INFECTION AND IMMUNITY, Aug. 1999, p. 4008–4013 Vol. 67, No. 8
0019-9567/99/$04.00+0

Cloning, Sequencing, and Role in Virulence of Two Phospholipases (A1 and C) from Mesophilic *Aeromonas* sp. Serogroup O:34

SUSANA MERINO,[1] ALICIA AGUILAR,[1] MARIA MERCEDES NOGUERAS,[1] MIGUEL REGUE,[2] SIMON SWIFT,[3] AND JUAN M. TOMÁS[1]*

Departamento de Microbiología, Facultad de Biología, Universidad de Barcelona, 08071 Barcelona,[1] and Departamento de Microbiología y Parasitología Sanitarias, Facultad de Farmacia, Universidad de Barcelona, 08028 Barcelona,[2] Spain, and School of Pharmaceutical Sciences, University of Nottingham, Nottingham NG7 2RD, United Kingdom[3]

Received 29 March 1999/Returned for modification 20 April 1999/Accepted 13 May 1999

Two different representative recombinant clones encoding *Aeromonas hydrophila* lipases were found upon screening on tributyrin (phospholipase A1) and egg yolk agar (lecithinase-phospholipase C) plates of a cosmid-based genomic library of *Aeromonas hydrophila* AH-3 (serogroup O34) introduced into *Escherichia coli* DH5α. Subcloning, nucleotide sequencing, and in vitro-coupled transcription-translation experiments showed that the phospholipase A1 (*pla*) and C (*plc*) genes code for an 83-kDa putative lipoprotein and a 65-kDa protein, respectively. Defined insertion mutants of *A. hydrophila* AH-3 defective in either *pla* or *plc* genes were defective in phospholipase A1 and C activities, respectively. Lecithinase (phospholipase C) was shown to be cytotoxic but nonhemolytic or poorly hemolytic. *A. hydrophila* AH-3 *plc* mutants showed a more than 10-fold increase in their 50% lethal dose on fish and mice, and complementation of the *plc* single gene on these mutants abolished this effect, suggesting that Plc protein is a virulence factor in the mesophilic *Aeromonas* sp. serogroup O:34 infection process.

Mesophilic motile *Aeromonas* species are opportunistic and primary pathogens of a variety of aquatic and terrestrial animals, including humans; the clinical manifestations range from gastroenteritis to soft tissue infections, including septicemia and meningitis (19). Serogroup O:34 strains of mesophilic *Aeromonas* spp. have been recovered from moribund fish (36) and from clinical specimens (40); O:34 is the most common *Aeromonas* serogroup (31), accounting for 26.4% of all infections. Previous investigations have documented O:34 strains as important causes of infections in humans (30, 31). The varied clinical picture of *Aeromonas* infections, and gastroenteritic illness in particular, suggests that complex pathogenic mechanisms occur in aeromonads.

Most aeromonads elaboratore a variety of extracellular enzymes: proteases, DNase, RNase, elastase, lecithinase, amylase, lipases, gelatinase, and chitinases; some of them are now confirmed as toxins (32, 38, 42): the cytotoxic/cytolytic enterotoxin (10), three different hemolysins (24, 25, 27) and cytotonic enterotoxins (34, 46, 48). Some of these toxins (for instance the aerolysin) are involved in septicemic infection (8). However, no clear information is available, to our knowledge, about the possible role of other extracellular enzymes (elastase, amylase, gelatinase, and chitinases) in *Aeromonas* pathogenesis. Clearly, it seems that the extracellular lipases play an important role in pathogenesis, for instance the glycerophospholipid-cholesterol acyltransferases from *A. hydrophila* and *A. salmonicida* (15, 53), which are implicated in the pathogenesis of this bacterium.

Phospholipases (PL) produced by bacteria are involved in different pathogenic process (14, 52) and are often associated with intestinal damage (5, 22, 54). Members of the family *Vibrionaceae* produced secreted PL, some of which act as hemolysins and some of which act as glycerophospholipid-cholesterol acyltransferases (47, 50, 53). Some of these PL have been cloned and sequenced (18, 49, 51), for instance the alpha-hemolysin (glycerophospholipid-cholesterol acyltransferase) of *A. hydrophila* (53). We report here the cloning, sequencing, identification of gene product, and role in virulence of two different genes of *A. hydrophila* AH-3 (serogroup O:34 [33]) encoding two different PL (PLA1 and PLC [lecithinase]).

* Corresponding author. Mailing address: Departamento de Microbiología, Facultad de Biología, Universidad de Barcelona, Diagonal 645, 08071 Barcelona, Spain. Phone: 34-93-4021486. Fax: 34-93-4110592. E-mail: juant@bio.ub.es.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown on Luria-Bertani LB Miller broth and LB Miller agar (6), while *Aeromonas* strains were grown on tryptic soy broth or agar (TSB and TSA) (37). Tributyrin-agar and egg yolk-agar were prepared as described in reference 6. Ampicillin (50 μg/ml), chloramphenicol (50 μg/ml), kanamycin (30 μg/ml), and/or tetracycline (20 μl/ml) was added to the different media when needed.

General DNA methods. DNA manipulations were carried out essentially as previously described (44). DNA restriction endonucleases, T4 DNA ligase, *E. coli* DNA polymerase (Klenow fragment), and alkaline phosphatase were used as recommended by the suppliers.

DNA sequencing. The primers used for DNA sequencing were purchased from Pharmacia LKB Biotechnology. Double-stranded-DNA sequencing was performed by the dideoxy-chain termination method of Sanger et al. (45) with the Abi Prism dye terminator cycle-sequencing kit (Perkin-Elmer).

