraIII
AATAAAAATAAAAGCGCATGAAAGAATCTTTTTACATAGAGGGAATGACTTGCACGGCGT
N  K  N  K  S  A  *
          copA  M  K  E  S  F  Y  I  E  G  M  T  C  T  A  C
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FIG. 2. Nucleotide and deduced amino acid sequences of the *pss* gene. Locations of putative −10 and −35 regions, a putative SD sequence, two restriction sites used for insertional mutagenesis with the CAT cassette, *Hin*dIII and *Dra*III (underlined), and the *copA* gene downstream of the *pss* gene (11) are indicated.

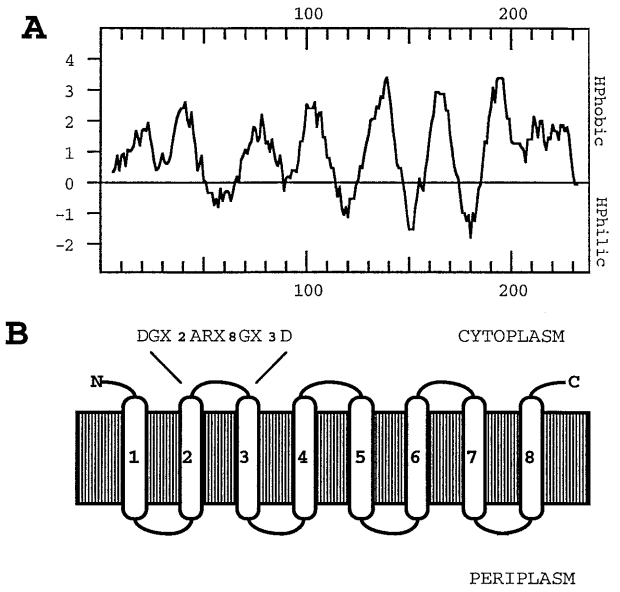

FIG. 3. Structural analysis of the *H. pylori* PSS. (A) Hydropathy profile of the PSS calculated by the method of Kyte and Doolittle (21); (B) predicted membrane topology of the PSS protein as analyzed by the 13 hydropathy algorithms described previously (47, 49). There was a consistent prediction of eight transmembrane segments as numbered. The motif highly conserved by several phospholipid synthases was located in the cytoplasmic loop as detailed in Results.

(putative) PSS sequences from the autotrophic archaeon *Methanococcus jannaschii* (5) (34% identity and 54% similarity), *B. subtilis* (32) (32% identity and 47% similarity), and *S. cerevisiae* (28) (22% identity and 44% similarity). A region spanning 77 residues, which is highly conserved among the PSSs of *H. pylori*, *M. jannaschii*, *B. subtilis*, and *S. cerevisiae* is presented in Fig. 4A. In this region, the degree of sequence homology among *H. pylori* and the other microorganisms was significantly greater: 45, 44, and 40% identity with *S. cerevisiae*, *M. jannaschii*, and *B. subtilis*, respectively. In addition, 44% sequence identity in this local region was also found between the *H. pylori* PSS and a putative *Mycobacterium tuberculosis* PSS (accession no. Z84724). The consensus sequence $DX_2DGX_2ARX_8GX_3DX_3D$ in these four enzymes is also present in the diacylglycerol cholinephosphotransferase (15), phosphatidylinositol synthase (28), and ethanolamine phosphotransferase of *S. cerevisiae* (16), the *E. coli* phosphatidylglycerolphosphate synthase (12, 48), and a putative phosphatidylglycerophosphate synthase from *Rhodobacter sphaeroides* (accession no. U29587). In addition, there are two regions close to the C terminus of PSS, one conserved among *H. pylori*, *M. jannaschii*, and *B. subtilis* (Fig. 4B) and another found only between *H. pylori* and *M. jannaschii* (Fig. 4C). Surprisingly, the *H. pylori* PSS exhibits little sequence similarity to the *E. coli* PSS, although they are both gram-negative bacteria. No sequence similarity was identified between the *H. pylori* PSS and its counterparts from *Haemophilus influenzae* RD, *Mycoplasma pneumoniae*, and *Mycobacterium genitalium*, whose whole genome sequences are now available. The hydropathy profile of the *H. pylori* PSS protein is also similar to those found within the *S. cerevisiae* and *B. subtilis* PSS polypeptides (28, 32). The amino acid composition among these four enzymes is primarily hydrophobic. The local hydrophilic stretch of the above-noted consensus sequence is likely located in the first cytoplasmic loop as shown in Fig. 3B. In contrast, the *E. coli* counterpart is primarily hydrophilic, in agreement with its cytoplasmic location (6).

