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Molecular Characterization of Enterobacterial *pldA* Genes Encoding Outer Membrane Phospholipase A

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The *pldA* gene of *Escherichia coli* encodes an outer membrane phospholipase A. A strain carrying the most commonly used mutant *pldA* allele appeared to express a correctly assembled PldA protein in the outer membrane. Nucleotide sequence analysis revealed that the only difference between the wild type and the mutant is the replacement of the serine residue in position 152 by phenylalanine. Since mutants that lack the *pldA* gene were normally viable under laboratory conditions and had no apparent phenotype except for the lack of outer membrane phospholipase activity, the exact role of the enzyme remains unknown. Nevertheless, the enzyme seems to be important for the bacteria, since Western blotting (immunoblotting) and enzyme assays showed that it is widely spread among species of the family *Enterobacteriaceae*. To characterize the PldA protein further, the *pldA* genes of *Salmonella typhimurium*, *Klebsiella pneumoniae*, and *Proteus vulgaris* were cloned and sequenced. The cloned genes were expressed in *E. coli*, and their gene products were enzymatically active. Comparison of the predicted PldA primary structures with that of *E. coli* PldA revealed a high degree of homology, with 79% of the amino acid residues being identical in all four proteins. Implications of the sequence comparison for the structure and the structure-function relationship of PldA protein are discussed.

Most bacterial outer membrane proteins are involved in the transport of nutrients across this membrane by forming pores or receptors. In addition, these membranes contain a few enzymes, e.g., the detergent-resistant outer membrane phospholipase A or PldA protein of *Escherichia coli*. Several activities reside in this enzyme, i.e., those of phospholipases A_1 and A_2 and of 1-acyl and 2-acyl lysophospholipase and lipase, with the phospholipase A_1 activity being six times greater than the phospholipase A_2 activity (24). The PldA protein is encoded by the *pldA* gene, the nucleotide sequence of which has been determined (23). This gene codes for a 30-kDa mature protein of 269 amino acid residues preceded by a signal sequence of 20 amino acid residues. The three-dimensional structure of the enzyme is unknown. Like other outer membrane proteins, PldA protein lacks hydrophobic sequences long enough to span the lipid bilayer. Therefore, its structure might be comparable with those of the porins which have recently been determined (10, 54, 55). In these outer membrane proteins, the polypeptide chain traverses the outer membrane repeatedly as antiparallel β-strands. A comparison of the outer membrane protein PhoE among three species of the family *Enterobacteriaceae* has revealed that during evolution some parts of the polypeptide have undergone more extensive divergence than others (49). For PhoE, these variable regions correspond to cell surface-exposed segments. A similar finding was reported for the OmpA protein (7). Thus, sequence comparisons can be helpful in predicting the topology of outer membrane proteins.

The exact function of the PldA protein is unknown. The protein has been shown to be required for efficient secretion of bacteriocins (27, 30, 36), although it is unlikely that this is the primary function of the protein. It seems that the PldA protein is dormant in normally growing cells (1). The activity of the enzyme must be well regulated because otherwise it would degrade the cell envelope. However, high outer membrane phospholipase activity can be induced by damaging the membrane, e.g., by phage-induced lysis (11) or temperature shock (15). Strains containing *pldA* mutations are normally viable (33), which indicates that the protein has no essential role under laboratory conditions. Nevertheless, the PldA protein might be essential for growth of the bacteria in their natural environment. In this case, the protein would be expected to be widely spread among species of *Enterobacteriaceae*. Thus far, outer membrane phospholipase activity has been reported only in *E. coli* and *Salmonella typhimurium* (5).

We are interested in elucidating the structure and the structure-function relationship as well as the physiological role of the PldA protein. As a part of these studies, we have characterized the *pldA* allele of the most commonly used *pldA* mutant strain S17 and constructed a *pldA* deletion mutant. Furthermore, we have investigated the presence of this protein in other species of *Enterobacteriaceae*. As an aid in predicting the topology of the protein and in identifying enzymatically important residues, the *pldA* genes of *S. typhimurium*, *Klebsiella pneumoniae*, and *Proteus vulgaris* were cloned and their nucleotide sequences were determined.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. Unless stated otherwise, bacteria were grown at 37°C in L broth (47) or minimal medium (29), supplemented when required with ampicillin (Ap) (50 to 100 μg/ml), kanamycin (Km) (50 μg/ml), tetracycline (Tc) (10 μg/ml), rifampin (40 μg/ml), or streptomycin (100 μg/ml).

DNA techniques. Plasmid DNA was isolated by the boiling

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TABLE 1. Bacterial strains and plasmids

