Molecular Characterization of *pldA*, the Structural Gene for a Phospholipase A from *Campylobacter coli*, and Its Contribution to Cell-Associated Hemolysis

KATHLEEN A. GRANT,¹ IBAN UBARRETXENA BELANDIA,² NIEK DEKKER,²
PETER T. RICHARDSON,¹ AND SIMON F. PARK^{1*}

Institute of Food Research, Reading Laboratory, Reading RG6 6BZ, United Kingdom,¹ and Department of Enzymology and Protein Engineering, Centre for Biomembranes and Lipid Enzymology, Institute of Biomembranes, Utrecht University, Utrecht, The Netherlands²

Received 21 August 1996/Returned for modification 22 October 1996/Accepted 15 January 1997

A gene (*pldA*) encoding a 35.0-kDa protein with significant homology to the *Escherichia coli* outer membrane phospholipase was identified upstream of an operon encoding an enterochelin transport system in *Campylobacter coli*. The results of this study suggest that this gene encodes an outer membrane phospholipase A in *C. coli*. First, expression of the *pldA* gene product in a PldA-deficient mutant of *E. coli* led to the restoration of phospholipase A activity. The recombinant product also partitioned to the outer membrane, suggesting that it may be similarly located in *C. coli*. Second, heterologous overexpression in *E. coli*, followed by in vitro folding and purification of *C. coli* PldA, resulted in pure protein which displayed calcium-dependent lysophospholipase and phospholipase A activities in vitro. Finally, mutants of *C. coli* in which the *pldA* gene had been inactivated by allelic exchange were deficient in phospholipase A activity. Phospholipases are associated with lysis of erythrocytes by a number of bacterial pathogens. The *pldA* mutant was shown to have a reduced hemolytic activity compared to the wild-type strain, suggesting a role for the phospholipase A in the lysis of erythrocytes by *C. coli*. Since hemolysins are intimately associated with the disease-causing potential of a number of bacterial pathogens, it is likely that the phospholipase A plays some role in *Campylobacter* virulence.

The thermophilic *Campylobacter* species, particularly *Campylobacter jejuni* and *Campylobacter coli*, are established as the most frequently isolated bacteria that cause acute diarrheal disease in humans (11, 27). In view of the incidence of *Campylobacter* infection, however, there still remains a general absence of information about the mechanisms involved in the pathogenesis of infection (36). Once ingested, the bacteria are thought to colonize the intestinal mucosa, with both flagella and their corkscrew shape enabling them to penetrate this viscous environment. The importance of flagella in colonization of the mucosa has been demonstrated in studies using aflagellate mutants (6, 43). *Campylobacters* are believed to adhere to and invade intestinal epithelial cells, and adhesion to cell lines has been demonstrated in vitro (18, 41). Although *C. jejuni* and *C. coli* have been reported to produce a variety of toxins (12, 24, 30), definitive reports have not yet appeared and the involvement of these proteins in the pathogenesis of infection remains controversial (13, 28).

Hemolysins are intimately associated with the virulence of a number of bacterial pathogens (20), and there is increasing evidence that campylobacters may produce one or more hemolysins (17, 23, 29). The modes of action of hemolysins are diverse, but there are several studies which correlate the ability of bacteria to lyse erythrocytes with phospholipase activity (1, 25). These enzymes cleave membrane phospholipids into lysophospholipids, lysophosphatides, and free fatty acids, leading to membrane lysis. In this study, we report the genetic characterization of a gene (*pldA*) encoding a phospholipase A from *C. coli*. In addition, we demonstrate that this phospholipase A

activity contributes to the cell-associated hemolytic activity of *C. coli*; it is likely, therefore, that PldA plays some role in *Campylobacter* virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. *C. coli* was grown routinely at 37°C on Mueller-Hinton (MH) agar or in MH broth in a microaerobic atmosphere of 5% H₂, 10% CO₂, and 85% N₂. *Escherichia coli* strains were grown aerobically at 37°C on L medium (21) supplemented when required with ampicillin (50 to 100 µg ml⁻¹), tetracycline (10 µg ml⁻¹), and kanamycin (50 µg ml⁻¹).

Recombinant DNA techniques and nucleotide sequencing. Standard techniques, including plasmid miniprepations, restriction endonuclease digestions, ligations, and transformation into *E. coli*, were performed according to standard procedures (21). Large-scale plasmid preparations were prepared by using affinity columns as instructed by the manufacturer (Qiagen, Studio City, Calif.). DNA sequencing was performed on an Applied Biosystems model 373A DNA sequencing system. A Taq Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, Warrington, United Kingdom) was used to generate fluorescence-based dideoxy sequence reactions containing plasmid DNA as the template. The region in pS2 containing the *pldA* gene was sequenced by using primers designed to bind to opposing strands at approximately 200-base intervals, allowing sequence data to be obtained independently from both strands. Sequence information was analyzed with either the Wisconsin Molecular Biology software package (10) or the Lasergene DNA software package (DNASTAR, Madison, Wis.).

The polypeptides encoded by pCPL3 were identified by using an *E. coli* in vitro coupled transcription-translation system (Promega). Proteins were radiolabelled by incubation with 20 µCi of [³⁵S]methionine (Amersham). Polypeptides were separated on sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gels (19), using Rainbow ¹⁴C-labeled methylated markers (Amersham) as molecular size standards. Radiolabelled bands were visualized by fluorography at –70°C.

Genomic DNA, isolated by the method of Pitcher et al. (31), was digested with *Bgl*II and electrophoresed on 0.8% agarose gels. Following depurination with 250 mM HCl, the DNA was transferred from the gel to a Hybond N+ nylon membrane (Amersham), using 0.4 M NaOH. Hybridizations were performed with the insert in pCPL1 as a probe, using a nonradioactive enhanced chemiluminescence gene detection kit (Amersham) according to the manufacturer's instructions.

PCR. PCR was used to confirm the recombinational event that generated the *pldA* mutation in *C. coli* CP200. Two oligonucleotide primers, PLDA1 (5'-AA

* Corresponding author. Mailing address: Institute of Food Research, Reading Laboratory, Earley Gate, Whiteknights Road, Reading RG6 6BZ, United Kingdom. Phone: 44 (0)118 9357227. Fax: 44 (0)118 9267917. E-mail: parks@bbsrc.ac.uk.