DNA and protein sequence analysis. The DNA sequence was translated in all six frames, and all open reading frames longer than 100 bp were inspected. Deduced amino acid sequences were compared with those of DNA translated in all six frames from the nonredundant GenBank and EMBL databases by using the BLAST network service at the National Center for Biotechnology Information (2). Multiple-sequence alignments and determination of putative terminator sequences were done with the PileUp and Terminator programs from the Genetics Computer Group package (GCG, Madison, Wis.) on a VAX 4300.

Construction of *pla* and *plc* mutant strains. To obtain mutants with defined insertion mutations in the *pla* and *plc* genes, a method based on the use of the suicide plasmid pFS100 was applied (43). Plasmid pBR-PLA2 was *Bgl*II digested and blunt ended by treatment with the Klenow fragment of DNA polymerase, and a *pla* internal DNA fragment (1,230 bp) was isolated, ligated to *Eco*RV-digested and dephosphorylated pFS100, and transformed into *E. coli* MC1061 (λ*pir*) to generate plasmid pFS-PLA.

Plasmid pBR-PLC was double digested with *Sal*I and *Kpn*I and blunt ended, and a *plc* internal DNA fragment (697 bp) was isolated, ligated to *Eco*RV-

TABLE 1. Bacterial strains, cosmids, and plasmids used in this study

Strain, cosmid, or plasmid	Relevant characteristics	Source or reference
E. coli		
DH5α	F$^-$ *endA hsdR17* (r_k^- m_k^+) *supE44 thi-1 recA1 gyr-A96* φ80*lacZ*	23
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* (F′ *proAB lacIZ* Δ*M15* Tn*10*)	Stratagene
MC1061	(λ*pir*), *thi thr-1 leu6 proA2 his-4 argE2 lacY1 galK2 ara14 xyl5 supE44*, λ*pir*	43
SM10	(λ*pir*), *thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu, Km^r, λ *pir*	43
A. hydrophila		
AH-3	Wild type, serogroup O:34	36
AH-405	Rifampin-resistant mutant derived from AH-3	39
AH-3PLA1	*pla* insertion AH-405 mutant obtained with pFS-PLA	This work
AH-3PLA4	*pla* insertion AH-405 mutant obtained with pFS-PLA	This work
AH-3PLC2	*plc* insertion AH-405 mutant obtained with pFS-PLC	This work
AH-3PLC3	*plc* insertion AH-405 mutant obtained with pFS-PLC	This work
Cosmids		
pLA2917	Tc^r Km^r	1
COS-PLA	pLA2917 with 20-kb chromosomal AH-3 *Sau*3A insert (*pla* gene)	This work
COS-PLC	pLA2917 with 20-kb chromosomal AH-3 *Sau*3A insert (*plc* gene)	This work
Plasmids		
pBR328	Ap^r Cm^r Tc^r	44
pBR-PLA1	pBR328 with 8.3-kb *Hin*dIII insert from COS-PLA (*pla* gene)	This work
pBR-PLA2	*Hpa*I-*Eco*RV 4.5-kb insert of *pla* gene derived from pBR-PLA1	This work
pBR-PLC	pBR328 with 3-kb *Hin*dIII insert from COS-PLC (*plc* gene)	This work
pBluescript SK	Ap^r *ori* of ColE1	Stratagene
pSK-PLA	pBluescript SK with insert of pBR-PLA2 (*pla* gene)	This work
pSK-PLC	pBluescript SK with insert of pBR-PLC (*plc* gene)	This work
pACYC184	Cm^r Tc^r	44
pACYC-PLA	pACYC184 with insert of pBR-PLA2 (*pla* gene)	This work
pACYC-PLC	pACYC184 with insert of pBR-PLC (*plc* gene)	This work
pFS100	pGP704 suicide plasmid, λ*pir* dependent, Km^r	43
pFS-PLA	pFS100 with an internal fragment (1,230 bp) of *pla*	This work
pFS-PLC	pFS100 with an internal fragment (697 bp) of *plc*	This work

digested and dephosphorylated pFS100, and transformed into *E. coli* MC1061 (λ*pir*) to generate plasmid pFS-PLC. Plasmid pFS-PLA and plasmid pFS-PLC were isolated, transformed into *E. coli* SM10(λ*pir*), and transferred by conjugation to *A. hydrophila* AH-405 (rifampin resistant) to obtain mutants with defined insertion mutations in the *pla* and *plc* genes, respectively.

Substrate specificity and enzyme activity measurements. The substrate specificity with neutral glycerides and glycerophospholipids was determined as previously described (16, 17). Lipase activity was initially determined with tributyrin by the method of Ihara et al. (28). PLC activity was tested initially with *p*-nitrophenylphosphorylcholine as described by Ingham and Pemberton (29).

Determination of extracellular activities. Hemolysin and cytotoxin assays were performed as previously described (37). Briefly, hemolysin activity was assayed with a 1% suspension of sheep, bovine, or rainbow trout erythrocytes (4) and cytotoxin activity was assayed on Vero cell monolayers (7) and EPC (epithelioma papulosum of carp, *Cyprinus carpium*) monolayers (57). Enterotoxin activity was assayed by the rabbit ligated ileal loop assay as described by Nishibuchi et al. (41). In some *E. coli* strains, these activities were also assayed with the periplasmic proteins released by osmotic shock (56).