Strain, species, or plasmid	Relevant characteristics[a]	Reference or origin[b]
E. coli strains		
AM1095	F^- *leu thi trp his pyrA lacY galK xyl ara mtl fhuA tsx rpsL recB21 recC22 sbcB15 sup*	18
CE1347	Δ*pldA*::Km^r derivative of AM1095	This study
PC1067	F^- *metE thyA lacY14 rha rpsL*	P.C.
JC10240	Hfr PO45, *srlC300*::Tn*10 recA56 thr-300 ilv-318 rpsE300*	12
CE1348	Δ*pldA*::Km^r *srlC300*::Tn*10 recA56* derivative of PC1067	This study
CE1302	F^- *fabB supD rpoB*	14
PC1602	F^- *metE thyA bio endA sup*	P.C.
CE1303	F^- *metE*$^+$ *pldA thyA*$^+$ *recA56* derivative of PC1602	14
MXR[pULB113]	Δ(*lac-pro*) *galE thi recA1* containing plasmid pULB113	51
Other species		
Shigella flexneri		W. Jansen
Salmonella typhimurium		21
Klebsiella pneumoniae		21
Enterobacter aerogenes		W. Jansen
Enterobacter cloacae		W. Jansen
Citrobacter freundii		W. Jansen
Serratia marcescens		W. Jansen
Edwardsiella tarda		W. Jansen
Proteus vulgaris		21
Yersinia enterocolitica		W. Jansen
Erwinia herbicola		W. Jansen
Providencia stuartii		21
Plasmids		
pUC4K	Ap^r Km^r	52
pUC18	Ap^r	57
pUC19	Ap^r	57
pPN100	Ap^r *pldA*$^+$	15
pRB1	Ap^r *pldA*$^+$	This study
pRB4	Ap^r Km^r Δ*pldA*	This study
pPI232	Cm^r *pldA*$^+$ *metE*$^+$	14
pULB113	RP4::miniMu3A	51
pSPH20	Ap^r Cm^r *corA*$^+$	20
pST103	4-kb *Hin*dIII fragment of pSPH20, containing *pldA* of *S. typhimurium* cloned in pUC19	This study
pKP002	R′ derivative of pULB113 containing *metE* and *pldA* of *K. pneumoniae*	This study
pKP102	2.0-kb *Hin*cII-*Pst*I fragment of pKP002 containing *pldA* cloned in pUC19	This study
pPV001	R′ derivative of pULB113 containing *metE* and *pldA* of *P. vulgaris*	This study
pPV101	5-kb *Sph*I fragment of pPV001 containing *pldA* cloned in pUC19	This study
pPV201	2.3-kb *Hpa*I fragment of pPV101 containing *pldA* cloned in pUC19	This study

[a] Cm^r, chloramphenicol resistance.

[b] P.C., Phabagen Collection, Department of Molecular Cell Biology, Utrecht University, The Netherlands; W. Jansen, the National Institute for Public Health and Environmental Protection, Bilthoven, The Netherlands.

method (22) or the alkaline extraction procedure (6), followed by anion-exchange chromatography on Qiagen pack 20 or pack 500 columns (Diagen, Düsseldorf, Germany).

The mutant gene was amplified by PCR using chromosomal DNA isolated (31) from strain CE1303 as template. Two oligonucleotides, B129 (5′-ATGAATTCCTTATCAATAATT TCG-3′) with a nonhybridizing *Eco*RI site and B130 (5′-AG AAGCTTCAACCACTCAACCGT-3′) with a nonhybridizing *Hin*dIII site, were used to hybridize with the mutant *pldA* allele immediately before the Shine-Dalgarno sequence and after the stop codon, respectively. Each PCR mixture contained 7.5 pmol of both primers, 200 μM (each) deoxynucleoside triphosphate, approximately 240 ng of template DNA, *Taq* DNA polymerase buffer (Promega), 1.5 mM $MgCl_2$, and 1 to 1.5 U of *Taq* DNA polymerase (Promega) in a total volume of 25 μl. Reactions were performed in 0.5-ml Eppendorf tubes with a Techne programmable Ori-block PHC-1. The thermal profile involved a first denaturing step at 94°C for 3 min followed by 25 cycles of denaturing at 94°C for 1 min, primer annealing at 48°C for 3 min, and extension at 65°C for 2 min (but for 12 min in the last cycle).

Restriction endonucleases, T4 DNA ligase, and calf intestinal alkaline phosphatase were obtained from Pharmacia LKB Biotechnology. DNA restriction fragments were subcloned into the multiple cloning site of vector pUC19 and sequenced on double-stranded template DNA by the dideoxy chain termination method (41) with the T7 DNA polymerase (deaza) sequencing kit (Pharmacia LKB Biotechnology).

Southern blots were made by using Hybond N^+ membranes (Amersham) and the model 785 vacuum blotter (Bio-Rad).

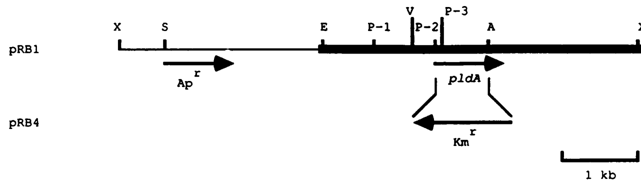

FIG. 1. Restriction maps of pRB1 and its derivative pRB4. Ap^r, ampicillin resistance gene; Km^r, kanamycin resistance gene. The thin line represents pUC18 DNA, and the thick line represents *E. coli* chromosomal DNA. In pRB4, the *Pst*I-2-*Afl*II fragment of pRB1 is replaced by a Km^r cassette. A, *Afl*II; E, *Eco*RI; P, *Pst*I; S, *Ssp*I; V, *Pvu*II; X, *Xba*I.

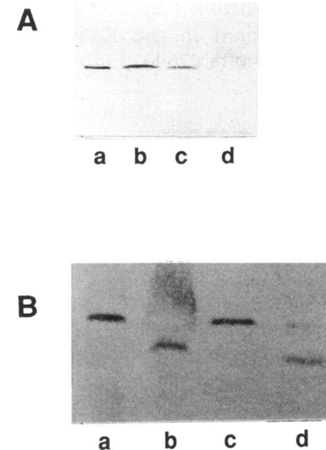

FIG. 2. (A) Western blot analysis of cell envelope fractions with anti-PldA serum. Applied was 0.005 μg of purified *E. coli* PldA protein (lane a) or cell envelope fractions of *pldA* mutant strain CE1303 (lane b), strain PC2254 (lane c), or *pldA* deletion mutant CE1347 (lane d). (B) Heat modifiability of wild-type and mutant PldA proteins, demonstrated by Western blot analysis of cell envelope fractions with anti-PldA serum. Lanes a and b, strain AM1095; lanes c and d, strain CE1303. Samples in lanes a and c were boiled, and those in lanes b and d were not boiled before electrophoresis.

