

Identification by microarray of a common pattern of gene expression in intact intestine and cultured intestinal cells exposed to virulent *Aeromonas hydrophila* isolates

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ABSTRACT

The genus *Aeromonas* comprises known virulent and avirulent isolates and has been implicated in waterborne disease. A common infection model of human gastroenteritis associated with *A. hydrophila* uses neonatal mice. The goal of this research was to evaluate whether a murine small intestinal cell line could provide comparable results to the gene expression changes in the neonatal mouse model. Changes in mRNA expression in host cell cultures and intestinal tissues were measured after exposure to virulent *Aeromonas hydrophila* strains. *A. hydrophila* caused the up-regulation of more than 200 genes in neonates and over 50 genes in cell culture. Twenty-six genes were found to be in common between the two models, of which the majority are associated with the innate immune response.

Key words | *Aeromonas hydrophila*, animal model, cell culture, microarray, virulence factors

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INTRODUCTION

Aeromonas hydrophila is a gram negative bacterium commonly found in surface water, groundwater, marine and estuarine environments, and even in chlorinated water supplies. This organism is currently listed on the U.S. Environmental Protection Agency's Candidate Contaminant List (CCL) (*U.S. Federal Register, March 2, 1998*) primarily because it has been implicated in waterborne disease and it is commonly found in source water. *A. hydrophila* can cause wound infections and septicemia in immuno-compromised

people and some evidence suggests that it causes gastrointestinal disease in healthy individuals. *Aeromonas* spp. contribute to biofilms (Gavriel *et al.* 1998), and experience regrowth in drinking water distribution systems (Gavriel *et al.* 1998; Smith & Cheasty 1998). *A. hydrophila* has been detected in hospital water supplies (Picard & Goulet 1987). Environmental occurrence varies by season, with increases seen in warmer summer months (Burke *et al.* 1984; Gavriel *et al.* 1998; Smith & Cheasty 1998). Infection rates are correlated with

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peak rates of occurrence in the environment. Two studies recovered more aeromonads in surface water than in groundwater (Burke *et al.* 1984; Legnani *et al.* 1998).

The consequences resulting from exposure of host cells to *Aeromonas* and their associated virulence factors (VFs) are not fully defined. The published literature presents confounding information on the relative importance of a particular *Aeromonas* VF or which VFs are absolutely essential for causing disease. Therefore, it is not currently possible to assess health risks associated with the large population of different *Aeromonas* isolates found in drinking water sources. A neonatal mouse model for *Aeromonas*, as described by Wong *et al.* 1996, has been used in past research to quantitate virulence of *A. hydrophila*. Gene Chip technology provides an innovative technique to monitor host cell transcriptome changes due to infection. Analyzing excised intestinal tissue after artificial infection will produce a holistic, yet very complicated, picture of host response. Epithelial cell cultures model the site of infection for many enteric pathogens. Results obtained from infection of an epithelial cell monolayer should reduce the complexity and be predictive of disease initiation. In a review of microarray research (Cummings & Relman 2000), examples are provided to support this approach with various known pathogens. Advantages noted include rapid detection of pathogen exposure and use of a single water sample to diagnose exposure to multiple disease agents.

In this work, a similar approach has been used to evaluate host cell exposure to *A. hydrophila* isolates. The intent was to demonstrate that the expression of specific host cell genes (previously reported to be up- or down-regulated in bacteria challenged host cells) can be similarly regulated in response to virulent *A. hydrophila*. An additional goal was to determine if the mRNA response in a mouse model of intestinal epithelial cells is comparable to the response seen in the whole animal's small intestinal tissue.

METHODS

Aeromonas strains

Aeromonas hydrophila isolate, EPA1 (40707D1), was obtained from a 2000–2001 water distribution system study conducted by the United States Environmental

Protection Agency (USEPA). *Aeromonas hydrophila* isolate, EPA2 (MC12723W), was a clinical isolate obtained from Dr. Amy Carnahan of the University of Maryland, Baltimore, USA. Pure *Aeromonas* cultures were stored long-term in 15% glycerol at -70°C . High virulence of both strains was demonstrated in immuno-compromised, adult Swiss Webster mice. Phenotypic assays showed each strain was positive for elastase and lipase activity, hemolytic to both sheep and rabbit erythrocytes, and cytotoxic to Vero cells (data not shown).