Virulence for fish and mice. The virulence of the strains grown at 20°C was measured by monitoring their 50% lethal dose (LD_{50}) by the method of Reed and Muench, as previously described (37).

(i) Fish. Rainbow trout (12 to 20 g) were maintained in 20-liter static tanks at 17 to 18°C. The fish were injected intraperitoneally with 0.05 ml of the test samples (approximately 10^9 viable cells). Mortality was recorded up to 2 weeks; all the deaths occurred within 2 to 8 days.

(ii) Mice. Albino Swiss female mice (5 to 7 weeks old) were injected intraperitoneally with 0.25 ml of the test samples (approximately 5×10^9 viable cells). Mortality was recorded up to 1 week; all the deaths occurred within 2 to 5 days.

Nucleotide sequence accession numbers. The nucleotide sequences of the genes described here have been assigned the following GenBank accession numbers: *pla*, AF092033; *plc*, AF092034.

RESULTS AND DISCUSSION

Mesophilic *Aeromonas* strains from different O serogroups, but mainly from serogroup O:34, seem to produce more than one enzyme with lipopolytic activity when grown on egg yolk medium according to Matos et al. (35). It is clear that in this medium these strains produce a precipitate zone (lecithinase reaction) and an iridescent film or "pearly layer," visible by reflected light, with lipase activity (35). Furthermore, O:34 strains were able to degrade trybutirin in a solid medium (lipolytic activity). We decided initially to call PLA the lipase activity on tributyrin medium and PLC the lecithinase activity on egg yolk medium. We decided to clone the corresponding genes from a single O:34 strain (AH-3) and see if they encoded two different PL activities.

Cloning of two *A. hydrophila* AH-3 genomic regions encoding PLA and PLC activities. *A. hydrophila* AH-3 (serogroup O:34) produces PLA and PLC activities. To determine the basis for these activities, a cosmid-based genomic library of *A. hydrophila* AH-3 was constructed and introduced into *E. coli* DH5α (21). Tetracycline-resistant (20 μg/ml) clones were screened independently on tributyrin and egg yolk-agar plates. We found two representative recombinant clones, COS-PLA and COS-PLC, with lipase activity on tributyrin and lecithinase activity on egg yolk-agar plates, respectively. It is important to note that COS-PLC showed only the precipitation zone, not the possible lipase activity (iridescent film) on egg yolk-agar plates.

To localize the genes involved in PLA (COS-PLA) and PLC (COS-PLC) activities, different subcloning experiments with some plasmid vectors were performed and the recombinant transformants were screened for the lipase (tributyrin) and lecithinase (egg yolk) activities, respectively. The strains and plasmids obtained are described in Table 1.

TABLE 2. Identity and similarity of the amino acid sequence of Pla1 and Plc proteins from *A. hydrophila* AH-3 to other proteins

Protein[a] and bacterium of origin	No. of amino acids	% identity	Overlap (amino acids)	Accession no.	Reference
Pla-1 (PLA1), *A. hydrophila* AH-3	805			AF092033	
LipE (extracellular lipase), *A. hydrophila*	684	52.1	696	P40600	3
Lip (extracellular lipase), *A. hydrophila*	751	50.4	746	O05484	12
Apl-1 (PLC), *A. hydrophila*	684	48.5	671	Q44055	29
Alt (cytotonic enterotoxin), *A. hydrophila*	368	96.0	326	Q44061	11
Plc (lecithinase, PLC), *A. hydrophila* AH-3	572			AF092034	
Hly1 (hemolysin), *A. salmonicida*	572	99.1	572	Q08675	26
LecD (PLD), *Vibrio damsela*	573	61.4	570	Q60079	Unpublished

[a] The percentages were obtained from pair comparisons, using the Gap program. Gap program settings: gap weight 12 and length weight 12.

Sequencing of the DNAs conferring PLA and PLC activities. The nucleotide sequences of 2,602 and 2,868 bp were determined in both directions from plasmid pSK-PLA (*pla*) and pSK-PLC (*plc*), respectively; oligonucleotides M13 universal, reverse M13, SK, and other sequence-derived oligonucleotides were used to complete the nucleotide sequence.

Analysis of the deduced sequence of pSK-PLA showed a potential ORF (*pla*) (nucleotides 176 to 2591), encoding a putative protein of 805 amino acid residues with a predicted molecular mass of 82.7 kDa. Sequence analysis of pSK-PLC showed a potential ORF (*plc*) (nucleotides 885 to 2691), encoding a putative protein of 572 amino acid residues with a predicted molecular mass of 64.8 kDa. Upstream of the *pla* and *plc* genes, sequences similar to the ribosomal binding site were found. Sequences similar to the −10 and −35 consensus sequences of *E. coli* promoter were found upstream of *plc* gene, and a palindromic sequence, which could be involved in transcription termination, was found downstream from *plc* gene.

Analysis of the *pla* and *plc* deduced amino acid sequences. The deduced 805-amino-acid sequence from *pla* showed amino acid similarities to three extracellular lipases (LipE, Lip, and Apl-1) and a heat-labile cytotonic enterotoxin (Alt) from *A. hydrophila* (Table 2). All five proteins show the lipase substrate binding signature sequence VHFLGHSL (13). Analyses of the Pla-1 amino acid sequence showed a putative lipoprotein signal sequence (residues 1 to 17) and a putative lipoprotein signal sequence cleavage site between residues 17 and 18. Another putative signal peptidase cleavage site was found between residues 48 and 49. Taken together, these two features strongly suggest that the Pla-1 is a secreted protein. Similar signal sequences and cleavage sites were previously found in the three extracellular lipases similar to Pla-1 but not in the heat-labile cytotonic enterotoxin. These results are in agreement with the previously reported similarity (47 to 51%) between Apl-1 and Alt (11). Furthermore, Alt also showed 54 and 52% of similarity to Lip (12) and LipE (3) extracellular lipases, respectively.

