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Stimulation of Platelet-Activating Factor (PAF) Synthesis in Human Intestinal Epithelial Cell Line by Aerolysin from *Aeromonas encheleia*

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Abstract *Aeromonas encheleia*, a potential human intestinal pathogen, was shown to infect a human intestinal epithelial cell line (Caco-2) in a noninvasive manner. The transcriptional profile of the Caco-2 cells after infection with the bacteria revealed an upregulated expression of genes involved in chloride secretion, including that of phospholipase A2 (PLA2) and platelet-activating factor (PAF) acetylhydrolase (PAFAH2). This was also confirmed by a real-time RT-PCR analysis. As expected from PLA2 induction, PAF was produced when the Caco-2 cells were infected with the bacteria, and PAF was also produced when the cells were treated with a bacterial culture supernatant including bacterial extracellular proteins, yet lacking lipopolysaccharides. Bacterial aerolysin was shown to induce the production of PAF.

Key words: *Aeromonas encheleia*, aerolysin, phospholipase A2, platelet-activating factor, PAF acetylhydrolase, Caco-2 cell

Many bacteria cause diarrhea that is induced by intestinal secretion based on the production of one or more toxins. This secretory and inflammatory diarrhea results from elevated intestinal epithelial fluid secretion by cyclic nucleotides, intracellular calcium, inflammatory mediators, such as interleukin-8, and the activation of phospholipase A2 (PLA2) [15, 39]. cAMP activates the Na/K/Cl cotransporter and calmodulin-dependent protein kinase [39], which results in an increase in the level of intracellular Ca^{2+} and decrease in the Na^{+} absorption in the intestinal epithelium, where the cotransport of Na^{+} and Cl^{-} occurs frequently. This modulation of permeability then induces Cl^{-} secretion [28]. Meanwhile, PLA2 residing in the phospholipid membrane induces intracellular mediators, such as prostaglandin E2 (PGE2) and the platelet-activating factor (PAF), where the latter is rapidly synthesized by cell stimulation. The choline-containing membrane phospholipid (PC) acts as a reservoir for the PAF precursor [30]. PG is synthesized by PLA2 via the liberation of arachidonic acid. Thus, PG and PAF are the two key regulators of gastrointestinal fluid secretion, which acts as a protective host response.

PAF (1-*O*-alkyl-2-acetyl-sn-glycerol-3-phosphatidylcholine) is induced by bacterial endotoxins (*e.g.*, lipopolysaccharide, LPS) and exotoxins (*e.g.*, *Vibrio cholerae* toxin, CTx) [6, 8, 20, 42]. Chronic inflammatory conditions and allergic conditions also elevate the PAF level [32]. Once PAF is activated, it is rapidly released to initiate the production of other inflammatory mediators, such as TNF-α, prostaglandins, and thromboxane [32]. The Cl^{-} secretion induced by PAF is known to be an important mechanism for inflammatory bowel diseases [23]. High levels of PAF have also been found in the stool of patients with ulcerative colitis (UC) and Crohn's disease [33], whereas the level of PAF-acetyl hydrolase (PAFAH), which degrades PAF, is known to be low in these patients [33]. Furthermore, CTX, a heat-stable enterotoxin from *Vibrio cholerae*, and a lipopolysachharide from Gram-negative bacteria have been shown to induce PAF and increase intestinal secretion by activating the production of adenylate kinase, which increases chloride ion secretion [22, 37]. Recently, PAF itself was shown to open a chloride channel in intestinal epithelial cells to stimulate chloride transport [7].

Among the *Aeromonas* spp., motile strains such as *A. hydrophila*, *A. caviae*, *A. sobria*, *A. jandaei*, and *A. veronii* are already known to be involved in gastroenteritis. Although other *Aeromonas* spp. are also believed to be involved in gastroenteritis [41], there are not enough case reports at present. When human intestinal epithelial cells (HT29) were treated with an *Aeromonas* cytotoxic enterotoxin (Act) from *Aeromonas* spp., a microarray analysis revealed that inflammation and a fluid secretory response were induced by interleukin-8, a cAMP responsive element-binding protein

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(CREBBP), and AP-1 [17]. Diarrhea was also reported to be induced by aerolysin, which is a pore-forming toxin, through cAMP and the calcium-dependent chloride secretory pathway [14, 45, 44]. β-Aerolysin from *A. hydrophila* showed the same effect [16]. Aerolysin is the main toxin produced from *Aeromonas spp.* and makes potassium voltage gates in membranes by forming pores after heptamerization [3]. These toxin-induced pores then change the ion transport in the intestine.