TABLE 1. Plasmids and bacterial strains used

Plasmid or strain	Genotype or relevant characteristics	Reference or source
Plasmids		
pS2	Ap ^r , contains 24-kb of DNA upstream of <i>ceuD</i> including <i>pldA</i>	35
pCPL1	Ap ^r , contains a 1,132-base <i>Hind</i> III fragment containing <i>pldA</i>	This study
pCPL2	Ap ^r Km ^r derivative of pCPL1 with Km ^r marker internal to <i>pldA</i>	This study
pCPL3	Ap ^r , contains a 1,404-base fragment generated by PCR containing <i>pldA</i>	This study
pT7BlueR	Ap ^r , PCR cloning vector	
pT7.7	Ap ^r , T7 expression vector	38
pND4	Ap ^r , T7.7 derivative expressing PldA	This study
<i>E. coli</i> strains		
BL21(DE3)	F ⁻ <i>ompT</i> r ⁻ m ⁻ DE3	37
AM1095	F ⁻ <i>leu thi trp his pyrA lacY galK xyl ara mlI fhuA tsx rpsL recB21 recC22 sbcB15 sup</i>	5
CE1347	Δ <i>pldA</i> :Km ^r derivative of AM1095	5
CE1302	F ⁻ <i>fabB supD rpoB</i>	8
<i>C. coli</i> strains		
UA585	Parental strain	42
CP200	Km ^r PldA-deficient mutant	This study
CCH1	Tc ^r <i>ceuE</i> ::pSP120	35
CATP1	Tc ^r <i>ceuD</i> ::pSP180	35
CFED1	Tc ^r Δ (<i>ceuB-ceuD</i>)	35

CATTCCTCACCTTTAG) and PLDA2 (5'-CTTCCTCCATAGCCATTGA), were designed to amplify a 453-bp fragment internal to the *pldA* gene of *C. coli* which included the *Eco*RI insertion site for the kanamycin cassette. In addition, the DNA fragment in pCPL3 containing the intact *C. coli pldA* gene was generated by using PCR and the oligonucleotides PLDA3 (5'-CCAAATCCTTC ACATAAACATCC-3') and PLDA4 (5' GCATTATTCCAAAAACAACAA GTA-3'). A commercial *Taq* DNA polymerase system (Perkin-Elmer Cetus) was used to amplify chromosomal DNA from *C. coli* UA585 and the PldA-deficient mutant CP200 in the presence of 200 μ M deoxynucleotides and a 0.1 μ M concentration of each primer. Following an initial denaturation at 95°C for 3 min, there were 30 cycles of amplification comprising denaturing at 95°C for 30 s, annealing at 46°C for 30 s, and extension at 72°C for 1 min. There was a final extension at 72°C for 5 min.

Construction of a PldA expression plasmid. To facilitate the subcloning of the *C. coli* PldA gene in a T7 expression plasmid, a synthetic duplex of oligonucleotides CC1 (5'-TATGTCTGATGATTTGAACAAGCTTGGGGGATCC-3') and CC2 (5'-AGCTGGATCCCCAAGCTTGTTCAAATCATCAGACA-3') was cloned into the *Nde*I/*Hind*III sites of pT7.7 (38), resulting in the plasmid pT7.linkerc. Plasmid pCPL3 was digested with *Hind*III and *Bam*HI, and the 1,140-bp fragment containing the *C. coli pldA* gene was subcloned in the *Hind*III/*Bam*HI-digested pT7.linkerc vector, resulting in pND4. The nucleotide sequence of the region between the *Nde*I and *Hind*III restriction sites was verified by double-stranded DNA sequencing. The resulting construct encodes a 37.3-kDa *C. coli* PldA, which is preceded by an N-terminal extension of 21 amino acids (MSDDFEQALEFEKKEQYKAM), under control of the T7f10 promoter.

Expression and purification of PldA. The procedure for the expression and purification of the *C. coli* PldA is a modification of the method described for *E. coli* PldA (9). For the large-scale production of PldA, a 200-ml overnight culture of BL21(DE3) containing plasmid pND4 in L medium with ampicillin (50 mg/ml) was used to inoculate 3 liters of the same medium. The culture was shaken at 37°C. When the optical density at 600 nm reached 0.65, expression was induced by adding isopropylthio- β -D-galactopyranoside to a final concentration of 0.4 mM. Cells were allowed to grow for another 4 h (final absorbance of 2.5) and harvested by centrifugation for 15 min at 4,500 $\times$ g. Subsequently, cells were resuspended in 80 ml of ice-cold lysis buffer (50 mM Tris-HCl [pH 8.0], 40 mM EDTA), to which 20 mg of sucrose and 16 mg of lysozyme were added. The lysate was left on ice for 30 min; then ice-cold lysis buffer (80 ml) was added to induce an osmotic shock, followed by another 30-min incubation on ice. The lysate was sonicated at intervals to prevent heating, with a Branson Sonifier-450 fitted with a large tip at 20 W, until the lysate was no longer viscous. After the addition of alkylpolyoxyethylene glycol (Brij 35) to a final concentration of 0.1% (by mass), the lysate was sonicated briefly. The inclusion bodies were collected by centrif-

ugation for 30 min at 4,500 $\times$ g, and the pellet was washed once with 100 ml of buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The inclusion bodies (40 mg) were dissolved in 100 ml of buffer (8 M urea, 20 mM glycine [pH 8.3]). Refolding was initiated by rapid dilution of the solution of inclusion bodies to final refolding conditions (5 mM glycine [pH 8.3], 2% Octyl-POE [Bachem], 0.1 mg of protein per ml, 2 M urea). Refolding was carried out for 16 h at room temperature. The refolding mixture was loaded onto a 20-ml DEAE column equilibrated with buffer A (10 mM *n*-dodecyl-*N,N*-dimethyl-1-ammonio-3-propanesulfonate [12-SB], 20 mM Tris-HCl [pH 8.3]). The column was washed with 5 column volumes of buffer A. PldA was eluted by applying a linear gradient of 0 to 1 M KCl in buffer A (twice; 200 ml). PldA eluted at 0.18 M KCl. Fractions containing enzymatic activity were pooled and dialyzed twice against 10 volumes of buffer B (2.5 mM 12-SB, 20 mM Tris-HCl [pH 8.3]) at 4°C. After dialysis, the protein was loaded onto a second 20-ml DEAE column equilibrated with buffer C (20 mM histidine, 10 mM 12-SB [pH 6.5]). The column was washed with 5 column volumes of buffer C and eluted by applying a linear gradient of 0 to 0.5 M KCl in buffer C (twice; 200 ml). Fractions containing enzymatic activity were pooled and dialyzed twice against buffer B at 4°C. The protein was concentrated by applying the dialyzed fraction onto a 1.5-ml fast-flow Q-Sepharose column (Pharmacia) pre-equilibrated with buffer B. The protein was eluted with 1 M KCl in buffer B. Fractions containing enzymatic activity were pooled, dialyzed twice against buffer B at 4°C, and then stored at -20°C. Protein concentrations were determined spectrophotometrically. SDS-polyacrylamide gel electrophoresis (PAGE) was used to analyze the purity of the protein at the various stages during purification. Samples were mixed with loading buffer and loaded either directly or after heating for 5 min at 100°C on an 11% acrylamide gel. Gels were stained with Coomassie brilliant blue. Wild-type *E. coli* PldA was purified as described previously (9).

In vitro phospholipase assay. Phospholipase activity was measured by determining the free fatty acids released by hydrolysis of the substrate with a Radiometer titration set as described by Horrevoets et al. (16). The assay compartment contained 10 ml of buffer (5 mM Tris-HCl [pH 8.3], 25 mM CaCl₂ or 5 mM EDTA) and 5 mM substrate solubilized with 12.8 mM Triton X-100. The substrate was soya bean phosphatidylcholine purified according to standard procedures. Lysophospholipase activity was measured spectrophotometrically with 2-hexadecanoyl-thioethane-1-phosphocholine as the substrate (9). One unit corresponds to the conversion of 1 μ mol of substrate/min.