Hybridization experiments were performed with part of the *E. coli pldA* gene (nucleotides 385 to 951; see Fig. 3) amplified by PCR, and subsequently labeled with a digoxigenin-11-dUTP DNA labeling kit (Boehringer Mannheim) as a probe. Hybridization temperatures were 68°C for *S. typhimurium* and *K. pneumoniae* DNA and 42°C for *P. vulgaris* DNA. Chemiluminescent detections were performed by using 3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)-phenyl-1,2-dioxetane (AMPPD; Boehringer Mannheim) as chemiluminescent substrate for alkaline phosphatase.

Genetic techniques. Competent bacterial cells were transformed with plasmid DNA by using a 75 mM $CaCl_2$–20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 6.0) solution (40). Mating experiments between donor strains carrying RP4::miniMu3A plasmid pULB113 and recipient strains were performed as described elsewhere (51).

Construction of *pldA* deletion mutants. An *E. coli* mutant in which 80% of the *pldA* gene was deleted from the chromosome and replaced by a Km^r gene was constructed by marker exchange using plasmid pRB4 (Fig. 1). To this end, the 4.25-kb *Eco*RI-*Xba*I fragment of plasmid pPN100 (Table 1), containing the *E. coli pldA* gene, was subcloned into a derivative of pUC18 lacking the *Pst*I site, resulting in plasmid pRB1 (Fig. 1). The *Pst*I-2-*Afl*II fragment of pRB1 was replaced by the Km^r gene of pUC4K via conventional recombinant DNA techniques (40). Strain AM1095 was transformed with 6 μg of *Eco*RI- and *Xba*I-digested pRB4 as described elsewhere (40) except that after the 2-min incubation at 42°C an extra 30-min incubation on ice was performed. Km^r transformants were tested both in vivo and in vitro for outer membrane phospholipase activity (see below). As expected, a Km^r, phospholipase-negative mutant, designated CE1347, did not produce any PldA protein detectable by Western blot (immunoblot) analysis (Fig. 2A, lane d). This mutation was transferred from strain CE1347 to *metE* strain PC1067 by P1 transduction (56). Km^r transductants were tested for outer membrane phospholipase activity in the in vivo assay and for the maintenance of the *metE* marker on minimal-medium agar. The *recA* mutation of strain JC10240 (12) was transferred to a *metE pldA* Km^r transductant by another P1 transduction, selecting for Tc-resistant transductants and screening for UV sensitivity (total dose, 300 erg/mm^2/sec). In this way *rpsL metE pldA* Km^r *recA* Tc^r strain CE1348 was obtained. Like strain CE1347, strain CE1348 did not produce any PldA protein detectable by Western blot analysis.

PldA antiserum. An antiserum was raised against PldA protein. Cell envelope proteins from strain CE1303 carrying *pldA* plasmid pPI232 (Table 1) were dansylated and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16). PldA protein was eluted from the gel (16) and used to immunize mice. The specificity of the polyclonal antiserum obtained was increased by affinity purification (8) using a sample of purified PldA protein (a generous gift of A. J. G. Horrevoets) (14) electroblotted onto a nitrocellulose filter.

Western blot analysis. Cell envelope proteins were separated by SDS-PAGE (28). The anti-PldA serum was raised against denatured PldA protein. To denature all proteins after SDS-PAGE and prior to Western blotting, the gel glass plate sandwich was wrapped in aluminum foil, sealed into a plastic bag, and heated for 8 min in steam. Subsequently, the proteins were electroblotted onto a nitrocellulose filter. After blocking in a solution containing 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 0.05% Tween 20, and 0.5% dried milk (Protifar), the filter was incubated with the mouse anti-PldA serum and with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G as second antibody as described elsewhere (2, 40).

Outer membrane phospholipase activity assays. A qualitative, in vivo assay for outer membrane phospholipase activity was performed as described elsewhere (15). Outer membrane phospholipase A_1 activity was determined quantitatively by using cell envelope fractions and a radioactive substrate with a nonhydrolyzable carbamoyl bond rather than an ester bond on the *sn*-2 position. Cell envelopes were isolated at 0°C as described elsewhere (28) and resuspended in 1 ml of a buffer containing 2 mM Tris-HCl and 3.5 mM Triton X-100, pH 8.0. The substrate, [1-^{14}C]palmitoyl-2-laurylcarbamyloxy-*sn*-glycero-3-phosphocholine (specific activity, 275 dpm/nmol), was prepared by acylation of the corresponding "lyso"-phospholipid via standard procedures (18). Outer membrane phospholipase A_1 activity was assayed in 450 μl of reaction buffer (25 mM Tris-HCl, 15 mM $CaCl_2$, and 0.4 mM Triton X-100, pH 8.0) containing 100 nmol of substrate together with 50 μl of cell envelope fraction. After 4 h of incubation at 37°C, the liberated ^{14}C-labeled palmitic acid was extracted from the reaction mixture by a modified Dole extraction procedure (48), and radioactivities were determined, in disintegrations per minute, by liquid scintillation spectrometry.

Nucleotide sequence accession numbers. The nucleotide

sequences of *S. typhimurium*, *K. pneumoniae*, and *P. vulgaris* *pldA* have been deposited in the EMBL data base under accession numbers X76900, X76901, and X76902, respectively.