However, in the present study, a microarray analysis [24–26] of human epithelial cells after infection with *Aeromonas* revealed another mechanism (involving PAF) that could lead to chloride secretion and diarrhea.

MATERIALS AND METHODS

Bacterial Strains and Human Cell Line

The *Aeromonas encheleia* used in this study was isolated from fish fin rot and shown to encode aerolysin, serine protease, lipase, nuclease, and lateral flagella (unpublished data). The bacteria were grown in an LB medium. Caco-2, human colonic epithelial cells, were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma, U.S.A.) at 37°C in the presence of 5% CO_2.

Adhesion and Invasion Assay

The *A. encheleia* was grown in an LB medium at 30°C for 19 h. After collecting the bacterial pellet using centrifugation, it was washed with phosphate-buffered saline (PBS) and dissolved in PBS. The Caco-2 cells were incubated in DMEM in a 24-well plate with a cover slip for 14 days. The medium was replaced with a fresh one before the bacterial infection. For the adhesion assay, the bacterial cells were added to the monolayer culture of Caco-2 cells at a final concentration of 10^7 CFU/ml. PBS was added as a negative control. The infected cells were further incubated for 60 or 120 min. Each well was then washed three times with PBS, fixed with methanol for 5 min, and stained with a Giemsa staining solution for 40 min, followed by rinsing with dH_2O before a microscopic analysis at 1,000×. For the invasion assay, the infected cells (at 60 or 120 min post infection) were washed three times with PBS and treated with gentamycin (100 mg/ml) for 90 min to remove any bacteria outside the Caco-2 cells. Next, the cells were washed with PBS and lysed with 0.01% Triton X-100, and then the lysate was grown on an LB plate and the number of intracellular bacteria counted [19].

DNA Microarray Hybridization

The total RNA was isolated with Trizol (Invitrogen, U.S.A.) according to the manufacturer's instructions, and the concentration of RNA measured using the RiboGreen reagent (Molecular Probe, U.S.A.). The DNA microarrays (GenePlorer TwinChip Human-8K) were purchased from Digital Genomics (Seoul, Korea) and immobilized with 8,170 PCR-amplified DNA fragments that cover 85% of the annotated open reading frames (ORFs) of the human genome. The microarrays were then prehybridized in a prehybridization buffer (25% formamide, 5× SSC, 0.1% SDS, 10 mg/ml BSA) for 45 min at 58°C, hybridized with 10 µg of a Cy3- and Cy5-labeled cDNA mixture (1:1) in a hybridization buffer (3 DNA 900, Genisphere, U.S.A.) for 16 h at 42°C, and washed once with a primary wash buffer (2× SSC, 0.1% SDS) for 5 min at 58°C and twice with a secondary wash buffer (0.1× SSC) for 5 min at room temperature. The other details were performed according to the manufacturer's protocol (http://www.digital-genomics.co.kr).

Scanning and Data Processing

The hybridized arrays were scanned with a GenePix 4000B laser scanner (Axon, Foster City, CA, U.S.A.), and laser lights with wavelengths of 532 nm and 635 nm were used to excite the Cy3 dye and Cy5 dye, respectively. The fluorescent images were captured as a multi-image tagged file format (TIFF) and analyzed with GenePix Pro 5.1 software (Axon). The signal intensity for each spot and its local background were determined, and the net signal intensity calculated by subtracting the median signal intensity for all the pixels within the local background area from the median signal intensity for all the pixels within the spot area.

The spots showing a signal-to-noise (S/N) ratio (foreground hybridization signal/background hybridization signal) of <3 were excluded from further analysis. When the Cy5 and Cy3 fluorescence signals were less than three-fold above the background, the signals were considered too weak to be analyzed quantitatively. Biases in the signal intensity between the two fluorescent dye channels in a microarray were normalized using a locally weighted linear regression analysis (LOWESS normalization) [9], using Acuity 3.1 software (Axon). Subsequent data analyses were conducted using Microsoft Excel. The relative expression level of a gene was calculated as $\log_2$ (Cy5/Cy3), where Cy5 and Cy3 were the normalized signal intensities from the test and reference cDNA. The means of the relative expression levels were calculated from three biological replicates (each comprised of two technical replicates). For a statistical analysis of the global gene expression, *P* values were calculated using a Students *t*-test based on the log-transformed normalized ratios.