Iron acquisition assay. The ability of the *C. coli pldA* mutant to utilize hemin and enterochelin was determined as described previously (35). Briefly, bacteria were seeded in MH agar containing 56 μ M ethylenediamine di-*o*-hydroxyphenylacetic acid (EDDHA; Sigma Chemical Co.). Filter paper discs impregnated with 10 μ l of 100 μ M or 1 mM hemin (Sigma) or enterochelin (kindly provided by J. B. Neillands, University of California) were placed on the agar, and the plates were incubated for 24 h to assess the growth stimulation.

In vivo phospholipase assay. The *in vivo* phospholipase A assay was based on the method of de Geus et al. (8). Cells of *C. coli* UA585 and CP200 were streaked across MH agar plates and incubated microaerobically overnight at 37°C. The streaks of bacterial growth were then overlaid with 4 ml of 1% P1 broth agar (21) containing 0.05% Triton X-100, 200 μ g of rifampin per ml, 10 mM CaCl₂, and approximately 10⁷ cells of *E. coli* CE1302 (a *fabB* temperature-sensitive mutant) per ml. The plates were dried briefly at 37°C and incubated aerobically at 40°C. Following overnight incubation, the plates were assessed for growth of the *E. coli* indicator strain in the soft agar above the streaks of *Campylobacter*.

Isolation and analysis of outer membrane proteins. A rapid microprocedure described by Carlone et al. (7) was used to isolate detergent-insoluble outer membrane proteins from both *C. coli* and *E. coli* cells. The concentration of protein in the samples was determined by using a Microprotein determination kit (Sigma) based on the method of Bradford (4). The outer membrane proteins were analyzed by SDS-PAGE. Aliquots containing 1 to 12.5 μ g of outer membrane and whole-cell preparations were electrophoresed on SDS-12.5% polyacrylamide gels under denaturing conditions (19). Following electrophoresis, the proteins were visualized by silver staining.

Hemolysin assay. A slight modification of the contact hemolysin assay described by Pickett et al. (29) was used to assess the ability of the wild-type *C. coli* and the PldA-deficient mutant to lyse sheep erythrocytes. Cells of *C. coli* were harvested from overnight cultures prepared on MH agar plates, washed in sterile phosphate-buffered saline (PBS), and resuspended to a concentration of 10⁹ bacteria ml⁻¹. A 500- μ l aliquot of washed bacterial cells was mixed with an equal volume of sheep erythrocytes (washed and resuspended in PBS to 10⁸ ml⁻¹). The reaction mixtures were then incubated aerobically at 42°C for 3 to 4 h. After this period, the intact cells were removed by centrifugation. Hemolysis was assessed by determining the absorbance of the supernatant fraction at 540 nm. The level of spontaneous hemolysis was determined by adding an equal volume of PBS to the sheep erythrocytes, and complete hemolysis was determined by adding an equal volume of sterile distilled water. The hemolytic activities of overnight, MH broth culture supernatants were also assessed by the same assay. When necessary, the composition of PBS was adjusted by adding 100 μ M CaCl₂.

Nucleotide sequence accession number. The *C. coli* UA585 gene has been assigned GenBank accession number Y11031.

```

CCCAAACCTCTTCACATAAACATCCCTTATGCGGATTTTATCACAGCTCTGTAGCTCACTTTTGTAAGA 70
ATTGCTAGAAAAAATGAACCAATAATCGCTTTATTATTTTCTAAAGCAAAGACCCACTTTGTAGATTTT 140
GAGGATGAAAGTCCTTTTTTAAATCTTAATTTTATCAAGAATATGAGCAATTTAAAGCGAGTACGAGT 210
GAAAAAATCTTTTTTTGATATTTTTTATAGTTTGGTATTTTGGCATGATTTGAACAAGCTTTAGAA 280
TTTGAGAAAAAAGGACAATATGAAAAAGCTATGCAAATTTATAAAAAACTAGCCTTGCAAAATCTCCTA 350
      M Q I Y K K L A L Q N S P
AACAGATTTAAAGAAAAATAATGCTTCTTTACTTTCAAGAATAAAGCACGAAACACAAAATACCCAAA 420
K Q D L K E N N A S L L S R I K H E T Q N T Q K
AACACCTAGCACAAAAGAGGATTTTCGCGCATTGCTTTGGCAAATTATTAGGAGAAAAATCAAGTTTT 490
      T P S T K E D F S R I A L A N Y L G E N S S F
AATCCCTTGAATCAGCTCATATAAGATGAATTATTTCTACCTTTTGCTTATAGTTTGGCTCATTAG 560
      N P L G I S S Y K M N Y F L P F A Y S F G S L
GCGGGGAAATAGAAAACTGAAATGAAATTTCAACTCAGTATTAAAAAAGATTATTTGAAGATTGTT 630
G G E N R K T E M K F Q L S I K K R L F E D L L
AGGATTAGGTGAAAAATATTATGTAGGCTACACTCAAACATCTTGGTGGCAAACTATAAACATTCCTCA 700
      G L G E K Y Y V G Y T Q T S W W Q N Y K H S S
CCCTTTAGAGAAACAAATTATCAACCTGAATTTTTTGTAGATATTCCTTTGCATTTTGAAGATTATAAA 770
      P F R E T N Y Q P E F F V D I P L H F E D Y K
TTCTTAATAATCTAAGAGTAGGAATTTTGCATGAAAGCAATGGCAAAGGAGATGAGAATTTAGAATCACG 840
F L N N L R V G I L H E S N G K G D E N L E S R
CTCTTGAATAGAATTTATGCTTCAAGCGTGTTTTTATATCAGCGTTTTTTATTTGTTCTAGAAATTTGG 910
      S W N R I Y A S S V F L Y Q R F L F V P R I W
TATAGAAATTCCTGAAACAGCGAAGATGATGATAATCCTGAAATCACACATTATATGGGAAATTTTGATA 980
      Y R I P E N S E D D D N P E I T H Y M G N F D
TCAATATAGGGTCTTTAGGAAATGATTATTTTATAAATCTCATGCTAAGAAATAATCTTGATTTCATGA 1050
I N I G S L G N D Y F I N L M L R N N L D F H D
CAATAAAGGCGCTGTGCAAGTGGATATAGGATATGATATTTTGTAAATGGAATTTATTGGTATTACAA 1120
      N K G A V Q V D I G Y D I F D N G I Y W Y L Q
TACTTCAATGGCTATGGAGGAAGCCTTATAGATTACAACAAACGCTTACAAAGACTTTCTACGGCATTTT 1190
      Y F N G Y G G S L I D Y N K R L Q R L S T A F
TGATTCATATTAATTTAATTTTCTTTTGTAGATTAAATTAACAATCAATTGAGATTTTATAAAT 1260
      L I S Y *
TTTTATAGTTTTTAAATTATTTTATTTTAAAAATACGATTTTATTTTGTATAATTATTACTATAAGG 1330
AAAAAATGCTTTTTTAACACTTGTAGTTTAAATATCTTTTAAATTTTACTTGTGTTTTGGAATTA 1400
TAAGCTTATTTATAGGGTTATTCCTATTA 1430

```

FIG. 1. Nucleotide sequence of the *C. coli* UA585 *pldA* gene. The predicted amino acid sequence is shown below the nucleotide sequence, and the termination codon is represented by an asterisk. Recognition sites for the restriction enzymes *Hind*III and *Eco*RI are underlined.