RESULTS

Characterization of a *pldA* mutant. Several attempts to isolate from local sewage a phage that uses the PldA protein as its receptor were unsuccessful. Therefore, we reasoned that the *pldA* mutant that was used to screen the isolated phages for PldA specificity, i.e., strain CE1303 (which carries the same *pldA* allele as strain S17), might still express an inactive mutant form of the PldA protein that is normally incorporated in the outer membrane. To test this possibility, cell envelope proteins from overnight cultures of strain CE1303 and of the wild-type strain AM1095 were analyzed by Western blotting. In the cell envelopes of the mutant strain, a band reacting with the PldA-specific antibodies that migrated with the same mobility in the gel as did the wild-type PldA protein (Fig. 2A, lanes a to c) was detected. Like the wild-type PldA protein (32), the mutant PldA protein was heat modifiable: when the samples were boiled before SDS-PAGE, the wild-type and mutant PldA protein bands migrated with an apparent molecular mass of 30 kDa, whereas in unboiled samples the molecular mass was 23 kDa (Fig. 2B). In the case of OmpA, it has been shown that such a heat-modifiable character is an indication of correct assembly of the protein into the outer membrane (17, 38). By analogy, the heat modifiability of the mutant PldA protein suggests that this protein is correctly folded and assembled in the outer membrane. Therefore, the mutation in the *pldA* gene of strain CE1303 is expected to be a very restricted one that affects activity. Sequence analysis was used to identify the site of the mutation. The mutant *pldA* allele was amplified by PCR and cloned in pUC19. The nucleotide sequence was determined on three recombinant plasmids, obtained after independent PCRs. The sequences were identical to that of the wild type (23), except for a single missense mutation changing a TCC codon into a TTC codon. As a result, serine 152 is replaced by phenylalanine, indicating that this residue might be important for enzymatic activity.

Construction of a *pldA* deletion mutant. Cell envelopes of *pldA* mutant strain CE1303 were tested in the in vitro assay for outer membrane phospholipase A activity. No activity was detected (data not shown), which is consistent with the conclusion of Ohki et al. (33) that the mutant protein is inactive. Nevertheless, because the mutant PldA protein is apparently normally incorporated into the outer membrane, the lack of a phenotype of the *pldA* mutant may be due to a low residual activity of the mutant enzyme. To investigate this possibility, deletion mutant CE1347, in which approximately 80% of the *pldA* gene is replaced by a Km^r gene, was constructed. The missense mutant strain CE1303 and the deletion mutant CE1347 grew comparably with their parental wild-type strains, i.e., PC1602 and AM1095, respectively, in L broth as well as in synthetic medium. When cell cultures were incubated for prolonged periods (up to 1 week) at 37°C, cell survival, as determined by plating, and the optical densities of the wild-type and mutant cultures declined at the same rate (results not shown). The latter results suggest that the PldA protein has no autolytic function. In addition, the wild-type and mutant cultures survived several 30-to-42°C and 0-to-37°C transitions to the same extent (results not shown). Apparently, PldA protein plays no important role in the repair of damage to the membrane brought about by temperature fluctuations.

TABLE 2. Outer membrane phospholipase A_1 activities in species of *Enterobacteriaceae*

Species or strain[a]	Phospholipase activity	
	In vivo[b]	In vitro (dpm)[c]
Shigella flexneri	+	2,046
Salmonella typhimurium	+	1,629
Klebsiella pneumoniae	+	2,975
Enterobacter aerogenes	+	719
Enterobacter cloacae	+	1,432
Erwinia herbicola	+	6,393
Citrobacter freundii	+	1,857
Serratia marcescens	+	1,567
Edwardsiella tarda	+	2,958
Proteus vulgaris	+	5,619
Providencia stuartii	+	6,760
Yersinia enterocolitica	+	6,059
AM1095	+	1,369
CE1347	−	0

[a] *E. coli* strain AM1095 and *pldA* mutant CE1347 were used as positive and negative controls, respectively.
[b] The results of a qualitative in vivo phospholipase assay are indicated; + or −, presence or absence, respectively, of detected phospholipase activity.
[c] Activity is expressed as disintegrations per minute of liberated [^{14}C]palmitic acid. The incubation mixture contained 100 nmol of [1-^{14}C]palmitoyl-2-lauryl-carbamyloxy-*sn*-glycero-3-phosphocholine (circa 14,000 dpm).

Detection of PldA proteins in species of *Enterobacteriaceae*. To test whether detergent-resistant outer membrane phospholipase is widely distributed among *Enterobacteriaceae*, several members of this family were tested both by the in vivo and in vitro outer membrane phospholipase activity assay. Detergent-resistant phospholipase A_1 activity was detected in the cell envelopes of all strains tested except for *pldA* mutant strain CE1347, although the levels of activity varied considerably (Table 2). Because of its low expression level, the PldA protein is undetectable by Coomassie brilliant blue staining of SDS-polyacrylamide gels containing cell envelope proteins. Therefore, it is not clear whether the variation in activity observed in the different strains is caused by different amounts of enzyme synthesized or by different specific activities of the enzymes. The detected activity, i.e., release of free fatty acid, is not necessarily caused by a protein structurally related to the PldA protein of *E. coli*. To test whether structurally related proteins are present in these strains, Western immunoblot analysis with anti-PldA serum was performed (Fig. 3). Bands were visible only in the M_r range of the PldA protein. An immunogenically related protein was found to be present in *S. typhimurium*, *Enterobacter aerogenes*, *Serratia marcescens*, *Shigella flexneri*, and *Citrobacter freundii*. The lack of a reaction in the other strains could indicate that the observed phospholipase activity in these strains is caused by a protein unrelated to PldA.

FIG. 3. Western blot analysis of cell envelope fractions with anti-PldA serum. Applied was 0.01 μg of purified *E. coli* PldA protein (a) or cell envelope fractions of *K. pneumoniae* (b), *C. freundii* (c), *Shigella flexneri* (d), *Erwinia herbicola* (e), *Serratia marcescens* (f), *Providencia stuartii* (g), *Edwardsiella tarda* (h), *Yersinia enterocolitica* (i), *Enterobacter aerogenes* (j), *S. typhimurium* (k), *P. vulgaris* (l), or *Enterobacter cloacae* (m).