Real-Time RT-PCR

The probes used for the assay were as follows: PLA2G1B, sense-5'CCGTACACCCACACCTATTC3', antisense-5'GTCGCAGTTGCAAATGAAGG3'; ITGAL, sense-5'CCAGAACACCTATCTGAGTGG3', antisense-5'TCG-AACCATCAAACAGAAATAC3'; ITPKC, sense-5'CCT-

TTGTGGTCTCCTTCCG3', antisense-5'TGCTCACACT-GACAGAAACG3'; CREBBP, sense-5'AACAACTGTC-GGAGCTTCTAC3', antisense-5'TGCTGGCGCTCACA-TTTC3'; PAFAH2, sense-5'TGACTTTGAAGGGCAAC-ATTG3', antisense-5'TCCAGAGGAAACATCCAAGC3'; ICAM4, sense-5'ACGTGACGCAGGTGTTCC3', antisense-5'GCTCCAGGCTTTCGGAATAG3'; SLC12A3, sense-5'CCTGGATCATCATCCTGCTG3', antisense-5'CAGA-CTCCGGGAGATGAGG3'; PTGES, sense-5'TGGCAGA-CACTTCCATTTAATG3', antisense-5'CAAGGTTTGGA-ACTGCAAATG3'; PTAFR, sense-5'CTTCCTGGGCGT-CATCAC3', and antisense-5'CAGGATGAGGAAGTAG-GATGC3'. An iScript cDNA synthesis kit (Bio-Rad, U.S.A.) was used for the reverse transcription and PCR, and an iQ SYBR Green supermix (Bio-Rad, U.S.A.) used for the fluorescent labeling of the PCR products. The mixture was incubated at 95°C for 3 min one time, followed by 45 cycles of incubation at 95°C for 30 sec and at 59.3°C for 30 sec in an iQ thermal cycler (Bio-Rad, U.S.A.). Glyceraldehyde-6-phosphate dehydrogenase (GAPDH) was used as the control. The primer sequences were as follows: sense-5'AAACCTGCCAAATATGATGACAT3' and antisense-5'GCCCAGGATGCCCTTGA3'.

Recovery of Bacterial Exoprotein

To inactivate any bacterial lipopolysaccharides (LPS) [29], the culture supernatant was treated with 250 U/ml of polymyxin B (Sigma, U.S.A.) at 30°C for 19 h. The mixture was then filtered through a membrane filter with 45-mM diameter pores, and the filtrate concentrated 10× using a Microcon (Amicon, U.S.A.).

Recombinant Proaerolysin

The gene encoding proaerolysin was amplified from *A. encheleia* using primers designed based on the proaerolysin gene from *A. hydrophila* (GenBank accession number M16495), as the sequence of the gene encoding the *A. encheleia* proaerolysin was not known; forward-5'-GCAGAGCCCGTCTATCCAGACCA-3', reverse-5'-CAC-GCTGAGGCTGACTTGTTGAA-3'. The PCR product was cloned into a pQE31 vector from Qiagen (U.S.A.) and transformed into *Escherichia coli* JM105. A 1-liter culture was then grown and induced with IPTG at a final concentration of 1 mM for 3 h. The protein was purified using an Ni-NTA column (Qiagen, U.S.A.) according to the manufacturer's manual. The bound protein was then eluted with 400 mM imidazole in a phosphate buffer, and the eluate dialyzed in PBS overnight.