RESULTS

Identification and cloning of the *C. coli* *pldA* gene. Earlier studies from this laboratory have described the characterization of a binding-protein-dependent transport system for the siderophore enterochelin in *C. coli* (26, 35). Plasmid pS2 was generated by plasmid rescue previously and contains part of the operon encoding the enterochelin transport system and the region upstream of this (35). To define the limits of this operon and to identify other possible virulence factors, we continued to sequence upstream of the *C. coli* chromosomal region characterized so far.

Analysis of the nucleotide sequence upstream of the *ceuB*

gene (35) revealed an open reading frame (ORF) which terminated 132 bases upstream of the ATG start codon of *ceuB* (Fig. 1). Although there is no clear translational initiation sequence for the ORF, the most likely start, based on the size of the protein product produced in in vitro transcription-translation systems, is the codon ATG at base 311 (Fig. 1). The corresponding ORF consists of 894 bases and has the potential to encode a protein of 297 amino acids with a molecular mass of 35.0 kDa. The G+C content of the coding sequence is 30.1%, which is in accordance with the low G+C content (32.5%) of all *Campylobacter* species (3). An alternative translational start for the ORF would be the codon GTG at position

FIG. 2. Comparison of the primary structure of the putative phospholipase A from *C. coli* with those of the proteins from *E. coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, and *Salmonella typhimurium* (5). Residues conserved in at least two of the amino acid sequences are shown in white type.

209; although this is an unusual start codon, its use in *C. jejuni* has been reported (22). However, this ORF would have the potential to encode a protein of 331 amino acids with a molecular mass of 39.2 kDa, which does not compare well the size of the protein product observed in expression studies. Furthermore, the 16 amino acids at the amino terminus of the predicted protein derived from this ORF have the characteristics of a lipoprotein signal sequence (32, 39). Accordingly, it consists of a valine residue followed by two lysine residues and then a predominantly hydrophobic region of 13 amino acids, ending with the sequence LVFC, which is distantly homologous to a signal peptidase II cleavage site. However, in vivo labelling studies using [3 H]palmitate have demonstrated that the recombinant protein is not expressed as a lipoprotein in *E. coli* (data not shown).

A search of the GenBank database with the predicted protein did not reveal significant homology to previously described components of siderophore transport systems. Instead, the putative gene product showed significant homology (27.4% identity) with the outer membrane, phospholipase A protein from *E. coli*, and those from other members of the family *Enterobacteriaceae* (5) (Fig. 2) and was accordingly designated *pldA*.

To confirm the nature and activity of the product of the *pldA* gene, it was necessary to subclone the region of pS2 containing *pldA*. Accordingly, the ORF and proximal DNA were amplified by PCR, using oligonucleotides PLDA3 and PLDA4, and cloned into pT7Blue to generate pCPL3.

Expression of PldA in *E. coli* and its localization within the outer membrane. The size of the protein specified by pCPL3 was determined by using an in vitro coupled transcription-translation system. pCPL3 specified a 35.0-kDa protein that was not expressed from the parental vector pT7Blue (Fig. 3a). The size of this protein is very similar to that predicted for the product of the *pldA* gene, and it is most likely that this band represents PldA. pCPL3 was next introduced into *E. coli* CE1347, a *pldA* mutant, to determine whether the *C. coli* *pldA* gene product could complement the PldA-deficient phenotype. The *E. coli* transformants were then assessed for phospholipase activity by using an in vivo phospholipase assay (8). Phospholipase A activity degrades phospholipids and releases free fatty acids. *E. coli* CE1302 is a fatty acid biosynthesis-defective mutant which lyses above 40°C if not provided unsaturated fatty acids. Consequently, the *E. coli* mutant will grow, at the nonpermissive temperature, as an indicator strain when it is cocultured with a bacterial strain expressing phospholipase A activity (see Fig. 5). No growth of the fatty acid biosynthesis mutant occurred above a streak of *E. coli* CE1347 cells containing the parental plasmid. However, when a streak of *E. coli* cells containing pCPL3 was overlaid with *E. coli* CE1302, growth of the indicator strain was apparent in the soft agar above the streak, demonstrating the expression of phospholipase A activity in this strain (Table 2).

The *E. coli* PldA protein is known to be localized in the outer membrane (15). Consequently, outer membrane prepa-

FIG. 3. Expression of the *C. coli* *pldA* gene. (a) Analysis of proteins expressed from plasmid pCPL3. The fluorograph shows [³⁵S]methionine-labelled proteins expressed in an in vitro coupled transcription-translation system using pCPL3 (lane A) and the parental plasmid pT7Blue (lane B) as templates. (b) Electrophoretic analysis of outer membrane proteins from *E. coli* CE1347 transformants. Bacterial outer membrane protein extracts prepared from cells containing pT7Blue (lane A) or pCPL3 (lane B) were separated on SDS-12.5% polyacrylamide gels and visualized by silver staining. The sizes of molecular size standards are shown at the left. The arrows mark the positions of the recombinant PldA.

rations from *E. coli* CE1347 cells containing pCPL3 were analyzed by SDS-PAGE. A 33.7-kDa protein was observed in the outer membrane protein extracts of *E. coli* transformants harboring pCPL3 but not in extracts derived from cells containing the parental plasmid pT7Blue (Fig. 3b). This finding suggests that the cloned PldA is located in the outer membrane of *E. coli* and, accordingly, may be similarly located in *C. coli*.