The putative Pla-1 lipoprotein had an apparent molecular mass of 83 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of plasmid pBR-PLA2 gene products with an *E. coli* S30 coupled transcription-translation system (Amersham) and [^{35}S]methionine (Amersham) (Fig. 1).

The deduced 572-amino-acid Plc protein was found to be nearly identical to hemolysin ASH1 from *A. salmonicida* ATCC14174 (26), and furthermore there were only four different nucleotides between the *plc* and this hemolysin structural gene, showing that the *plc* gene is practically identical in both *Aeromonas* species. The Plc protein was also found to be similar to the unpublished PLD from *Vibrio damsela* (Table 2). However, no significative similarity was detected between Plc and a previously reported PLC from *A. hydrophila* (Apl-1), which is similar to Pla-1 (Table 2). Analyses of the Plc amino acid sequence showed a putative signal sequence (residues 1 to 19) and a putative signal sequence cleavage site between residues 19 and 20. In vitro coupled transcription-translation experiments on plasmid pBR-PLC showed a polypeptide of about 65 kDa, similar in size to the expected *plc* mature product (Fig. 1).

PLA and PLC are two different enzymes with PL activity. *E. coli* strains harboring the *pla* gene in the COS-PLA, pBR-PLA1, pBR-PLA2, and pSK-PLA plasmids were able to degrade tributyrin but unable to show any lecithinase activity on egg yolk medium. *E. coli* DH5α and the same strain carrying the same plasmids used as vectors without the DNA insert from AH-3 showed no activity in either trybutirin or egg yolk media. *E. coli* strains harboring COS-PLA, pBR-PLA1, pBR-PLA2, pACYC-PLA, and pSK-PLA plasmids were able to degrade other neutral glycerides (di- or triolein) or natural glycerophospholipids carrying a 1-acyl bond (phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, or phosphatidylglicerol) but unable to degrade neutral glycerides like cholesteryloleate or *p*-nitrophenylacetate or substituted (at position 1-acyl) glycerophospholipids like 1-alkyl-2-acyl-*sn*-glyero-3-phosphocholine. For all these reasons, we concluded that this activity found in tributyrin plates is a PLA1 activity (15, 16).

E. coli strains harboring the *plc* gene in the Cos-PLC, pBR-PLC, pACYC-PLC, and pSK-PLC plasmids were able to pre-

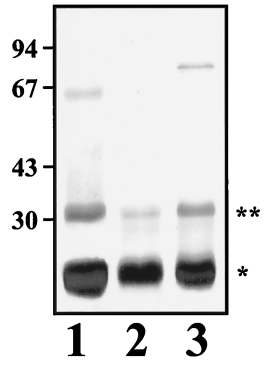

FIG. 1. In vitro *E. coli* S30 coupled transcription-translation system with [^{35}S]methionine performed as specified by the supplier (Amersham). Lanes: 1, pBR-PLC plasmid DNA (*plc* gene); 2, pBR328 plasmid DNA (cloning vector); 3, pBR-PLA2 plasmid DNA (*pla* gene). *, chloramphenicol acetyltransferase (25 kDa) from the Cm^r determinant of pBR328 plasmid DNA; **, β-lactamase (31.5 kDa) from the Ap^r determinant of pBR328 plasmid DNA. Molecular mass standards are shown in kilodaltons.

TABLE 3. Determination of some extracellular and periplasmic activities in different *E. coli* and *A. hydrophila* strains as described in Materials and Methods

Strain	Cellular fraction[a]	Hemolysis on erythrocytes of:			Cytotoxicity on:	
		Sheep	Bovine	Rainbow trout	Vero cells	EPC cells
E. coli						
DH5α	S	—[b]	—	—	—	—
	P	—	—	—	—	—
DH5α + pACYC-PLA	S	—	—	—	—	—
	P	—	—	—	—	—
DH5α + pACYC-PLC	S	—	—	1/8	1/4	1/16
	P	—	—	1/16	1/64	1/128
A. hydrophila						
AH-405 (AH-3 Rif^r)	S	1/64	1/64	1/128	1/64	1/128
AH-3PLA1; *pla* insertion mutant from AH-405	S	1/64	1/64	1/128	1/64	1/128
AH-3PLA4; *pla* insertion mutant from AH-405	S	1/64	1/64	1/128	1/64	1/128
AH-3PLA1(pACYC-PLA); mutant *pla* complemented	S	1/64	1/64	1/128	1/64	1/128
AH-3PLA4(pACYC-PLA); mutant *pla* complemented	S	1/64	1/64	1/128	1/64	1/128
AH-3PLC2; *plc* insertion mutant from AH-405	S	1/64	1/64	1/128	1/4	1/16
AH-3PLC3; *plc* insertion mutant from AH-405	S	1/64	1/64	1/128	1/4	1/16
AH-3PLC2(pACYC-PLC); mutant *plc* complemented	S	1/64	1/64	1/128	1/32	1/64
AH-3PLC3(pACYC-PLC); mutant *plc* complemented	S	1/64	1/64	1/128	1/32	1/128

[a] S, supernatant; P, periplasmic fraction.
[b] —, not detected.