Extraction and Detection of PAF

The Bligh and Dyer technique was used [4]. Briefly, the Caco-2 cells pretreated with the bacterial culture supernatant or recombinant proaerolysin were detached with trypsin-EDTA treatment and homogenized in PBS. Chloroform/methanol (50:50, v/v) was added to the homogenate and the mixture subjected to centrifugation at 15,000 ×*g* for 10 min. The organic phase was then recovered. The PAF contained in the extract was polar-extracted according to Denizot *et al.* [10]. The recovered organic phase was evaporated and redissolved in chloroform. The lipid extract was then applied to a minicolumn (Amprep, 100 mg-octadecyl minicolumn; Amersham, U.S.A.) to isolate the C_{18} lipid. The minicolumn was first rinsed with chloroform, then washed with methanol, a polar solvent, and eluted with chloroform, a nonpolar solvent. Thereafter, the extract was separated on a silica plate (Silica gel 60 F_{254}; Merck, Germany) using a solvent of chloroform/methanol/water (65:35:6, v/v). Finally, the plate was sprayed with H_2SO_4/methanol (50:50, v/v) and heated at 150°C for 10 min. PAF and lyso-PAF (Sigma, U.S.A.) were purchased and used as the standards.

RESULTS

Aeromonas Infection to Caco-2 Cells

The surface of the Caco-2 cells after infection with *A. encheleia* was observed 30, 60, and 120 min post infection. Many bacteria were shown to adhere to the cell surface after 30 min and a morphological change in the host cells was observed after 60 min (Figs. 1A and 1C). Vacuolation was also observed (Fig. 1E), and a near complete lysis of the host cells was seen after 120 min (Fig. 1D). Therefore, it was decided to isolate the host cell mRNA for a microarray analysis 60 min post infection. The bacteria did not show any invasion according to an invasion assay (data not shown), which is a characteristic of an extracellular pathogen.

cDNA Microarray Analysis and Real-Time RT-PCR

DNA microarrays comprised of 8 K cDNAs from the human genome were used to monitor the differences in the mRNA composition between the human Caco-2 cells grown with or without *Aeromonas* infection. In triplicate experiments for each culture condition, 7,292 (86.1%) spots passed the spot-quality control criteria and were analyzed statistically. The standard deviation (SD) of the log ratios was 0.30, and 28 (0.4%) and 368 (5.1%) genes showed 1 SD below and above the mean $\log_2$ ratio, respectively (Fig. 2). Genes that showed a significant (P<0.01 by t-test) change in their relative mRNA levels of greater than 2-fold or less than 0.5-fold (+1 or −1 on $\log_2$ scale) were considered to be differentially expressed. Although none of the genes were identified as downregulated, 131 genes showed a $\log_2$ ratio of >1 with a P value of <0.01 and were assumed to be upregulated (Table 1). These 131 genes were then subjected to a real-time RT-PCR analysis to confirm the expression change (Fig. 3).