Expression and purification of PldA. Recently it has been shown that *E. coli* PldA can be efficiently produced in the T7 expression system (9). A similar approach was used for the overexpression and purification of *C. coli* PldA. Accordingly, the *pldA* gene was subcloned in a pT7.7 derivative, resulting in a construct encoding PldA with a short N-terminal extension. Protein expression in strain BL21(DE3) resulted in the accumulation of PldA as inclusion bodies. The expression level was estimated from SDS-PAGE to be 13 mg/liter. To isolate the

inclusion bodies, cells were lysed and the insoluble inclusion bodies were collected by centrifugation. PldA is the major protein in this fraction and migrates at around 39 kDa on SDS-PAGE. To derive enzymatically active protein from the inclusion bodies, PldA had to be folded in vitro. The structure of *E. coli* PldA is very stable, and after mixing with SDS-PAGE sample buffer, the protein remains folded. Only after boiling does the protein completely unfold. As a result, the folded protein migrates faster on SDS-PAGE than the unfolded form (5), a phenomenon generally referred to as heat modifiability (14). *C. coli* PldA displayed heat modifiability, indicating similarities in protein structure. The heat modifiability allowed for the separation of the folded from the unfolded form after the in vitro folding step on an analytical scale. In this way, the efficiency of the refolding was analyzed, and this step was optimized toward the protein, detergent, and urea concentrations (data not shown). The optimal refolding conditions were very similar to those for *E. coli* PldA, but only 10% folding was achieved. On a large scale, 40 mg of PldA was refolded, and SDS-PAGE revealed a faint band for the folded protein at 30 kDa (data not shown). On the first DEAE column, the protein eluted as a single broad peak. Enzymatic activity could be measured in the chromogenic assay, and fractions with active protein were pooled and dialyzed. A total of 14 mg of protein was obtained, of which 50% was folded. The unfolded material present could be removed on the second DEAE column, after which the PldA was over 90% pure. The protein was concentrated on Q-Sepharose and stored at -20°C . The purification method resulted in the isolation of 7.3 mg of *C. coli* PldA from 3 liters of culture. The protein was over 90% pure and fully heat modifiable.

Enzymatic activity of *C. coli* PldA. Enzymatic activity of the purified *C. coli* PldA was measured with soya bean lecithin to assay the phospholipase A activity. The *C. coli* PldA had a specific activity of 6 U/mg, which is 30% of the specific activity of *E. coli* PldA under these conditions (28 U/mg). The lyso-phospholipase activity of *C. coli* PldA was measured by using a mono-acyl phospholipid analog (2-hexadecanoyl-thioethane-1-phosphocholine) as a substrate. A specific activity of 18 U/mg, which is 25% of the activity of the *E. coli* enzyme in this assay (70 U/mg), was determined for *C. coli* PldA. When in the assays calcium was replaced by EDTA, the enzymatic activity of *C. coli* PldA was completely abolished, indicative of an absolute calcium requirement for enzymatic hydrolysis.

TABLE 2. Phospholipase A and hemolytic activities in strains of *E. coli* and *C. coli*

Strain	Characteristic	Phospholipase A activity ^a	Hemolytic activity ^b	
			PBS	PBS + 100 μM Ca^{2+}
<i>E. coli</i> AM1095	PldA ⁺	+	0.3 $\pm$ 0.1	ND
<i>E. coli</i> /pT7Blue.CE1347	PldA ⁻	-	1.0 $\pm$ 0.1	ND
<i>E. coli</i> /pCPL3.CE1347	PldA ⁺	+	1.4 $\pm$ 0.2	ND
<i>C. coli</i> strains ^c				
UA585	Parental strain	+	12.9 $\pm$ 1.4	0.2 $\pm$ 0.1
CP200	PldA ⁻	-	0.6 $\pm$ 0.1	0.2 $\pm$ 0.1
CCH1	<i>ceuE</i>	+	20.4 $\pm$ 3.4	ND
CATP1	<i>ceuD</i>	+	16.4 $\pm$ 3.3	ND
CFED1	$\Delta(\text{ceuB-ceuD})$	+	24.4 $\pm$ 4.1	ND

^a The results of an in vivo phospholipase assay are indicated as positive (+) and negative (-).

^b Measured as the A_{540} of supernatant solutions and expressed as a percentage of complete hemolysis. Values represent means and standard deviations of triplicate assays from a typical experiment. ND, not determined.

^c CCH1, CATP1, and CFED1 have mutations in different genes of the enterochelin uptake operon (35). Complete hemolysis had a measured A_{540} of 4.06 ± 0.060 .

FIG. 4. Confirmation of the disruption of the *C. coli* *pldA* gene in CP200 by Southern analysis (a) and by PCR analysis (b). (a) Plasmid pCPL1, containing the cloned *C. coli* *pldA* gene, was labelled and used to probe genomic DNA digested with *Bgl*III from *C. coli* UA585 (lane A) and *C. coli* CP200 (lane B). (b) PCR was performed with the *pldA*-specific primers PLDA1 and PLDA2 with 100 ng of purified genomic DNA derived from *C. coli* UA585 (lane A) and *C. coli* CP200 (lane B). The sizes of molecular size standards are shown at the left.

Generation of a PldA-deficient mutant of *C. coli* by allelic exchange. To confirm the nature and activity of the product of the *pldA* gene in *C. coli*, the ORF was disrupted by the introduction of an antibiotic resistance cassette. A *Hind*III fragment, containing the *pldA* gene sequence, was excised from pS2 (35) and ligated into the vector pGEM-13ZF (Promega), to give the plasmid pCPL1. A 1.5-kb *Eco*RI kanamycin resistance cassette (17a) was then inserted into the unique *Eco*RI site at position 915 (Fig. 1). The resultant plasmid, pCPL2, was then introduced into *C. coli* UA585 by natural transformation as described previously (42). Kanamycin-resistant transformants were obtained at a frequency of 268 transformants per μ g of DNA. Genomic DNA from one of the *C. coli* transformants, designated CP200, and from the parental *C. coli* strain was digested with *Bgl*III and probed with the DNA insert present in pCPL1. The probe hybridized to a single *Bgl*III fragment in the DNA of both strains, as is expected since there are no sites for *Bgl*III internal to *pldA*. However, with the DNA derived from CP200, the fragment was 1.5 kb larger (Fig. 4). This can be explained if allelic exchange of the wild-type *pldA* DNA with the disrupted sequence in pCPL2, containing the 1.5-kb kanamycin resistance cassette, has occurred as anticipated. Further evidence of the mutant genotype was provided by PCR analysis. Oligonucleotide primers PLDA1 and PLDA2 were designed to amplify the region of the *pldA* gene containing the inserted kanamycin resistance cassette. When genomic DNA from *C. coli* CP200 was used as a target in a PCR containing the two primers, an amplification product of approximately 2,000 bases was obtained. In contrast, a PCR product of approximately 500 bases was obtained with wild-type DNA (Fig. 4). Again this difference is consistent with the presence of the 1.5-kb kanamycin resistance cassette in the *pldA* gene of the mutant.