cipitate the egg yolk medium (lecithinase activity) but unable to degrade tributyrin. Also, these strains were able to rapidly degrade the typical synthetic substrate for PLC, *p*-nitrophenylphosphorylcholine, at pH 7.2 with 2 mM $CaCl_2$, and this activity was inhibited by spermine tetrahydrochloride (Calbiochem). The PL activity was dramatically reduced (<5% of the activity) on the same substrate at pH = 5.7 and 0.5 M $CaCl_2$ (typical conditions for measuring PLD activity [20]). Two different assays were performed to differentiate PLC and PLD activities, and the PL activity determined by the *plc* gene showed (i) the presence of the diglyceride when incubated with lecithin as determined by thin-layer chromatography (petroleum ether-diethyleter-acetic acid [90:10:1, vol/vol]) and visualized by UV light as described by Grossman et al. (20) and (ii) hydrolysis of phosphatidylcholine and release of P_i in a low-P_i medium (33). The release of P_i was measured spectophotometrically by the method of Chen et al. (9), in either concentrated supernatants (AH-3 = 18 μg of P_i; AH-3PLC2 [*plc* insertion mutant] = 0.15 μg of P_i) or periplasmic fractions (DH5α = 0.3 μg of P_i; DH5α with the pACYC-PLC plasmid [*plc*] = 22 μg of P_i). From these results, we concluded that the lecithinase activity in these strains on egg yolk medium is mainly a PLC activity.

Construction of defined *pla* and *plc* insertion mutants. Plasmid pFS-PLA, a replication *pir*-dependent plasmid, carrying an internal fragment of the *pla* gene was transferred by mating to a rifampin-resistant *A. hydrophila* strain, AH-3 (AH-405 [39]), and Rif^r and Km^r colonies were selected. We obtained mutants AH-3PLA1 and AH-3PLA4 unable to degrade tributyrin but still able to show lecithinase (PLC) activity. The insertion of plasmid pFS-PLA in these mutants was confirmed by Southern blot analysis with appropriate DNA probes. Complementation of these mutants with COS-PLA or pACYC-PLA restored the PLA1 activity on tributyrin medium.

Plasmid pFS-PLC carrying a *plc* gene internal fragment was used in identical way to that mentioned above to generate mutants AH-3PLC2 and AH-3PLC3 (also confirmed by Southern blot analyses), which were unable to show any lecithinase (PLC) activity on egg yolk medium but were able to degrade tributyrin (PLA1 activity). Complementation of these mutants with COS-PLC or pACYC-PLC restored the lecithinase activity on egg yolk medium. All these results indicate that *pla* and *plc* are different genes in *A. hydrophila* AH-3 and also are unique genes for PLA1 and PLC activities in this strain.

PLC (lecithinase) is cytotoxic. As we previously shown *A. hydrophila* AH-3, as well as other strains from serogroup O:34, are hemolytic, cytotoxic, and enterotoxic (37, 38). As shown in Table 3, neither *E. coli* strains carrying *pla* nor *A. hydrophila* AH-3 *pla* insertion mutants were altered in their hemolytic or cytotoxic activities (no activities found for *E. coli* strains in either supernatants or periplasmic cellular fractions). Also, nonenterotoxic activity was found in *E. coli* strains carrying the *pla* gene and reduced enterotoxicity was found in *pla* insertion mutants of *A. hydrophila* AH-3, despite the high homology between Alt (heat-labile cytotonic enterotoxin [11]) and Pla in 33% of the last protein. Then, we concluded that Pla is an extracellular lipase with PLA1 activity but is nonhemolytic, noncytotoxic, and nonenterotoxic.

However, *E. coli* strains carrying the *plc* gene showed a clear cytotoxic activity on Vero and EPC cells (by assaying either supernatant or periplasmic fraction). Furthermore, *plc* insertion mutants of *A. hydrophila* AH-3 were reduced in their cytotoxic activity, and this activity was fully restored in these mutants by complementation with COS-PLC or pACYC-PLC (Table 3). These results for the cytotoxicity of Plc suggested that the wild-type *A. hydrophila* strain has several cytotoxic factors, with Plc being one of them. The results observed for the hemolytic activity of *E. coli* strains carrying the *plc* gene, *plc* insertion mutants of *A. hydrophila* AH-3, and the mutants complemented with COS-PLC or pACYC-PLC (Table 3) suggested the following. (i) Plc is nonhemolytic on sheep or bovine erythrocytes and only slightly on rainbow trout erythrocytes. These results are mainly in agreement with those reported for Hly1 of *A. salmonicida* (26), which has 99% identity to Plc (Table 2). The authors concluded that Hly1 (termed "hemolysin" despite its poorly hemolytic activity) found in *A. salmonicida* ATCC 14174 did not originate from this bacterium because of its low GC content, the codon usage frequency, and

TABLE 4. Virulence for rainbow trout and mice of *A. hydrophila* AH-3 (serogroup O:34), defined insertion mutants, and mutants complemented by *pla* or *plc*