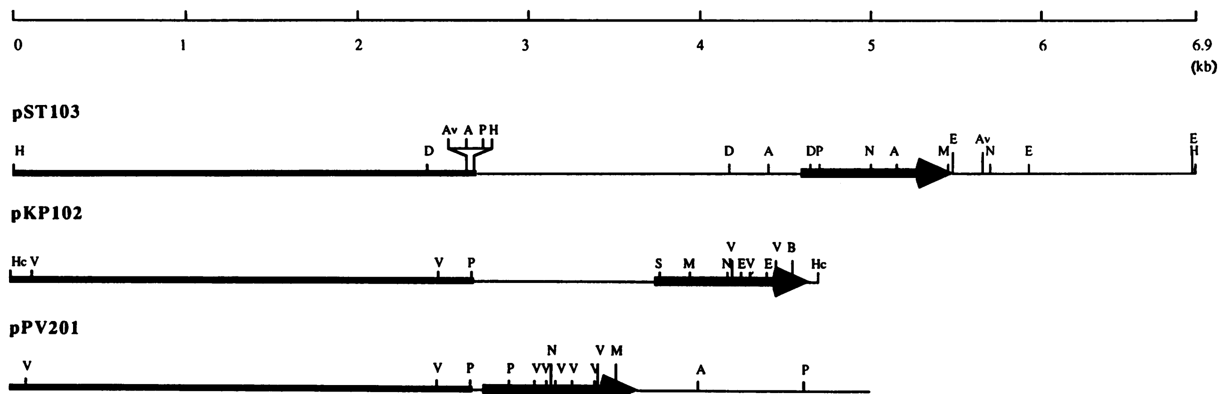

FIG. 4. Restriction maps of pST103, pKP102, and pPV201. The thick lines indicate pUC19 DNA. The thin lines indicate chromosomal DNA, and the thick arrows indicate the *pldA* genes of *S. typhimurium*, *K. pneumoniae*, and *P. vulgaris*, respectively. A, *Acc*I; Av, *Ava*I; B, *Bgl*II; D, *Nde*I; E, *Eco*RV; H, *Hin*dIII; Hc, *Hin*cII; M, *Pml*I; N, *Nru*I; P, *Pst*I; S, *Sph*I; V, *Pvu*II.

Alternatively, the immunodominant epitopes of PldA may not be conserved. Sequencing results (see below) favor the second possibility. From these results, it can be concluded that a PldA protein is present in many different enterobacterial strains.

Cloning of *pldA* genes. The *pldA* gene of *S. typhimurium* was obtained from pSPH20, which contains the *corA* gene of *S. typhimurium* together with approximately 8 kb of flanking chromosomal DNA (19). Since the *corA* and *pldA* genes are only 2.1 kb apart on the *E. coli* chromosome (13), the presence of the *S. typhimurium pldA* gene on this plasmid was considered. Transformants of *pldA* mutant strain CE1348 containing pSPH20 reacted positively in the in vivo outer membrane phospholipase activity assay. Apparently, pSPH20 contains the *S. typhimurium pldA* gene.

We also attempted to clone the *pldA* genes of strains of two species, *K. pneumoniae* and *P. vulgaris*, that did not react with the anti-PldA serum in the Western blot (Fig. 3). The putative *pldA* genes of these strains were obtained by using an in vivo cloning procedure with the RP4::miniMu3A plasmid pULB113 (51). This plasmid renders cells resistant to Ap, Km, and Tc. To select for a cloned *pldA* gene, we took advantage of the location of the *pldA* gene on the *E. coli* chromosome near the selectable marker *metE* (3, 13). Plasmid pULB113 was transferred by conjugation from donor strain MXR(pULB113) to spontaneous rifampin-resistant derivatives of *K. pneumoniae* and *P. vulgaris*, selecting for Ap^r and rifampin-resistant transconjugants. Several transconjugants were subsequently used as donor strains in a second mating with *rpsL metE pldA recA* strain CE1348 as the recipient. All streptomycin-resistant $MetE^+$ transconjugants obtained were tested in the in vivo outer membrane phospholipase activity assay. Two transconjugants that displayed phospholipase activity were further analyzed. The R′ plasmids in these transconjugants, designated pKP002 and pPV001, apparently contain, in addition to the *metE* gene, the *pldA* gene of *K. pneumoniae* or that of *P. vulgaris*, respectively. Before sequence analysis, the *S. typhimurium*, *K. pneumoniae*, and *P. vulgaris pldA* genes were subcloned from pSPH20, pKP002, and pPV001, respectively, into the multicopy cloning vector pUC19. To this end, restriction fragments containing the *pldA* genes were identified by Southern hybridization experiments using a digoxigenin-labeled part of the *E. coli pldA* gene as a probe (results not shown). Reacting fragments were purified from the gels and subcloned in pUC19, resulting in pST103, pKP102, and pPV101 (Table 1). The *pldA* gene of *P. vulgaris* was further subcloned, resulting in pPV201 (Table 1). Plasmids pST103, pKP102, and pPV201 all contained an intact *pldA* gene, since transformants of strain CE1348 carrying these plasmids all reacted positively in the in vivo outer membrane phospholipase activity assay (results not shown). Restriction maps of these plasmids were established and are depicted in Fig. 4.