Suspensions of EPA1 and EPA2 were prepared by growing the cells in tryptic soy broth (TSB) (Becton Dickinson, Franklin Lakes, NJ, USA) for 16 hours at 35°C . This broth culture was swabbed across the entire surface of a sheep blood agar (SBA) plate (Becton Dickinson, Franklin Lakes, NJ, USA) and incubated for 5 hours at 37°C . The resultant lawn of bacteria was suspended in 20 ml of phosphate buffered saline (PBS) at pH 7.4 to give a concentration of 10^9 CFU/ml. In the tissue culture tests, *Aeromonas* suspensions were diluted to about 10^6 CFU/ml in PBS (1:1000 dilution). Viable cell counts were performed to confirm actual dosage levels. The initial concentrations for bacterial cell suspensions harvested from the SBA plates were 1.9×10^9 and 1.8×10^9 for EPA1 and EPA2, respectively, for the tissue culture experiments and 6.5×10^9 and 6.3×10^9 for EPA1 and EPA2, respectively, for the neonatal experiments.

Controls consisted of either sterile PBS or combined UV and heat killed suspensions of EPA1 (heating at 50°C for 20 minutes while exposed to UV light) (Model 11SC-1, Spectronics Corp., Westbury, NY, USA). The loss of viability was confirmed by a culture assay.

Neonatal mice

All animal experiments were performed under a protocol approved by the US EPA Animal Facility Oversight Committee. Timed pregnant Swiss Webster dams were received at 15 days gestation (Charles River Laboratories, Wilmington, MA, USA). Five neonates, aged 4–6 days, were intubated with $20 \mu\text{l}$ of 10^9 CFU/ml suspension of either EPA1, EPA2, sterile 1X PBS (as control), or UV/Heat killed suspension (as control) through a $100 \mu\text{l}$ Hamilton syringe fitted with a 24 gauge neonatal mouse intubation canula.

Neonates were euthanized by asphyxiation in CO₂ chamber (w/ 5% O₂) five hours post-exposure and necropsied, or necropsied within 0.5 hours of death. Small intestines were excised and rinsed in pre-chilled 1X PBS and then minced into sections no longer than 0.5 cm. The processed intestines were stored at 4°C in RNALater solution (Ambion, Austin, TX, USA).

Cell cultures

In vitro experiments were performed using an intestinal murine cell line (mIC_{c12}) which have been derived from isolated crypts of small intestinal villi of a L-PK/Tag1 transgenic mouse (Bens *et al.* 1996). Previous studies have demonstrated that these cells expressed many features of crypt epithelial cells from which they were derived (Peng *et al.* 1999; Hornef *et al.* 2002; Luangsay *et al.* 2003). Cells were cultured in 25 cm² canted, vented cell culture flasks (Corning Inc., Corning, NY, USA) with 5 ml of Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (D-MEM/F12, GIBCO, Grand Island, NY, USA) supplemented with 2% fetal bovine serum (FBS). Growth factors additions included (all from Sigma, St. Louis, MO, USA) insulin (5 µg/ml), dexamethasone (5×10^{-8} M), selenium (60 nM), transferrin (5 µg/ml), triiodothyronine (10^{-9} M), EGF (10 ng/ml), sodium bicarbonate (1.2 g/l), D-glucose (22.4 ml/l of 10% solution) and antibiotics (100 U penicillin/ml; 100 µg streptomycin/ml). Cells were maintained at 37°C in a humidified incubator containing 7% CO₂.

Prior to use, cells became 90–100% confluent. The cells were rinsed twice with 5 ml of antibiotic-free and serum-free D-MEM/F-12 medium. The mono-layers were then refreshed with 5 ml of antibiotic-free and serum-free D-MEM/F-12 medium. Control flasks (n = 5) were treated with 0.1 ml of sterile 1X PBS or killed cell suspension. Each experimental flask (n = 5) was inoculated with suspension of EPA1 or EPA2 at a ratio of approximately 1:100 (bacteria: host cells). Exposed and control flasks were centrifuged for 10 minutes at 1,000 rpm (Sorvall Instruments, Model RC3B, Newtown, CT, USA). All flasks were incubated at 37°C in humidified air containing 7% CO₂ for five hours. Following incubation, the medium in each flask was decanted and samples extracted using TRIzolTM.

RNA extraction/isolation

All samples were extracted for RNA content within 7 days with TRIzolTM (Invitrogen, Carlsbad, CA, USA), as per manufacturer's instructions. Intestinal samples were disrupted in TRIzolTM using a ten second burst with a homogenizer (Fisher Scientific, Model PowerGen700, Pittsburg, PA) but this was not necessary for the mICc12 cells. The RNA concentrations were determined spectrophotometrically (Perkin Elmer UV/VIS, Model Lambda 20, Wellesley, MA, USA) at 260 nm (260/280 ratios also determined). Total RNA samples were stored at –20°C until purified using a glass-fiber procedure (RNAqueous, Ambion, Austin, TX, USA). RNA quality was assessed for each sample using an Agilent 2100 bioanalyzer and associated RNA LabChip kits (Agilent Corp., Palo Alto, CA, USA). Following RNAqueous purification, samples were stored at –20°C.