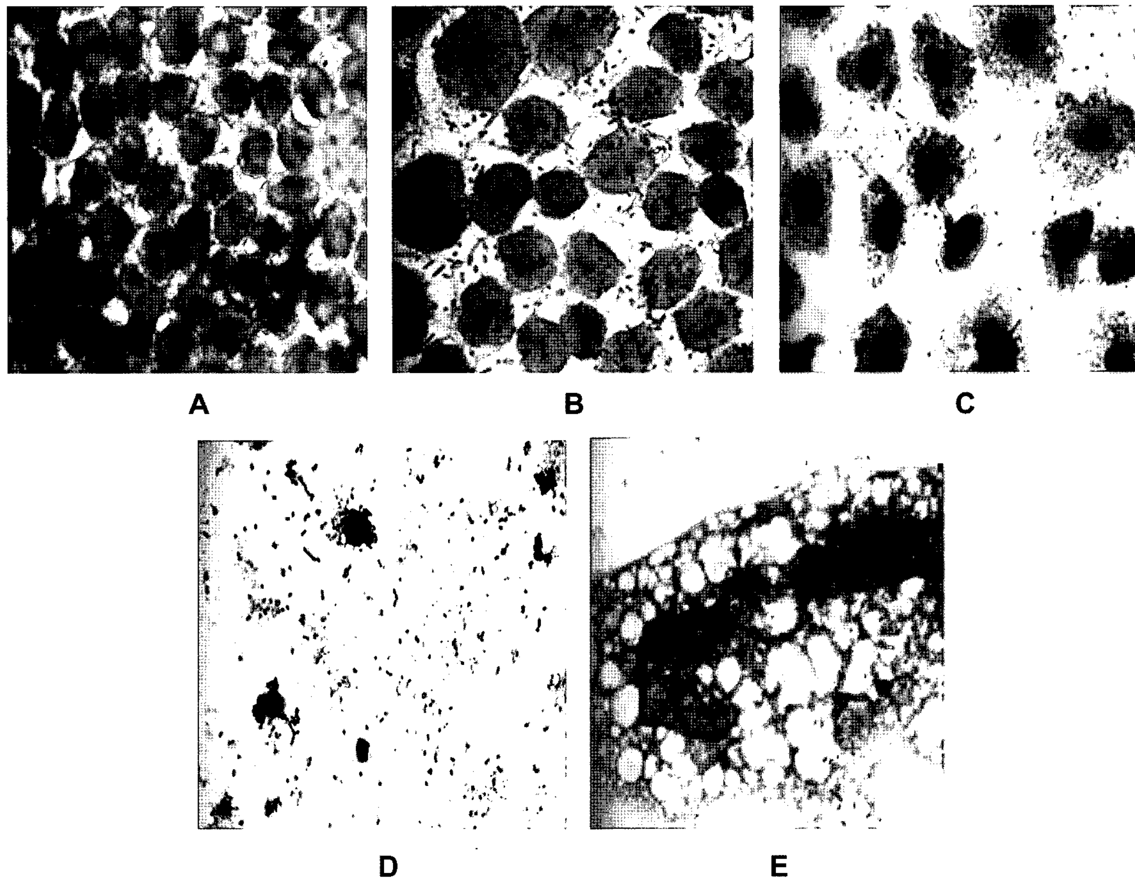

Fig. 1. Light micrographs of Giemsa-stained monolayers of Caco-2 cells infected with *A. encheleia*.
A. Confluent monolayer of Caco-2 cells without bacterial infection. **B.** Caco-2 cells after bacterial infection for 30 min. **C.** Caco-2 cells after bacterial infection for 60 min. **D.** Caco-2 cells after bacterial infection for 120 min, where bacteria could be seen adhering to the glass coverslip after destruction of cell the monolayer. **E.** Vacuolated Caco-2 cells after bacterial infection for 60 min.

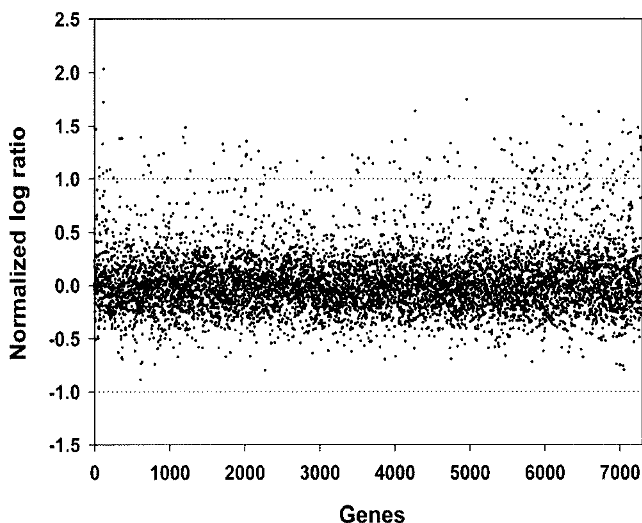

Fig. 2. Global gene expression profile of human Caco-2 cells infected with *Aeromonas*.
Normalized log_2 ratios (infected culture/aseptic culture) are plotted on the y axis, and individual genes are arbitrarily plotted on the x axis. The dotted lines indicate the log_2 ratio of +1 and −1, respectively.

It is noteworthy that the upregulated genes included the genes involved in intestinal epithelium fluid secretion (Table 1 and Fig. 3). First of all, there was an increased expression of phospholipase A2 (PLA2G1B), which is known to stimulate the synthesis of lipid inflammatory response mediators, prostaglandin and PAF [35, 36, 38]. Accordingly, the expression of prostaglandin E synthase (PTGES) was also upregulated. Prostaglandin is made through arachidonic acid metabolism by PTGES [12], whereas PAF is made by PLA2 and degraded to lyso-PAF by PAF acetylhydrolase (PAFAH2). Thus, the expression of PAFAH2 was also shown to increase. In a previous study, PAF-AH activity was found to be higher in a dead patient than in a surviving patient after sepsis [13], whereas in another study, the PAF-AH level in a sepsis patient was reduced to half of that in a healthy person [18]. Therefore, in the present study, the higher production of PAF was possibly reduced by PAF-AH.