Sequence analysis. The nucleotide sequences of the *pldA* genes of *S. typhimurium*, *K. pneumoniae*, and *P. vulgaris* were completely determined in both directions. These sequences are depicted in Fig. 5 and compared with the published sequence of the *E. coli pldA* gene (23). Upstream of the coding regions of the four *pldA* genes, a well conserved Shine-Dalgarno sequence (*rbs* in Fig. 5) can be discerned. Approximately 40 bp upstream of this ribosome-binding site, a putative −10 region is located. However, no DNA stretch with homology to a consensus −35 region could be identified in the appropriate position.

Daniels and coworkers (13) have analyzed the nucleotide sequence of the 84.5- to 86.5-min region of the *E. coli* genome. Upstream and oriented divergently to the *pldA* gene, an open reading frame (ORF; f161) was identified with its promoter overlapping the *pldA* promoter. The ORF, with unknown function, was assumed to encode a protein of 161 amino acid residues. In the nucleotide sequences upstream of the *S. typhimurium*, *K. pneumoniae*, and *P. vulgaris pldA* genes, a similar ORF could be identified. A start codon different from the one indicated by Daniels and coworkers (13) but conserved in all four sequences and preceded by a potential ribosome-binding site is indicated in Fig. 5. Consequently, the ORF would encode in *E. coli* a protein of only 155 amino acid residues. The putative −10 and −35 regions in the promotors of these four ORFs f161 are also highly conserved (Fig. 5). Two closely spaced or overlapping but divergently transcribed promoters are frequently found in prokaryotes and often indicate some coordinated expression.

Downstream of the stop codon of the *E. coli pldA* gene, a perfect 8-bp inverted repeat was identified, followed by a poly(T) stretch (23). Such a sequence is indicative of a rho-independent transcriptional terminator (39). The −10 region of the *recQ* gene (GAAAAT) (26) has been located in the middle of this terminator. The 8-bp inverted repeats and the intervening −10 region of *recQ* are completely conserved in the other three *pldA* sequences (Fig. 5). Also, the putative −35 region in the *recQ* promoter (TTGCAG) (26) could be identified in all four sequences, indicating that in the *S.*