Strain and main characteristics	LD_{50}[a] for:	
	Rainbow trout	Mice
AH-3; wild-type	$0^{5.3}$	$10^{7.4}$
AH-405; AH-3 rifampin-resistant mutant	$10^{5.4}$	$10^{7.6}$
AH-3PLA1; *pla* insertion mutant from AH-405	$10^{5.6}$	$10^{7.7}$
AH-3PLA4; *pla* insertion mutant from AH-405	$10^{5.5}$	$10^{7.5}$
AH-3PLA1(pACYC-PLA); mutant *pla* complemented	$10^{5.7}$	$10^{7.5}$
AH-3PLA4(pACYC-PLA); mutant *pla* complemented	$10^{5.5}$	$10^{7.6}$
AH-3PLC2; *plc* insertion mutant from AH-405	$10^{7.4}$	$>10^{9.0}$
AH-3PLC3; *plc* insertion mutant from AH-405	$10^{7.3}$	$>10^{9.0}$
AH-3PLC2(pACYC-PLC); mutant *plc* complemented	$10^{5.6}$	$10^{7.7}$
AH-3PLC3(pACYC-PLC); mutant *plc* complemented	$10^{5.7}$	$10^{7.6}$

[a] The values are the averages of three independent experiments, and the maximum deviation was always $<10^{0.4}$.

the negative results obtained by a DNA probe for this gene; unfortunately, they did not test if this protein showed any lecithinase activity (26). We suggest that this gene originates from *A. hydrophila*. (ii) Plc is not a major hemolytic factor on *A. hydrophila* AH-3, because no major differences could be observed between the *plc* insertion mutants and the wild-type strain.

Finally, nonenterotoxic activity was found in *E. coli* strains carrying the *plc* gene and reduced enterotoxicity was found in *plc* insertion mutants of *A. hydrophila* AH-3. We therefore concluded that Plc is nonenterotoxic.

PLC (lecithinase) is an important virulence factor. As pointed out by Vipond et al. (55), the major *A. salmonicida* secreted proteins (toxins) are not essential for the virulence of this bacterium, as they demonstrated with defined deletion mutants. We therefore decided to study the contribution to *A. hydrophila* pathogenesis of our defined *pla* and *plc* insertion mutants. We tested the virulence of the wild-type strain and the corresponding *pla* and *plc* insertion mutants (LD_{50}), as shown in Table 4. As can be observed, no differences were found in mortality between the wild-type strain (or they rifampin-resistant mutant) and mutants AH-3PLA1 and AH-3PLA4, which suggests that the PLA1 activity is not essential for virulence on these strains or that the mutation is unstable in vivo. However, mutants AH-3PLC2 and AH-3PLC3 showed a higher LD_{50} (an increase of 1 to 2 log units) in both fish and mice than the wild-type strain did. Complementation of these insertion mutants with COS-PLC or just with pACYC-PLC (carrying the single *plc* gene) completely restored their virulence for fish or mice (similar LD_{50} to the wild-type strain [Table 4]). These results suggested that Plc (lecithinase) is an important virulence factor for mesophilic *Aeromonas* (serogroup O:34) pathogenesis.

ACKNOWLEDGMENTS

This work was supported by grants from DGICYT and Plan Nacional de I+D (Ministerio de Educación y Cultura, Madrid, Spain). A.A. and M.M.N. are predoctoral fellows from the same institution and Generalitat de Catalunya, respectively.

We thank Maite Polo for her technical assistance.