Other genes involved in chloride secretion were also upregulated, including the CREB-binding protein (CREBBP) and inositol triphosphate kinase C (ITPKC) that increases intracellular calcium and reduces the absorption of chloride,

Table 1. Genes differentially expressed in *Aeromonas encheleia*-infected human intestinal epithelial cells identified by using microarrays.

Rank	Gene			Log_2 ratio (with/without infection)
	Name	Accession number	Characteristics and function	
Induction				
1	RPS3	AA593872	Ribosomal protein 3, structural constituent of ribosome	+2.03
2	**PLA2G1B***	M21054	Calcium-dependent cytosolic phospholipase A2	+1.74
3	ANXA2	AA164741	Annexin A2, calcium-dependent phospholipid-binding activity	+1.48
4	ACTB	X00351	Actin-beta, structural constituent of cytoskeleton	+1.43
5	**ITGAL**	NM_00220	Integrin, alpha L, cell adhesion receptor activity	+1.35
6	EIF3S2	AA306891	Eukaryotic translation initiation factor 3, translation initiation factor activity	+1.35
7	SLC25A5	AA314562	Solute carrier family 25, transporter activity	+1.34
8	**ITPKC**	D38169	Inositol-trisphosphate 3-kinase	+1.33
9	EGFL4	AB011541	EGF-like domain, multiple 4, calcium ion-binding activity	+1.33
10	HMGCS1	L25798	Hydroxymethylglutaryl-CoA synthase 1	+1.32
11	MDM2	Z12020	Mdm2, transformed 3T3 cell double minute 2, negative regulation of cell proliferation	+1.27
12	**CREBBP**	U47741	CREB-binding protein, histone acetyltransferase activity	+1.26
13	**PAFAH2**	D87845	Platelet-activating factor acetylhydrolase 2	+1.23
14	**ICAM4**	L27671	Intercellular adhesion molecule 4, cell adhesion molecule activity	+1.23
15	KCNJ13	AJ006128	Potassium inwardly-rectifying channel, voltage-gated ion channel activity, potassium channel activity	+1.20
16	ZNF297B	AB007874	Zinc finger protein 297B	+1.19
17	TSC2	NM_02105	Tuberous sclerosis 2, RAB GTPase activator activity	+1.18
18	CORO2A	NM_00338	Coronin, actin-binding protein 2A, intracellular signaling cascade	+1.17
19	IL10	M57627	Interleukin 10, apoptosis inhibitor activity	+1.16
20	SLC17A5	AA258257	Solute carrier family 12, hydrogen:sugar symporter-transporter activity	+1.16
21	TGFBI	AI08687	Transforming growth factor, beta-induced, transcription-activating factor	+1.16
22	UGT2B11	AF016492	UDP glycosyltransferase 2 activity	+1.16
23	GCNT2	Z19550	Glucosaminyltransferase activity	+1.14
24	MYO1C	X98507	Myocin IC, ATP-binding activity, calmodulin-binding activity	+1.14
25	**SLC12A3**	X91220	Solute carrier family 12, sodium:chloride symporter activity	+1.12
26	CD84	AJ223324	CD84, lymphocyte antigen	+1.11
27	CAMTA2	AB020716	Calmodulin-binding transcription activator 2	+1.11
28	CHES1	AA777764	Checkpoint suppressor 1, DNA damage checkpoint	+1.11
29	MCCC2	BC14897	Methylcrotonoyl-Coenzyme A carboxylase	+1.10
30	**PTGES**	AI421214	Prostaglandin E synthase	+1.10
31	IFIT4	AF083470	Interferon-induced protein with tetratricopeptide repeats	+1.09
32	CTSS	M90696	Cathepsin S, immune response	+1.08
33	PRKWNK1	AB002342	Protein kinase	+1.07
34	LOC51326	AI027434	ARF protein	+1.07
35	CAPRI	AB011110	Ca^{2+}-promoted Ras inactivator	+1.07
36	COG7	AF070508	Component of oligomeric golgi complex 7, protein transporter activity	+1.06
37	KCNQ3	AF033347	Voltage-gated potassium channel activity	+1.06
38	PITPNB	D30037	Phosphatidylinositol transfer protein, beta	+1.05
39	AK1	NM_00047	Adenylate kinase activity	+1.01
40	BAT2	NM_00463	HLA-B-associated transcript 2, MHC-interacting protein	+1.01
41	SWAP70	AF134894	SWAP-70 protein, calcium ion-binding activity	+1.00

Table 1. Continued.