Biochemical characterization of *C. coli* *pldA* isogenic mutant. The nucleotide sequence encoding the *C. coli* *pldA* gene lies immediately upstream of the operon encoding components

of the enterochelin uptake system (35). Because of its location, it was necessary to determine whether the putative *pldA* gene encoded a protein with a role in enterochelin acquisition. The ability of the *pldA* mutant to utilize enterochelin and hemin as iron sources was therefore assessed by adding cells to MH agar containing EDDHA, which binds iron, making it unavailable to *C. coli*. Both mutant and wild-type cells were able to grow when either 1 mM enterochelin or 100 μ M hemin was provided as an exogenous source of iron (data not shown), demonstrating that PldA does not play a role in the uptake of iron-containing compounds. The phenotype of CP200 cells was next assessed using the *in vivo* phospholipase assay. When a streak of wild-type *C. coli* was overlaid with *E. coli* CE1302, growth of the indicator strain was apparent in the soft agar above the streak, demonstrating the expression of phospholipase activity in these *Campylobacter* cells. In contrast, no growth of the fatty acid biosynthesis mutant occurred above the streak of *C. coli* CP200 cells, confirming the loss of phospholipase activity in the *pldA* mutant (Fig. 5). Since the recombinant protein partitions into the outer membrane when it is expressed in *E. coli*, it seems likely that PldA is localized in the *C. coli* outer membrane. However, we were unable to detect any differences in outer membrane protein profiles of wild-type and PldA-deficient cells (data not shown), suggesting that the protein is expressed at low levels. To eliminate the possibility that the reduction of phospholipase activity was due to a polar effect of the *pldA* mutation on the expression of downstream genes, three *C. coli* strains with mutations in different genes of the enterochelin uptake operon were assayed for phospholipase activity, since these lie immediately downstream of *pldA*. There was no loss of phospholipase activity with any of the three mutant strains compared with the wild-type *C. coli* (Table 2).

A hemolytic activity associated with PldA. Phospholipases can cleave membrane-bound phospholipids into lysophosphatides and free fatty acids, leading to membrane lysis. Consequently, hemolytic activity has often been associated with phospholipases in other bacterial pathogens (1, 25, 44). To assess whether PldA from *C. coli* was responsible for a cell-associated hemolytic activity reported in campylobacters (29), the ability of the wild-type and *pldA* mutant strains to hemolyze sheep erythrocytes was examined by using a contact hemolysin assay. Wild-type cells of *C. coli* were hemolytic, giving rise to 12.9 U of hemolytic activity (Table 2). In contrast, the *pldA* mutant was significantly less hemolytic and gave rise to

FIG. 5. Confirmation of the phospholipase-deficient phenotype of CP200 in an *in vivo* phospholipase assay. Streaks of growth of *C. coli* UA585 (A) and *C. coli* CP200 (B) were overlaid with 4 ml of 1% P1 broth agar containing 10^7 cells of *E. coli* CE1302 per ml. Growth of the *E. coli* indicator strain occurs only in the presence of cells possessing phospholipase A activity.

only 0.6 U of hemolytic activity, demonstrating that the phospholipase-deficient phenotype affected the ability of *C. coli* cells to lyse sheep erythrocytes. The parental PldA⁺ strain of *E. coli* gave rise to 0.3 U of hemolytic activity, compared to 1.0 U measured for the isogenic PldA-deficient mutant CE1347, and thus the *E. coli* PldA does not appear to be involved in hemolysis. Cells of CE1347 containing pCPL3, and expressing *C. coli* PldA, gave rise to 1.4 U of hemolytic activity and therefore were not significantly hemolytic.

As calcium is reported to be an absolute requirement for the activity of phospholipase A in *E. coli* (8), the hemolysin assays were repeated in the presence of 100 μ M CaCl₂. In the presence of calcium, however, the hemolytic activity of the wild-type cells was markedly reduced. Accordingly, hemolytic activity in the presence of calcium was 0.2 U, compared to 12.9 U measured in the absence of this cation (Table 2). It is interesting that inhibition of bacterial hemolysis by the presence of calcium has been reported previously for *Helicobacter pylori* (1).

When tested for the presence of hemolysins, culture supernatants from broth-cultured cells of wild-type *C. coli* and the PldA-deficient mutant gave rise to 16.1 and 11.0 U, respectively, of hemolytic activity (data not shown). This result suggests that there is an additional hemolytic activity expressed by *C. coli* cells that is independent of PldA and is not cell associated but secreted into the medium. Some bacterial hemolysins are expressed fully only in cells growing in the logarithmic phase, not in stationary-phase cells. Accordingly, hemolysis was assessed in cells isolated from these growth phases. The hemolytic activity of *C. coli* cells was not, however, growth phase dependent, as cells representative of the logarithmic phase and of the stationary phase had similar hemolytic titers (data not shown).

The disruption of the *pldA* gene with an antibiotic marker did not affect enterochelin acquisition and does not, therefore, have a negative effect on the expression of downstream genes. Furthermore, there is a transcriptional terminator between the end of the *ceuBCDE* operon and the likely transcriptional start for the only gene (*orfA*) downstream of *ceuE* that is in an orientation which is likely to be affected by a mutation in *pldA* (35). This finding suggests that this gene is transcribed independently of both *pldA* and *ceuBCDE*. Nevertheless, to eliminate the possibility that the reduction in hemolytic activity was due to a polar effect of the *pldA* mutation on the expression of downstream genes, three *C. coli* strains with mutations in different genes of the enterochelin uptake operon were assayed for hemolytic activity. There was no loss of hemolytic activity with any of the three mutant strains compared with the wild-type *C. coli* (Table 2). This result is further evidence that the reduction in hemolytic activity in the *pldA* mutant is due to the absence of phospholipase A activity.

DISCUSSION

To define the upstream limit of the operon encoding an enterochelin transport system and to identify other possible virulence factors, we have characterized the chromosomal region upstream of the genes encoding the part of the system identified previously (35). Analysis of the nucleotide sequence upstream of the *ceuB* gene revealed an ORF, the predicted protein sequence of which did not reveal any significant homology to previously described outer membrane siderophore receptors, demonstrating that the gene encoding this component must lie elsewhere in the chromosome. The putative gene product did, however, show significant homology with the

outer membrane, phospholipase A protein from *E. coli* (5) (Fig. 2).

Very few phospholipase A proteins from bacteria have been characterized in detail (40). An exception is the phospholipase A encoded by the *pldA* gene of *E. coli*, which is known to be an outer membrane protein (15) with a broad specificity range. Accordingly, several activities reside in this enzyme, including those of phospholipase A₁, phospholipase A₂, 1-acyl and 2-acyl lysophospholipases, and lipase (16). Although the precise function of this enzyme in *E. coli* is unknown, it appears to be well conserved among *Enterobacteriaceae*, including pathogenic species, and may be essential for growth in the natural environment or during the infection of host cells (5). A number of lines of evidence point to the fact that the *C. coli pldA* gene encodes a phospholipase A with an activity similar to that of the *E. coli* protein. First, expression of the cloned *C. coli pldA* gene in a PldA-deficient strain of *E. coli* results in the restoration of phospholipase A activity in the mutant. Second, to demonstrate the phospholipase activity of *C. coli* PldA, the protein was overexpressed in *E. coli*, in vitro folded, and purified to homogeneity. The purified *C. coli* PldA displayed calcium-dependent lysophospholipase and phospholipase A activities. Finally, mutants of *C. coli* in which the *pldA* gene has been inactivated by allelic exchange are deficient in phospholipase A activity. Although the precise location of the PldA protein within *C. coli* has yet to be determined, the finding that the recombinant product partitioned to the outer membrane of *E. coli* suggests that it may be similarly located in *C. coli*. In *E. coli*, the PldA protein is thought to span the outer membrane by a series of antiparallel, amphipathic β -strands (5). In this respect, the predicted amino acid sequence of the *C. coli* PldA protein has hydrophobic regions analogous to the short hydrophobic regions in the *E. coli* PldA protein, suggesting that it traverses the outer membrane in a similar manner.