REFERENCES

1. **Allen, L. N., and R. S. Hanson.** 1985. Construction of broad host-range cosmid cloning vector: identification of genes necessary for growth of *Methylobacterium organophilum* on methanol. J. Bacteriol. **161:**955–962.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Anguita, J., L. B. Rodriguez Aparicio, and G. Naharro.** 1993. Purification, gene cloning, amino acid sequence analysis, and expression of an extracellular lipase from an *Aeromonas hydrophila* human isolate. Appl. Environ. Microbiol. **59:**2411–2417.
4. **Asao, T., S. Kozaki, K. Kato, Y. Kinoshita, K. Otsu, T. Uemura, and G. Sakaguchi.** 1986. Purification and characterization of an *Aeromonas hydrophila* hemolysin. J. Clin. Microbiol. **24:**228–232.
5. **Asnani, N., and P. J. Asnani.** 1991. Structural and functional changes in rabbit ileum by purified extracellular phospholipase A of *Salmonella newport*. Folia Microbiol. **36:**572–577.
6. **Atlas, R. M.** 1993. *In* L. C. Parks (ed.), Handbook of microbiological media. CRC Press, Inc., Boca Raton, Fla.
7. **Blanco, J., M. Blanco, E. A. Gonzalez, M. P. Alonso, and J. L. Garabal.** 1990. Comparative evaluation of three tests for the detection of *Escherichia coli* cytotoxic necrotizing factors (CNF1 and CNF2) using filtrates of cultures treated with mitomycin C. FEMS Microbiol. Lett. **69:**311–316.
8. **Chakraborty, T., B. Huhle, H. Hof, H. Bergbauer, and W. Goebel.** 1987. Marker exchange mutagenesis of the aerolysin determinant in *Aeromonas hydrophila* demonstrates the role of aerolysin in *A. hydrophila*-associated systemic infections. Infect. Immun. **55:**2274–2280.
9. **Chen, P. S., Jr., T. Y. Toribara, and H. Warner.** 1956. Microdetermination of phosphorus. Anal. Chem. **28:**248–254.
10. **Chopra, A. K., C. W. Houston, J. W. Peterson, and G. F. Jin.** 1993. Cloning, expression and sequence analysis of a cytolytic enterotoxin gene from *Aeromonas hydrophila*. Can. J. Microbiol. **39:**513–523.
11. **Chopra, A. K., J. W. Peterson, X. J. Xiu, D. H. Coppenhaver, and C. W. Houston.** 1996. Molecular and biochemical characterization of a heat-labile cytotonic enterotoxin from *Aeromonas hydrophila*. Microb. Pathog. **21:**357–377.
12. **Chuang, Y. C., S. F. Chiou, J. H. Su, M. L. Wu, and M. C. Chang.** 1997. Molecular analysis and expression of the extracellular lipase of *Aeromonas hydrophila* MCC-2. Microbiology **143:**803–812.
13. **Derewenda, Z. S., and A. M. Sharp.** 1993. News from the interface: the molecular structures of triacylglyceride lipases. Trends Biochem. Sci. **18:**20–25.
14. **Diener, M., C. Egleme, and W. Rummel.** 1991. Phospholipase C-induced secretion and its interaction with carbachol in the rat colonic mucosa. Eur. J. Pharmacol. **299:**267–276.
15. **Eggset, G., R. Bjornsdottir, R. M. Leifson, J. A. Arnesen, and D. H. Coucheron.** 1994. Extracellular glycerophospholipid-cholesterol acyl transferase from *Aeromonas salmonicida*—activation by serine protease. J. Fish Dis. **17:**17–29.
16. **Fauvel, J., H. Chap, V. Roques, L. Sarda, and L. Douste-Blazy.** 1984. Substrate specificity of two cationic lipases with high phospholipase A1 activity purified from guinea pig pancreas. I. Studies on neutral glycerides. Biochim. Biophys. Acta **792:**65–71.
17. **Fauvel, J., H. Chap, V. Roques, and L. Douste-Blazy.** 1984. Substrate specificity of two cationic lipases with high phospholipase A1 activity purified from guinea pig pancreas. I. Studies on glycerophospholipids. Biochim. Biophys. Acta **792:**72–78.
18. **Fiore, A. E., J. M. Michalski, R. G. Russell, C. L. Sears, and J. B. Kaper.** 1997. Cloning, characterization, and chromosomal mapping of a phospholipase (lecithinase) produced by *Vibrio cholerae*. Infect. Immun. **65:**3112–3117.
19. **Freij, B. J.** 1984. *Aeromonas*: biology of the organism and diseases in children. Pediatr. Infect. Dis. J. **3:**164–175.
20. **Grossman, S., G. Oestreicher, P. K. Hogue, J. G. Cobley, and T. P. Singer.** 1974. Microanalytical determination of the activities of phospholipases A, C, and D and of their mixtures. Anal. Biochem. **58:**301–309.
21. **Guasch, J. F., N. Piqué, N. Climent, S. Ferrer, S. Merino, X. Rubirés, J. M. Tomás, and M. Regué.** 1996. Cloning and characterization of two *Serratia marcescens* genes involved in core lipopolysaccharide biosynthesis. J. Bacteriol. **178:**5741–5747.
22. **Gustafson, C., and C. Taggesson.** 1990. Phospholipase C from *Clostridium perfringens* stimulates phospholipase A_2-mediated arachidonic acid release in cultured intestinal epithelium cells (INT 407). Scand. J. Gastroenterol. **29:**243–247.
23. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**577–580.
24. **Hanes, D. E., and D. K. F. Chandler.** 1993. The role of a 40-megadalton plasmid in the adherence and hemolytic properties of *Aeromonas hydrophila*. Microb. Pathog. **15:**313–317.
25. **Hirono, I., and T. Aoki.** 1991. Nucleotide sequence and expression of an extracellular hemolysin gene of *Aeromonas hydrophila*. Microb. Pathog. **11:**189–197.
26. **Hirono, I., and T. Aoki.** 1993. Cloning and characterization of three hemolysin genes from *Aeromonas salmonicida*. Microb. Pathog. **15:**269–282.
27. **Howard, S. P., and J. T. Buckley.** 1985. Activation of the hole-forming toxin aerolysin and extracellular processing. J. Bacteriol. **163:**336–340.