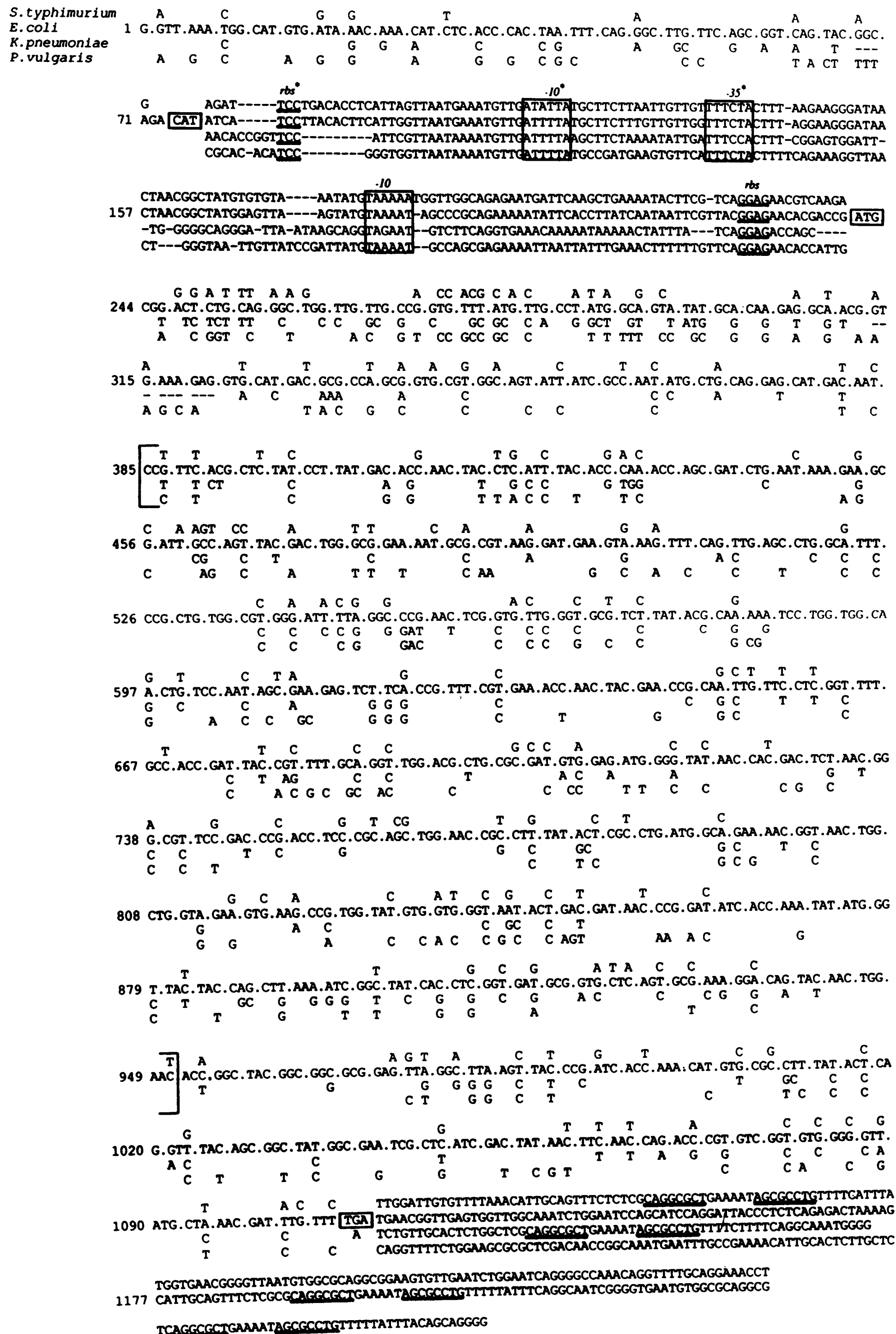

FIG. 5. Comparison of the nucleotide sequences of *pldA* genes, including their promoter areas and part of ORF f161, from *S. typhimurium*, *K. pneumoniae*, and *P. vulgaris* with the *E. coli* sequence. In the coding regions of both *pldA* and ORF f161, only those nucleotides that differ from *E. coli pldA* and ORF f161 are indicated. In the noncoding regions, complete sequences for all the genes are given. The Shine-Dalgarno sequence (*rbs*) is underlined. The translational start and stop signals as well as the putative −10 and −35 regions are boxed. Putative ribosome-binding site (*rbs*) and the −10 and −35 regions of ORF f161 are indicated by asterisks. The inverted repeats in the putative transcriptional terminators are also underlined. The part of the *E. coli pldA* gene used as probe in the Southern hybridization experiments is indicated between brackets.

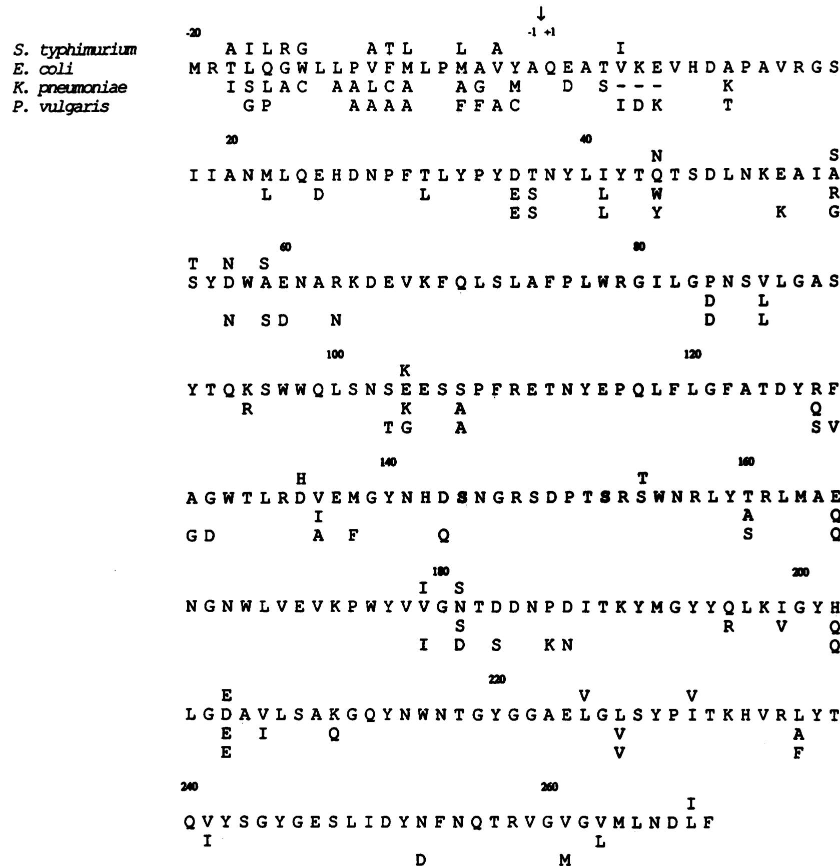

FIG. 6. Comparison of the primary structures of the PldA proteins from *S. typhimurium*, *K. pneumoniae*, and *P. vulgaris* with the primary structure of *E. coli* PldA protein. Only residues that differ from the latter sequence are shown. Dashes represent deletions of amino acids. Residues −20 to −1 represent the signal sequences. The arrow indicates the putative signal peptidase cleavage site. The serine 144 and 152 residues, discussed in the text, are indicated in boldface.

typhimurium, *K. pneumoniae*, and *P. vulgaris* genomes the *recQ* gene is located immediately downstream of the *pldA* gene.

Comparison of the primary structures of the four PldA proteins. The deduced amino acid sequences of the different PldA proteins were compared with that of the *E. coli* PldA protein (23) as depicted in Fig. 6. The N-terminal segment of 20 amino acid residues of each of the four PldA proteins has the properties of a prokaryotic signal sequence (35). Among the signal sequences of the four PldA proteins, there is a very high degree of sequence variation, without disturbing the overall character of signal sequences. In contrast, a high degree of homology was found in the mature parts of the proteins in spite of the lack of immunological detection of PldA in the cell envelopes of *K. pneumoniae* and *P. vulgaris* in the Western blots (Fig. 3). Compared with *E. coli* PldA, 94%, 88%, and 85% of the amino acid residues are identical in *S. typhimurium*, *K. pneumoniae*, and *P. vulgaris* PldA, respectively, with 79% of the amino acid residues being conserved in all four PldA proteins. The *K. pneumoniae* PldA protein has a single deletion of three amino acids in the N-terminal part of the mature protein.

DISCUSSION

Horrevoets and coworkers (25) have identified the Ser-144 residue of PldA as a catalytic center residue and suggested that it is part of a "classical" Asp-His-Ser catalytic triad found for the serine hydrolases. In all four PldA proteins, the Ser-144 residue is present. Furthermore, 4 of 5 histidine residues and 9 of 16 aspartic acid residues present in *E. coli* PldA are conserved. Since a Glu-His-Ser catalytic triad has been found recently in the lipase of *Geotrichum candidum* (42) and the *Torpedo californica* acetylcholinesterase (45), we cannot exclude a possible involvement of glutamic acid instead of aspartic acid in the catalytic center of the PldA protein. Of the 15 glutamic acid residues present in *E. coli* PldA, 8 are conserved in all four PldA proteins. Consequently, there are 17 possible candidates for the acidic component in the catalytic triad. In addition to the Ser-144 residue, Ser-152 seems to be important, since a Ser-152→Phe substitution was deleterious for enzymatic activity. This serine residue is conserved in the three other PldA proteins (Fig. 6), which underscores the notion that this residue has an important role. In view of the findings of Horrevoets et al. (25), it seems unlikely that Ser-152 is the catalytic center residue. Although folding and outer membrane assembly of the Ser-152→Phe mutant protein did not seem to be affected, it is possible that the replacement of this serine residue, close to the catalytic center, by the bulky phenylalanine results in some local distortion of protein conformation, leading to enzyme inactivation. Site-directed mutagenesis will be carried out to identify the catalytic center residues and the exact role of the Ser-152 residue.