Although PSSs from *H. pylori*, *M. jannaschii*, *B. subtilis*, and *S. cerevisiae* display significant sequence and structure similarity, some marked divergence exists among these polypeptides. First, their sizes are significantly variable; *S. cerevisiae*, *H. pylori*, *M. jannaschii*, and *B. subtilis* PSS proteins consist of 275, 237, 201, and 177 amino acids, respectively. Second, there are eight predicted hydrophobic stretches within the *H. pylori* PSS protein, whereas six and four hydrophobic stretches were predicted in the *B. subtilis* and *S. cerevisiae* PSS proteins, respectively (28, 32). Third, the N-terminal sequence of the *S. cerevisiae* PSS appears to be hydrophilic; in contrast, those of the *B. subtilis* and *H. pylori* counterparts are relatively hydrophobic.

Identification of the *pss* gene product. The polypeptide encoded by the *H. pylori pss* gene was expressed in a derivative of maxicell strain CSR603 (40), namely, CSRDE3, carrying the P_{lac}-controlled T7 RNA polymerase gene on the chromosome (52). Initially, the wild-type *pss* gene was amplified from pBSHpC10 and cloned into pBluescript KS−. In CSRDE3 cells, no detectable gene product was expressed (data not shown). This could be due to the fact that there is a weak ribosomal binding site, as found in the sequence (Fig. 2), to initiate synthesis of this protein. Therefore, a strong ribosomal binding site preceding the putative start codon was engineered into the *pss* gene by PCR. There was no misincorporation of nucleotides found in the PCR-generated *pss* gene sequence as determined by sequencing. A plasmid carrying such a *pss* gene, pBKHpPss, as well as its derivatives pBKHpPssH and pBSHpPss (Table 1) were introduced into CSRDE3 cells. A

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A

Hp PSS  42 ASLILDGLDGRVARLTNTTSKFGIEFDSLADVIAFGVAPSLITYFYVGYNFGRIGMAVSALFVIFGAIRLARFNIST 118
Mj PSS  37 LSIIFDSLDGYVARKTGTVSDFGAELDSISDVVSFGVAPAYLLYNNFESNLALI---SAIIFCLCGALRLARFGILN 109
Sc PSS 122 LGMCFDFLDGRVARLRNRSSLMGQELDSLADLVSFGVAPAAIAFAIGFQTTFDV--MILSFFVLCGLARLARFNVTV 196
Bs PSS  37 TGMFLDFFDGMAARKLNAVSDMGRELDSFADLVTFGVAPSMLAYSVALYTLPFIGILCALTYSICGMLRLSKFNIEQ 113
              .  *  **  **  .  *  * * ** .*...*****. . .     .   .       . . *  **..*..

B

Hp PSS 123 PYSFIGIPIPAAAVLVVLCVLLDNKYHFLEGNTEKLFLSFIVLLGVLMVSNIRYPNFK 180
Bs PSS 116 LPTFIGMPIPFAGMCLVILSFTYNPILLAIG---------TCGLSYLMVSKIKFPHFK 164
Mj PSS 110 VKGFIGLPIPAGALLLVGFCQLINSYLINS--------ILAILIGLLMISDIKYPKYP 159
               ***.***   . .*      *                  .  **.* *..* .