28. **Ihara, F., Y. Kageyama, M. Hirata, T. Nihira, and Y. Yamada.** 1991. Purification, characterization, and molecular cloning of lactonizing lipase from *Pseudomonas* species. J. Biol. Chem. **266:**18135–18140.
29. **Ingham, A. B., and J. M. Pemberton.** 1995. A lipase of *Aeromonas hydrophila* showing nonhemolytic phospholipase C activity. Curr. Microbiol. **31:**28–33.
30. **Janda, J. M., L. S. Guthertz, R. P. Kokka, and T. Shimada.** 1994. *Aeromonas* species in septicemia: laboratory characteristics and clinical observations. Clin. Infect. Dis. **19:**77–83.
31. **Janda, J. M., S. L. Abbott, S. Khashe, G. H. Kellogg, and T. Shimada.** 1996. Further studies on biochemical characteristics and serologic properties of the genus *Aeromonas*. J. Clin. Microbiol. **34:**1930–1933.
32. **Kirov, S. M.** 1997. *Aeromonas* and *Pleisomonas* species, p. 265–287. *In* M. P. Doyle, L. R. Beuchat, and T. J. Montville (ed.), Food microbiology: fundamentals and frontiers. ASM Press, Washington, D.C.
33. **Kurioka, S., and P. V. Liu.** 1967. Improved assay method for phospholipase C. Appl. Microbiol. **15:**551–555.
34. **Ljungh, Å., P. Enroth, and T. Wadström.** 1982. Cytotonic enterotoxin from *Aeromonas hydrophila*. Toxicon **20:**787–794.
35. **Matos, J. E., R. J. Harmon, and B. E. Langlois.** 1995. Lecithinase reaction of *Staphylococcus aureus* strains of different origin on Baird-Parker medium. Lett. Appl. Microbiol. **21:**334–335.
36. **Merino, S., V. J. Benedí, and J. M. Tomás.** 1989. *Aeromonas hydrophila* strains with moderate virulence. Microbios **59:**165–173.
37. **Merino, S., S. Camprubí, and J. M. Tomás.** 1992. Effect of the growth temperature on outer membrane components and virulence of *Aeromonas hydrophila* strains of serotype O:34. Infect. Immun. **60:**4343–4349.
38. **Merino, S., X. Rubires, S. Knøchel, and J. M. Tomás.** 1995. Emerging pathogens: *Aeromonas* spp. Int. J. Food Microbiol. **28:**157–168.
39. **Merino, S., X. Rubirés, A. Aguilar, and J. M. Tomás.** 1997. The role of flagella and motility in the adherence and invasion to fish cell lines by *Aeromonas hydrophila* serogroup O:34 strains. FEMS Microbiol. Lett. **151:** 213–217.
40. **Misra, S. K., T. Shimada, R. K. Bhadra, S. C. Pal, and G. B. Nair.** 1989. Serogroups of *Aeromonas* species from clinical and environmental sources in Calcutta, India. J. Diarrhoeal Dis. Res. **7:**8–12.
41. **Nishibushi, M., A. Fasano, R. G. Russell, and J. B. Kaper.** 1992. Enterotoxigenicity of *Vibrio parahaemolyticus* with and without genes encoding thermostable direct hemolysin. Infect. Immun. **60:**3539–3545.
42. **Pemberton, J. M., S. P. Kidd, and R. Schmidt.** 1997. Secreted enzymes of *Aeromonas*. FEMS Microbiol. Lett. **152:**1–10.
43. **Rubirés, X., F. Saigi, N. Piqué, N. Climent, S. Merino, S. Albertí, J. M. Tomás, and M. Regué.** 1997. A gene (*wbbL*) from *Serratia marcescens* N28b (O4) complements the *rfb-50* mutation of *Escherichia coli* K-12 derivatives. J. Bacteriol. **179:**7581–7586.
44. **Sambrook, J., E. F. Fristch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
45. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
46. **Seidler, R. J., D. A. Allen, H. Lockman, R. R. Colwell, S. W. Joseph, and O. P. Daily.** 1980. Isolation, enumeration and characterization of *Aeromonas* from polluted waters encountered in diving operations. Appl. Environ. Microbiol. **39:**1010–1018.
47. **Shaw, J. F., R. C. Chang, K. H. Chuang, Y. T. Yen, and F. G. Wang.** 1994. Nucleotide sequence of novel arylesterase gene from *Vibrio mimicus* and characterization of the enzyme expressed in *E. coli*. Biochem. J. **298:**675–680.
48. **Shimada, T., R. Sakazaki, K. Horigome, Y. Ueska, and K. Niwano.** 1984. Production of cholera-like enterotoxin by *Aeromonas hydrophila*. Jpn. J. Med. Sci. Biol. **37:**141–144.
49. **Shinoda, S., H. Matsuoaka, T. Tsuchie, S. I. Miyoshi, S. Yamamoto, H. Taniguchi, and Y. Mizuguchi.** 1991. Purification and characterization of a lecithin-dependent haemolysin from *Escherichia coli* by a *Vibrio parahaemolyticus* gene. J. Gen. Microbiol. **137:**2705–2711.
50. **Taniguchi, H., H. Otha, O. Ogawa, and Y. Mizuguchi.** 1985. Cloning and expression in *Escherichia coli* of *Vibrio parahaemolyticus* thermostable direct hemolysin and thermolabile genes. J. Bacteriol. **162:**510–515.
51. **Taniguchi, H., H. Hirano, S. Kobomura, K. Higashi, and Y. Mizuguchi.** 1986. Comparison of the nucleotide sequences of the genes for the thermostable direct hemolysin and thermolabile hemolysin from *Vibrio parahaemolyticus*. Microb. Pathog. **1:**425–432.
52. **Thelestam, M., and R. Mollby.** 1975. Sensitive assay for the detection of toxin-induced damage to the cytoplasmic membrane of human diploid fibroblasts. Infect. Immun. **12:**225–232.
53. **Thornton, J., S. P. Howard, and J. T. Buckley.** 1988. Molecular cloning of a phospholipid-cholesterol acyltransferase from *Aeromonas hydrophila*. Sequence homologies with lecithin-cholesterol acyltransferases and other lipases. Biochim. Biophys. Acta **959:**153–159.
54. **Titball, R. W.** 1993. Bacterial phospholipases C. Microbiol. Rev. **57:**347–366.
55. **Vipond, R., I. R. Bricknell, E. Durant, T. J. Bowden, A. E. Ellis, M. Smith, and S. MacIntyre.** 1998. Defined deletion mutants demonstrate that the major secreted toxins are not essential for the virulence of *Aeromonas salmonicida*. Infect. Immun. **66:**1990–1998.
56. **Willis, R. C., R. G. Morris, C. Cirakoglu, G. D. Schellenberg, N. H. Gerger, and C. E. Furlong.** 1974. Preparation of the periplasmic binding proteins from *Salmonella typhimurium* and *Escherichia coli*. Arch. Biochem. Biophys. **161:**64–75.
57. **Wolf, K., and J. A. Mann.** 1980. Poikilotherm vertebrate cell lines and viruses: a current list for fishes. In Vitro **16:**168–179.

Editor: J. T. Barbieri