Rank	Gene			Log2 ratio (with/without infection)
	Name	Accession number	Characteristics and function	
Repression				
1	GNA15	M63904	Guanine nucleotide-binding protein, heterotrimeric G-protein GTPase	-0.68
2	UGT2B17	U59209	UDP glycosyltransferase 2, guanine nucleotide-binding protein (G protein), GTPase activator activity	-0.68
3	PTPN4	M68941	Protein tyrosine phosphatase activity	-0.67
4	MAPKAPK3	U09578	Mitogen-activated protein kinase activated protein, ATP-binding activity	-0.64
5	GNG2	AI422400	Guanine nucleotide-binding protein (G protein)	-0.63
6	CLCN7	AI026618	Chloride channel 7, voltage-gated chloride channel activity	-0.62

*Bold character indicates genes involved in chloride secretion.

inducing intestinal fluid secretion [33]. The *Aeromonas* cytotoxic enterotoxin (Act) and CTx are also both known to induce this mechanism [44]. SLC12A3 is also a solute carrier family protein involved in chloride transport, which could lead to chloride secretion [40]. Since there was no cDNA probe for the PAF receptor in the microarray used in the present study, a real-time RT-PCR analysis was conducted for the PAF receptor (PTAFR), and its upregulation confirmed (Fig. 3). PTAFR is a G-protein coupled receptor and induces inflammatory mediators, such as TNF-α, PG, and thromboxane, upon interaction with PAF [5, 6, 31, 40]. In addition, it activates a cascade of kinase systems, including inositol triphosphate kinase C (ITPKC) [11], which was also confirmed by the real-time RT-PCR analysis in the present study (Fig. 3). The upregulation of ICAM expression by PG is also known [21], and was confirmed by the real-time PCR analysis in the present experiment (Fig. 3), along with the upregulated expression of ITGAL, an adhesion molecule receptor.

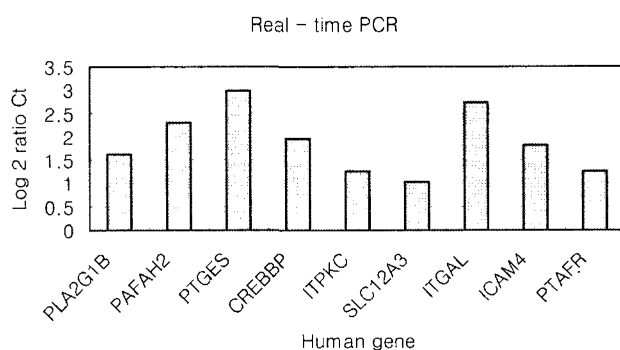

Fig. 3. Confirmation of selected upregulated genes after *Aeromonas* infection of human intestinal epithelial cells using real-time RT-PCR.

PLA2G1B, phospholipase A2; PAFAH2, platelet-activating factor acetylhydrolase 2; PTGES, prostaglandin E synthase; CREBBP, CREB-binding protein; ITPKC, 1D-myo-inositol-trisphosphate 3-kinase C; SLC12A3, solute carrier family 12; ITGAL, integrin alpha L; ICAM4, intercellular adhesion molecule 4; PTAFR, platelet-activating factor receptor.

PAF Production from Caco-2 Cells After Aeromonas Infection

As expected from the microarray results, PAF production was observed when the Caco-2 cells were infected with *Aeromonas* (Fig. 4B, lane 2). Yet, since bacterial LPS, a