C

Hp PSS 185 NLKLFILVLIFLSLVFVRPLEALSVFMGLYLIYGIIRWLFLMVKIIFNKNKSA 237
Mj PSS 160 NKIFIYIFAVSLCLAIVGIPHFALMLCLIYAIYGIIKYIRGD---------- 201
           *     .  . *.*  *       .    .* *****. .
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FIG. 4. Sequence alignment of the *H. pylori* PSS protein (Hp) with three known PSS proteins from *M. jannaschii* (Mj) (5), *B. subtilis* (Bc) (32), and *S. cerevisiae* (Sc) (29), using the program CLUSTAL W (46). Identical residues and conservative replacements at the corresponding positions of these proteins are marked by asterisks and dots, respectively. Numbers represent amino acid positions. Gaps were introduced to optimize alignment. (A) The conserved region shared by all four PSS proteins; (B) the conserved region among the *H. pylori*, *M. jannaschii*, and *B. subtilis* PSS proteins; (C) the conserved region between the *H. pylori* and *M. jannaschii* PSS proteins.

protein with a molecular mass of 26 kDa was specified by pBKHpPss, which contains an intact *pss* gene (Fig. 5, lane 1). In contrast, there was no corresponding product expressed from either pBKHpPssH carrying the partially deleted *pss* gene (Fig. 5, lane 2) or pBSHpPss containing the intact *pss* gene separated from the T7 promoter (Fig. 5, lane 3). In addition, the size of the expressed polypeptide is in agreement with that (26,617 Da) calculated from the deduced amino acid sequence of the *pss* gene. As a result, the evidence presented here indicates that the predicted ORF represents a complete coding region specifying a polypeptide of ~26 kDa.

***H. pylori* PSS activity is membrane associated.** To investigate if the cloned gene actually encodes the *H. pylori* PSS, membrane and cytoplasmic fractions of *E. coli* cells expressing the putative *H. pylori* PSS protein were prepared. Subsequently, the PSS activity in these samples was examined by using a procedure developed for the *B. subtilis* PSS (32). The data (Table 2) showed that PSS activity was present only in the membrane fraction of the *E. coli* cells (CSRDE3/pBKH pPss) expressing the intact *H. pylori* PSS protein. Such an activity was Mn^{2+} dependent, since without this divalent ion the PSS activity was significantly reduced (Table 2). In addition, the truncated PSS protein expressed from pBKHpPssH has no PSS activity in this assay (Table 2). In contrast, no manganese-dependent PSS activity was found in either the cytoplasmic or membrane fractions in cells either harboring the vector or with no plasmid. It should be noted that a manganese-sensitive activity associated with the presence of pBluescript KS exists in the membrane fraction. The reason for this result is unknown at present. Nevertheless, the evidence presented here demonstrates that the gene cloned from *H. pylori* UA802 indeed encodes the PSS.

Lethal effect of the knockout *pss* gene on the growth of *H. pylori*. To examine if the *pss* gene is essential for viability of *H. pylori* cells, we constructed plasmid pBKHpPssHm by inserting the CAT cassette into a *Hin*dIII site which is located in the middle region of the *pss* gene on plasmid pBKHpPss (Fig. 1). In addition, we constructed pBKHpPm/C, which contains ~0.6- and 2-kb regions flanking the insertion site of the CAT cassette. Both pBKHpPssHm and pBKHpPm/C were introduced into *H. pylori* cells by natural competence. However, we were unable to obtain any knockout *pss* mutant of *H. pylori* by plating on BHI-YE agar containing chloramphenicol (50 μg/

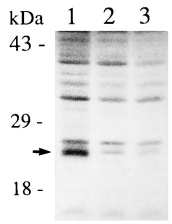

FIG. 5. Overexpression of *H. pylori pss* gene in *E. coli* CSRDE3 cells. Proteins were synthesized as described in Materials and Methods; plasmid constructs are described in Table 1. Equal amounts of protein extracts as determined by the turbidity of the cultures were separated on a sodium dodecyl sulfate–15% polyacrylamide gel. Lanes: 1, pBKHpPss; 2, pBKHpPssH; 3, pBSHpPss. Positions of molecular mass markers (Gibco BRL) are labeled on the left; the arrow marks the overexpressed protein.