Phospholipases have often been implicated as virulence factors in other bacterial pathogens. For example, in *Rickettsia prowazekii*, an obligate intracellular parasite, phospholipase A is thought to be involved in bacterial escape from the host cell phagosome (25). Furthermore, there are several reports which correlate the ability of bacteria to lyse erythrocytes with phospholipase activity (1, 25, 44). There is now increasing evidence that campylobacters may produce one or more hemolysins (17, 23, 29). As part of this study, we have demonstrated that the phospholipase A activity contributes to the cell-associated hemolytic activity of *C. coli*. Accordingly, PldA-deficient cells exhibited a 20-fold decrease in hemolytic activity compared to cells of the parental strain. Furthermore, the hemolytic activity affected by the *pldA* mutation appears to be cell associated, consistent with the probable location of PldA in the *C. coli* outer membrane. It seems likely, therefore, that the reduced hemolytic activity of the PldA-deficient mutant results from the loss of phospholipase A which acts directly on the phospholipids of the erythrocyte membrane to induce damage and lysis. Alternatively, and less likely, the reduced hemolytic activity of the PldA-deficient mutant may reflect differences in cell lysis or in the secretion of other cellular components. In *E. coli*, for example, the PldA protein has been implicated in generating small holes in the outer membrane to allow the escape of bacteriocins. Accordingly, PldA-deficient mutants of *E. coli* excrete bacteriocins less efficiently (33). If, however, the *pldA* mutation in *C. coli* reduced the excretion of a hemolysin in an analogous manner, then a difference in the hemolytic activities of the culture supernatants would be expected. Since no significant difference in the hemolytic titers of the supernatants from wild-type and PldA-deficient cells was apparent, it is unlikely that PldA mediates its effect on hemolysis by effecting

the secretion of a hemolysin. Furthermore, it is unlikely that the variation in hemolysis is a result of differences in bacterial growth or in the spontaneous lysis of cells during the assessment of hemolysis since viable counts of the parental strain and the *pldA* mutant were equivalent when measured after the period of the assay (data not shown). When the *C. coli* PldA was expressed in *E. coli*, it appeared to partition into the outer membrane and was enzymatically active. However, *E. coli* cells expressing the recombinant protein did not demonstrate increased hemolytic activity, suggesting that the hemolytic activity requires additional factors, other than PldA, in *C. coli*.

Calcium ions are an absolute requirement for phospholipase A activity (2, 16). Indeed, the activity of the *C. coli* PldA showed an absolute requirement for this cation. Consequently, the presence of an excess of calcium may be expected to enhance the hemolytic activity of *C. coli* cells if this is due to the phospholipase A. In contrast, the hemolytic activity of the wild-type cells was actually reduced in the presence of Ca^{2+} . The reason for this reduction in hemolytic activity is unclear at present. It seems likely, however, that in the absence of exogenous Ca^{2+} , there are sufficient concentrations of this cation, in the buffers used for the hemolysis assay or at the cell surface, to allow phospholipase A activity. It is possible, therefore, that Ca^{2+} interferes with the attachment of *C. coli* cells to the erythrocytes, a necessary step for hemolysis since the phospholipase A appears to be cell associated. It is interesting, however, that the inhibition of bacterial hemolysis by the presence of calcium has been reported previously for *H. pylori* (1).

The level of hemolysis measured in the parental strain *C. coli* UA585 is lower than those measured for some bacterial pathogens. However, campylobacters are considered to be only weakly hemolytic (17, 23, 29) and do not form readily identifiable zones of hemolysis on blood agar plates (17). Indeed, when a number of *Campylobacter* strains were assessed for hemolytic activity, they all gave rise to levels of hemolysis which were statistically equivalent to that measured for *C. coli* UA585 (data not shown). Furthermore, the hemolytic activity measured for *C. coli* UA585 is similar to those reported previously for a number of campylobacter strains (29). Given this broad equivalence in hemolytic titers, it seems likely that PldA also contributes to the hemolytic activities of other strains of *Campylobacter*.

In view of the incidence of *Campylobacter* diarrheal disease, there still remains a general absence of information about the mechanisms involved in the pathogenesis of infection. Since hemolysins are intimately associated with the disease-causing potential of a number of bacterial pathogens, it is likely that PldA plays some role in *Campylobacter* virulence. This study represents the first step toward the characterization of phospholipase A activity and its association with pathogenicity in campylobacters.

ACKNOWLEDGMENTS

We are grateful to the Department of Health, London, England, for supporting this work.

We thank Ronald Brok for useful discussions.