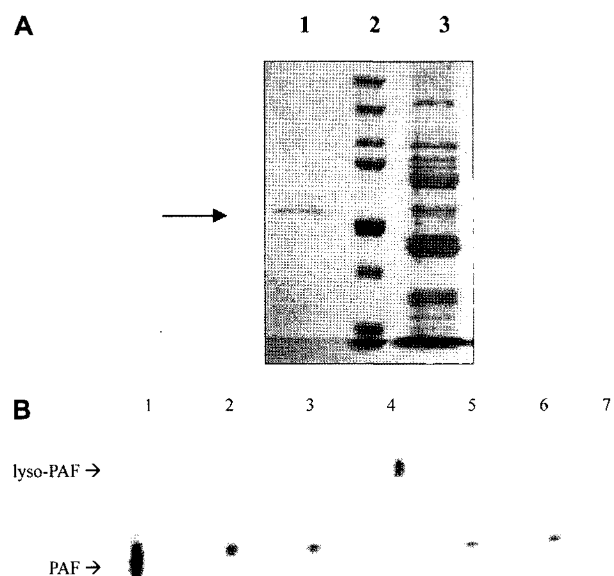

Fig. 4. A. Purification of recombinant proaerolysin. Lane 1, purified recombinant proaerolysin; lane 2, molecular weight marker; lane 3, crude extract of induced cells expressing proaerolysin. Recombinant aerolysin was >99% pure as determined by densitometry. **B.** TLC plate analysis of PAF. Lane 1, standard PAF (1 mg/ml); lane 2, PAF in lipid extract from Caco-2 cells infected with *Aeromonas* for 60 min; lane 3, PAF in lipid extract from Caco-2 cells treated with bacterial supernatant lacking LPS for 90 min; lane 4, standard lyso-PAF (0.5 mg/ml); lane 5, PAF in lipid extract from Caco-2 cells treated with purified recombinant proaerolysin (1 μg/ml) for 90 min.

The purified protein was subjected to polymixin B treatment to inactivate any contaminating bacterial LPS. Lane 6, PAF in lipid extract from Caco-2 cells treated with CTx (1 μg/ml), a known inducer, for 90 min; lane 7, PAF in lipid extract from Caco-2 cells treated with the eluate of Ni-affinity column loaded with cell extracts of *E. coli* harboring empty pQE vector (control).

well-known inducer of PAF, may have been present in the culture supernatant, the supernatant was treated with polymixin B to inactivate LPS. The Caco-2 cells were then treated with a bacterial culture supernatant lacking LPS, and PAF production was still observed (Fig. 4B, lane 3).

PAF Production from Caco-2 Cells After Treatment with Recombinant Proaerolysin

As the culture supernatant was devoid of LPS, a further exploration was conducted to determine which extracellular protein made by the bacteria was responsible for the PAF induction. *Aeromonas* spp. are known to produce virulence factors, including aerolysin, serine protease, lipase, nuclease, and lateral flagella [2, 34]. Since the main toxin produced by *Aeromonas* spp. is aerolysin, which makes potassium voltage gates in the membrane by forming pores, followed by changes in the ion transport in the intestine, the involvement of aerolysin in the production of PAF was checked.

Recombinant proaerolysin was cloned, expressed, and purified (Fig. 4A, lane 1). Given that the DNA sequences encoding the aerolysin from *Aeromonas encheleia* are unknown, the DNA sequences encoding the aerolysin from the closely related *Aeromonas hydrophila* were used. Proaerolysin is known to convert to aerolysin by host furin [1]. When the Caco-2 cells were incubated with the purified recombinant proaerolysin, PAF production was observed (Fig. 4B, lane 5). The same effect was also observed when the cells were treated with a cholera toxin (Fig.4B, lane 6). No PAF was produced without bacterial infection or a toxin (Fig. 4B, lane 7).

DISCUSSION

We showed that *Aeromonas* infection to Caco-2 cells changed the host's gene expression profile. Among the genes differentially regulated are those involved in chloride secretion. Since the intestinal epithelial cells are the first line of defense against bacterial infection, an inflammatory response is expected. A case of diarrhea involving cAMP and a calcium-dependent chloride secretory pathway resulting from *A. sobria* aerolysin has been reported [14, 44, 45], and β-aerolysin from *A. hydrophila* has also been shown to have the same effect [14]. The inflammatory and secretory diarrhea induced by *Aeromonas* infection is known to be mediated by IL-8, cAMP, and AP-1 [17]. However, the gene expression profile in the current study indicated PLA2 upregulation, which was also confirmed by a real-time RT-PCR analysis. PAF production from *Aeromonas* infection was demonstrated, too. Aerolysin is the most abundant extracellular toxin produced from *Aeromonas*. In addition to forming pores on the host cell surface, we found another virulence mechanism of this toxin in this study. The involvment of other extracellular virulence factors of *Aeromonas* spp. related to PAF induction remained to be explored. The present results would seem to support the possibility of intestinal fluid secretion by PAF through aerolysin-induced PAF production.

Acknowledgments

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