TABLE 2. Specific activities of PSS in *E. coli* CSRDE3 cells with or without plasmids containing the *H. pylori pss* gene[a]

Strain	Sp act (nmol of labeled serine incorporated/min/mg of protein)			
	With Mn^{2+} (10 mM)		Without Mn^{2+}	
	Membrane	Soluble	Membrane	Soluble
CSRDE3	<0.15	<0.15	<0.15	<0.15
CSRDE3/pBluescript II KS	<0.15	<0.15	0.8	<0.15
CSRDE3/pBKHpPssH	<0.15	<0.15	0.75	<0.15
CSRDE3/pBKHpPss	6.1	0.4	0.68	<0.15

[a] Overnight cultures of the cells were reinoculated in LB broth supplemented with or without ampicillin (100 μg/ml) and then grown for 3.5 h. Growth of the cells was continued for an additional hour in the presence of 1 mM IPTG, and the cells were harvested. The membrane and soluble fractions were prepared and the activity assay was performed as described in Materials and Methods. Values were averaged from duplicate experiments in which the difference in activities for the samples tested was within 10%.

ml). In contrast, our previous studies have shown that various *copA*-disrupted *H. pylori* mutants can be easily generated by using the same procedure and that a ~400-nt flanking region was sufficient for homologous recombination in *H. pylori* (9, 11). In addition, as a control, over 100 of the *copA*-disrupted *H. pylori* transformants were obtained by using the *copA*-inactivated plasmid pBHpcopADm as donor DNA. This plasmid was created by inserting a CAT cassette into a *Dra*III site which is only 240 nt downstream of the *Hin*dIII site (11). BHI-YE medium containing 10 or 20 mM Mg^{2+} was also used to screen the *pss*-inactivated *H. pylori* mutants, since it was reported that medium containing 20 mM Mg^{2+} was able to support the growth of the *pss*-disrupted *E. coli* cells (6); however, the putative *pss* mutant was still not viable. Therefore, our failure to obtain a *pss*-inactivated *H. pylori* mutant is likely due to the requirement of this gene product for cell viability.

DISCUSSION

In the DNA sequence of the *H. pylori pss* gene, only a weak ribosomal binding site (SD sequence) preceding the putative AUG codon was found. This observation was supported by the fact that there was no detectable product overexpressed in the maxicells by using the native *pss* gene, as shown in the Results, which might be expected since the PSS protein could be constitutively produced in low amounts as found in *E. coli* (22).

Although *H. pylori* is a gram-negative bacteria, its PSS appears structurally and functionally similar to the PSS enzymes from the gram-positive bacterium *B. subtilis* and the lower eukaryote *S. cerevisiae* but not to its *E. coli* counterpart. First, the amino acid sequence of the *H. pylori* PSS exhibits significant homology with the *B. subtilis* and *S. cerevisiae* PSS proteins but none with the *E. coli* PSS. Second, the *H. pylori* PSS, like *B. subtilis* and *S. cerevisiae* PSS proteins, appears to be membrane bound (references 7 and 24 and this study), whereas the *E. coli* PSS is likely to be membrane associated primarily through electrostatic binding (25). Such a difference in the interaction between PSS proteins and the membrane within *H. pylori* and *E. coli* cells could be explained by their amino acid compositions. In *H. pylori*, *B. subtilis*, and *S. cerevisiae*, PSS proteins consist primarily of hydrophobic residues which potentially form several transmembrane segments (references 28 and 32 and this study). In contrast, the *E. coli* PSS is primarily hydrophilic except for two relatively hydrophobic domains (6). Finally, the *H. pylori* PSS contains a region whose residues have been highly conserved by these *B. subtilis* and *S. cerevisiae* PSS proteins as well as several other phospholipid synthases and phosphotransferases as described above (Fig. 4C). This conserved domain may contribute to the common reaction mechanism utilized by these enzymes (32). In *B. subtilis*, a sequential bi-bi mechanism (in a reaction involving two products and two reactants, the presence of one of the products can stimulate an exchange reaction between a product and one of the reactants when the other reactant is present) appears to be followed by the PSS reaction (7). The same mechanism was also proposed for *S. cerevisiae* PSS and *E. coli* phosphatidylglycerophosphate synthase (1, 14). In contrast, the *E. coli* PSS reaction has characteristics of ping-pong mechanisms in which one or more products are released before all substrates have been added (22, 34).