REFERENCES

1. Ansorg, R., R. Rein, A. Spies, and G. von Reckling. 1993. Cell-associated hemolytic activity of *Helicobacter pylori*. Eur. J. Clin. Microbiol. Infect. Dis. 12:98–104.
2. Bekkers, A. C. A. P. A., P. A. Franken, E. Toxopeus, H. M. Verheij, and G. H. de Haas. 1991. The importance of glycine-30 for enzymatic activity of phospholipase A₂. Biochim. Biophys. Acta 1076:374–378.
3. Belland, R. J., and T. J. Trust. 1982. Deoxyribonucleic acid sequence relationship between members of the genus *Campylobacter*. J. Gen. Microbiol. 128:2515–2522.
4. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.
5. Brok, R. G. P. M., E. Brinkman, R. van Bostel, A. C. A. P. A. Bekkers, H. M. Verheij, and J. Tommassen. 1994. Molecular characterization of enterobacterial *pldA* genes encoding outer membrane phospholipase A. J. Bacteriol. 176:861–870.
6. Caldwell, M. B., P. Guerry, E. C. Lee, J. P. Burans, and R. I. Walker. 1985. Reversible expression of flagella in *Campylobacter jejuni*. Infect. Immun. 50:941–943.
7. Carlone, G. M., M. L. Thomas, H. S. Rumschlag, and F. O. Sottnek. 1986. Rapid microprocedure for isolating detergent-insoluble outer membrane proteins from *Haemophilus* species. J. Clin. Microbiol. 24:330–332.
8. de Geus, P., I. van Die, H. Bergmans, J. Tommassen, and G. de Haas. 1983. Molecular cloning of *pldA*, the structural gene for outer membrane phospholipase of *E. coli* K12. Mol. Gen. Genet. 190:150–155.
9. Dekker, N., K. Merck, J. Tommassen, and H. M. Verheij. 1995. *In vitro* folding of *Escherichia coli* outer-membrane phospholipase A. Eur. J. Biochem. 232:214–219.
10. Devereux, J., P. Haeberli, and P. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12:387–395.
11. Griffiths, P. L., and R. W. A. Park. 1990. *Campylobacter* associated with human disease. J. Appl. Bacteriol. 69:281–301.
12. Guerrant, R. L., C. A. Wanke, R. A. Pennie, L. J. Barret, A. A. M. Lima, and A. D. O'Brien. 1987. Production of a unique cytotoxin by *Campylobacter jejuni*. Infect. Immun. 55:2526–2530.
13. Guerrant, R. L., G. Fang, R. A. Pennie, and R. D. Pearson. 1992. *In vitro* models for studying *Campylobacter jejuni* infections, p. 160–167. In I. Nachamkin, M. J. Blaser, and L. S. Tompkins (ed.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D.C.
14. Heller, K. B. 1978. Apparent molecular masses of a heat-modified protein from the outer membrane of *Escherichia coli* in gels with different acrylamide concentrations. J. Bacteriol. 134:1181–1183.
15. Homma, H., T. Kobayashi, N. Chiba, K. Karasawa, H. Mizushima, I. Kudo, K. Inoue, H. Ikeda, M. Sekiguchi, and S. Nojima. 1984. The DNA sequence encoding *pldA* gene, the structural gene for detergent-resistant phospholipase A of *Escherichia coli*. J. Biochem. 96:1655–1664.
16. Horrevoets, A. J. G., T. M. Hackeng, H. M. Verheij, R. Dijkman, and G. H. de Haas. 1989. Kinetic characterization of *Escherichia coli* outer membrane phospholipase A using detergent-lipid micelles. Biochemistry 28:1139–1147.
17. Hossain, A., D. E. S. Stewart-Tull, and J. H. Freer. 1993. Heat-labile and heat-stable hemolysins of *Campylobacter jejuni*. FEMS Immunol. Med. Microbiol. 6:331–340.
- 17a. Ketley, J. M. (University of Leicester). Unpublished data.
18. Konkel, M. E., and L. A. Jones. 1989. Adhesion to and invasion of HEp-2 cells by *Campylobacter* spp. Infect. Immun. 57:2984–2990.
19. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.
20. Ludwig, A., and W. Goebel. 1991. Genetic determinants of cytolytic toxins from Gram-negative bacteria, p. 117–186. In J. E. Alouf and J. H. Freer (ed.), Source book of bacterial protein toxins. Academic Press, London, England.
21. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. Miller, S., E. C. Pesci, and C. L. Pickett. 1994. Genetic organization of the region upstream from the *Campylobacter jejuni* flagellar gene *flhA*. Gene 146:31–38.
23. Misawa, N., K. Hirayama, K. Itoh, and E. Takahashi. 1995. Detection of alpha- and beta-hemolytic-like activity from *Campylobacter jejuni*. J. Clin. Microbiol. 33:729–731.
24. Moore, M. A., M. J. Blaser, G. I. Perez-Perez, and A. D. O'Brien. 1988. Production of Shiga-like cytotoxin by *Campylobacter*. Microb. Pathog. 4:455–462.
25. Ojcius, D. M., M. Thibon, C. Mounier, and A. Dautry-Varsat. 1995. pH and calcium dependence of hemolysis due to *Rickettsia prowazekii*: comparison with phospholipase activity. Infect. Immun. 63:3069–3072.
26. Park, S. F., and P. T. Richardson. 1995. Molecular characterization of a *Campylobacter jejuni* lipoprotein with homology to periplasmic siderophore-binding proteins. J. Bacteriol. 177:2259–2264.
27. Penner, J. L. 1988. The genus *Campylobacter*. A decade of progress. Clin. Microbiol. Rev. 1:157–172.
28. Perez-Perez, G. I., N. D. Taylor, P. D. Echeverria, and M. J. Blaser. 1992. Lack of evidence of enterotoxin involvement in pathogenesis of *Campylobacter* diarrhea, p. 184–192. In I. Nachamkin, M. J. Blaser, and L. S. Tompkins (ed.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D.C.
29. Pickett, C. L., T. Auffenberg, E. C. Pesci, V. L. Sheen, and S. S. D. Jusuf. 1992. Iron acquisition and hemolysin production by *Campylobacter jejuni*. Infect. Immun. 60:3872–3877.
30. Pickett, C. L., E. C. Pesci, D. L. Cottle, G. Russel, A. N. Erdem, and H.

- Zeytin.** 1996. Prevalence of cytolethal distending toxin production in *Campylobacter jejuni* and relatedness of *Campylobacter* sp. *cdtB* genes. *Infect. Immun.* **64**:2070–2078.
31. **Pitcher, D. G., N. A. Saunders, and R. J. Owen.** 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett. Appl. Microbiol.* **8**:151–156.
 32. **Pugsley, A. P.** 1993. The complete general secretory pathway in gram-negative bacteria. *Microbiol. Rev.* **57**:50–108.
 33. **Pugsley, A. P., and M. Scharwitz.** 1984. Colicin E2 release: lysis, leakage or secretion? Possible role of phospholipase. *EMBO J.* **3**:2393–2397.
 34. **Renetseder, R., S. Brunie, B. W. Dijkstra, J. Drenth, and P. B. Sigler.** 1985. A comparison of the crystal structures of phospholipase A₂ from bovine pancreas and *Crotalus atrox* venom. *J. Biol. Chem.* **260**:11627–11634.
 35. **Richardson, P. T., and S. F. Park.** 1995. Enterochelin acquisition in *Campylobacter coli*: characterization of components of a binding-protein-dependent transport system. *Microbiology (Reading)* **41**:3181–3191.
 36. **Ruiz-Palacios, G. M.** 1992. Pathogenesis of *Campylobacter* infection: in vitro models, p. 158–159. *In* I. Nachamkin, M. J. Blaser, and L. S. Tompkins (ed.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D.C.
 37. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**:113–130.
 38. **Tabor, S.** 1990. Expression using the T7 RNA polymerase/promotor system, p. 16.2.1–16.2.11. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), *Current protocols in molecular biology*. Greene Publishing and Wiley Interscience, New York, N.Y.
 39. **von Heijne, G.** 1989. The structure of signal peptides from bacterial lipoproteins. *Protein Eng.* **2**:531–534.
 40. **Waite, M.** 1987. Bacterial acyl hydrolases (phospholipases A, B, and lysophospholipases), p. 17–28. *In* D. J. Hanahan (ed.), *Handbook of lipid research*, vol. 5. The phospholipases. Plenum Press, London, England.
 41. **Walker, R. L., M. B. Caldwell, E. C. Lee, P. Guerry, T. J. Trust, and G. M. Ruiz-Palacios.** 1986. Pathophysiology of *Campylobacter* enteritis. *Microbiol. Rev.* **50**:81–93.
 42. **Wang, Y., and D. E. Taylor.** 1990. Natural transformation in *Campylobacter* species. *J. Bacteriol.* **172**:949–955.
 43. **Wassenaar, T. M., N. M. C. Bleumink-Pluym, and B. A. M. van der Zeijst.** 1991. Inactivation of *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that *flaA* but not *flaB* is required for invasion. *EMBO J.* **10**:2055–2061.
 44. **Winkler, H. H., and E. T. Miller.** 1980. Phospholipase A activity in the hemolysis of sheep and human erythrocytes by *Rickettsia prowazekii*. *Infect. Immun.* **29**:316–321.

Editor: P. E. Orndorff