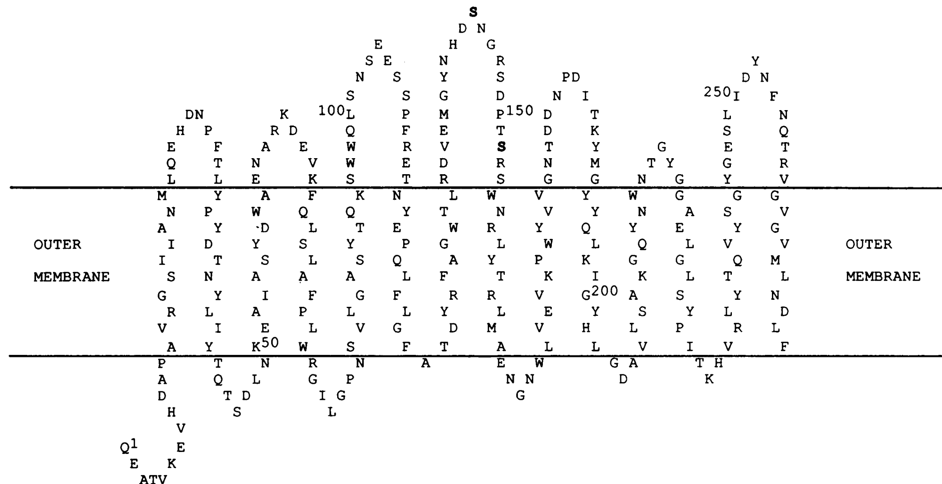

FIG. 7. Putative topology model for the *E. coli* PldA protein. The numbers refer to amino acid positions in the mature PldA protein. The serine 144 and 152 residues, discussed in the text, are indicated in boldface.

Calcium ions are absolutely required for the catalytic activity of the PldA protein (24), a property that this enzyme shares with eukaryotic phospholipases A_2 (53). In phospholipases A_2, a Gly-X-Gly-Gly sequence which forms part of a calcium-binding loop and is important for activity is present (4, 37). Interestingly, a Gly-Tyr-Gly-Gly sequence is present in all four PldA proteins (residues 219 to 222). Mutagenesis experiments will be carried out to determine whether this Gly-Tyr-Gly-Gly sequence in PldA plays a role in calcium-binding and enzymatic activity.

Because PldA protein, like the porins, lacks hydrophobic sequences long enough to span the lipid bilayer, it probably belongs to the same class of β-sheet-structured (integral) outer membrane proteins. These proteins span the lipid bilayer by several antiparallel amphipathic β-strands (10, 54). For most outer membrane proteins, like the porins (49) and OmpA (7), the highest degree of variability is located in the surface-exposed parts, with the membrane-spanning segments being rather well conserved. In the four PldA amino acid sequences, no hypervariable regions could clearly be distinguished. Previously, a model for the topology of porin PhoE has been proposed (46, 50). The recent resolution of the crystal structure (10) confirmed this topology model with respect to the number and position of the membrane-spanning β-strands. By using the same criteria as for PhoE (46), we constructed a working model for the topology of the *E. coli* PldA protein (Fig. 7). Seven hydrophilic peaks could be distinguished in the hydrophilicity profile (not shown), which were postulated to correspond to the cell surface-exposed domains. Two potential amphipathic β-strands of approximately 10 residues in length were discerned between each hydrophilic peak, and they were assumed to span the membrane (Fig. 7). All of the seven regions placed at the cell surface and most of the regions placed at the periplasmic side of the membrane contained turn predictions, according to the criteria of Paul and Rosenbusch (34). Only one turn was predicted in the membrane-spanning segments (R-G-S in the first membrane-spanning segment). In the PldA proteins of *S. typhimurium*, *K. pneumoniae*, and *P. vulgaris*, several substitutions which do not affect the hydrophobicity of the putative β-barrel are present. However, there are two exceptions, alanine 54, which is replaced by arginine in *K. pneumoniae* PldA, and glycine 130, which is replaced by aspartic acid in *P. vulgaris* PldA. These substitutions introduce hydrophilic residues in the center of the hydrophobic side of putative membrane-spanning segments. For PhoE, it has been shown that the introduction of single hydrophilic residues in the external hydrophobic core of the β-barrel was tolerated (43). On the basis of these results, we propose that the substitutions do not affect the structure and function of the PldA protein. Future investigations, e.g., by the epitope insertion method (9, 44), will be carried out to determine whether this topology model for PldA protein is correct.

The *pldA* gene can be deleted from the *E. coli* chromosome without changing the phenotype except for the lack of outer membrane phospholipase activity. Apparently, the protein is not required for *E. coli* under laboratory conditions. However, sequence analysis of several enterobacterial *pldA* genes demonstrated that the protein is well conserved among *Enterobacteriaceae*, including pathogenic species, suggesting that PldA protein might be essential for growth of the *Enterobacteriaceae* in their natural environment, possibly during the infection of host cells. Future experiments will be carried out to investigate this possibility.

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