PSS catalyzes the formation of phosphatidylserine from CDP-diacylglycerol and serine (20). Phosphatidylserine serves as a precursor for a major phospholipid in bacteria, PE, and two major phospholipids in yeast, PE (3) and phosphatidylcholine (4). The previous studies suggested that PSS in both prokaryotes and eukaryotes plays an important role in many biological processes as discussed in the introduction, but the essentiality of its function for cell viability has been questioned. In *E. coli*, temperature-sensitive mutant cells lacking the functional *pss* gene stop division at a nonpermissive temperature (42 or 44°C) (6, 31, 37, 42). This phenotype of the mutant cells can be suppressed by the addition of magnesium and sucrose to the growth medium without restoring normal PE synthesis. Under these conditions, the phospholipid content in the membrane did not show a significant difference between the mutant and wild-type cells (6). However, in the mutant cells, the amount of cardiolipin was markedly increased (6, 30). This result suggested that cardiolipin in the presence of magnesium may be able to replace the PE. This proposal was further supported by the evidence demonstrating that it is impossible to obtain a mutant carrying both the interrupted *cls* and *pss* genes (6, 30). Likely, the yeast strain containing the disrupted *CHO1* gene encoding PSS is capable of growing to a limited extent in medium supplemented with either ethanolamine or choline. Accumulation of PE to a significant level in such mutant cells grown on choline suggests that there is a secondary pathway involving in the synthesis of PE (12, 33). The finding that addition of *myo*-inositol to the medium elevates the cellular level of phosphatidylinositol and stimulates the growth of the mutant indicates that phosphatidylinositol complements the role of phosphatidylserine, probably by restoring superficial negative charges of the membranes to a level favorable for growth of the mutant (13). It should be noted that growth of the *CHO1*-disrupted *S. cerevisiae* mutant in medium supplemented with the above-mentioned chemicals is still slower than that of the wild-type strain, demonstrating that the normal function of PSS is necessary for optimally growing *S. cerevisiae*. The *PEL1* gene product, whose protein sequence is homologous to that of the *CHO1* gene product (17), regulates the cardiolopin content in yeast cells (18). Disruption of the *PEL1* gene has a lethal effect on the viability of the *cho1* null mutant lacking the main phosphatidylserine synthesis (18). These lines of evidence suggest that *E. coli* and yeast cells have evolved alternative pathways to compensate for the functions of PSS in the event that lethal mutations occur in this enzyme.

Our results obtained from using knockout mutagenesis of the *H. pylori pss* gene suggest that the *pss* gene product is essential for cell viability. Such an essential role of the *pss* gene product in this microorganism could be explained by the lack of an alternative route(s) to substitute for or complement the functions of PSS. In fact, the *H. pylori* genome contains 1.67×10^3 to 1.74×10^3 kb (19) and thus is much smaller than the *E. coli* genome ($\sim 4 \times 10^3$ kb). Also additional requirements for serum and a microaerobic atmosphere for growth indicate that this organism has lost some genetic counterparts existing in *E. coli*. When a plasmid vector is available for this organism, temperature-sensitive or inducible promoter-controlled *pss* mutants can be constructed. Such studies would significantly increase our understanding of the precise roles of the *pss* gene in *H. pylori.*

In summary, the *pss* gene was isolated from *H. pylori* and is likely essential for cell viability. The lines of evidence presented in this study indicated that the *H. pylori* PSS is a membrane-bound protein and that its optimal activity is required with the presence of divalent ion manganese. These features of this enzyme as well as its amino acid sequence are similar to those of the PSS proteins from the archaebacterium *M. jannaschii*, gram-positive bacterium *B. subtilis*, and lower eukaryote *S. cerevisiae* rather than the PSS from *E. coli*. Our previous studies showed that the *H. pylori* FtsH (10), CopA (9, 11), and 23S rRNA (45) tend to have higher sequence homology with their counterparts from either eukaryotes or gram-positive

bacteria than from *E. coli*. As a result, such sequence similarity implies that the *H. pylori* genome has, at least in part, a close evolutionary relatedness to archaebacteria, gram-positive bacteria, and eukaryotes.

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