Gene chips

High quality double-stranded cDNA was created from total RNA using a SuperScriptTM Double-Stranded cDNA Synthesis kit (Invitrogen, Carlsbad, CA, USA) using manufacturer's protocol. Biotinylated cRNA targets were produced *in vitro* from cDNA using a Enzo BioArrayTM HighYieldTM RNA Transcript labeling kit (Affymetrix, Santa Clara, CA, USA) using manufacturer's protocol. The cRNA targets were then hybridized to prefabricated Mouse 430A and B GeneChip probe arrays (Affymetrix, Santa Clara, CA, USA) and scanned according to the manufacturer's protocol.

Microarray data processing and analysis

To account for differences attributable to non-biological factors (e.g., sample processing that might lead to different starting concentrations of mRNA) data were normalized using a log scale, robust multi-array analysis (RMA) method (Irizarry *et al.* 2003; reviewed by Saviozzi & Calogero 2003). Normalization was performed using the RMA module of an R-based program language, specific to Affymetrix applications, downloaded from the Bioconductor web site (www.bioconductor.org).

Once data were normalized, mRNA intensities were assessed using significant analysis of microarrays (SAM) (Version 1.21) obtained from Stanford University's web site (<http://www-stat.stanford.edu/~tibs/SAM/>) (Tusher *et al.* 2001). The SAM program was used to select an acceptable false discovery rate of 5% (FDR) for this study.

RESULTS AND DISCUSSION

Up- and downregulated genes

The number of genes with at least 2-fold change in expression when compared to a phosphate buffered saline (PBS) control is shown in Table 1. For this study, a 2-fold change in gene expression is considered significant. None of the UV, heat-killed controls showed any significant changes in gene expression. The larger number of significantly changed genes in neonates compared to the cell cultures was likely due to the use of the entire intestine for the analysis. However, there were 26 genes that were upregulated and two genes downregulated in both the neonates and the cell culture, after exposure to these virulent strains of *A. hydrophila* (Table 2). These 26 upregulated genes are considered as possible indicators of *Aeromonas* virulence.

The observation that a greater number of genes are upregulated compared to downregulated in response to exposure to a bacterial pathogen was also reported by Cohen *et al.* (2000) using infection of promyelocytic cells

with *Listeria monocytogenes*. Their explanation was that mRNA levels decrease from events such as repression of basal transcriptional machinery and mRNA turnover, but these events are less likely to cause a large change in mRNA when compared to a positive regulatory event such as induction of transcription.

In the present study, the absolute number of up- and downregulated genes was similar when fold change was not a criterion (data not shown). However, when a fold change of greater than 2 was applied, most downregulated genes were discarded. If a criterion of greater than 3 fold change was applied, the downregulated genes almost completely disappear. As a modified t-test, SAM is sensitive to variability. Small fold changes between the control and experimental replicate average responses, combined with the variation inherent in true replicates (as were used in this research), causes downregulated genes with small fold changes to be considered as not significant by SAM. When Cohen assessed reproducibility using independent infections (i.e., true replicates) the percent genes commonly found between the replicates were 57% and 21% for up- and downregulated genes, respectively. If we consider EPA1 and EPA2 as replicate experiments (both being *A. hydrophila*, but acknowledging that they are not clonal), similar percentages are observed. These similar percentages are in light of the different microarray systems used (Affymetrix oligo arrays versus cDNA spotted arrays [200–500 bp]). Reproducibility of this magnitude is encouraging considering the complexity of the assay involved.

Table 1 | Numbers of genes up- and downregulated from *A. hydrophila* infection

	Control, UV, heat-killed	EPA1	EPA2	Common genes, EPA1 and EPA2	Common to both animal and cell culture
Number of upregulated genes in animal model	0	427	316	239	26
Number of upregulated genes in cell culture	0	58	58	37	
Number of downregulated genes in animal model	0	97	221	46	2
Number of downregulated genes in cell culture	0	245	125	72	

Table 2 | Common genes up-regulated and down-regulated in cell culture and neonates. Fold changes for EPA1 are listed first. EPA2 are presented in parentheses

Probe Set ID	Gene title	Gene symbol	Public ID	F.C. cell culture	F.C. neonates
<i>Signaling molecules</i>					
1418930_at	chemokine (C-X-C motif) ligand 10	Cxcl10 ^a	NM_021274	4.5 (4.8)	8.3 (9.6)
1419209_at			NM_008176	10 (7.3)	67 (55)
1441855_x_at	chemokine (C-X-C motif) ligand 1	Cxcl1 ^b	BB554288	5.0 (3.5)	3.3 (3.4)
1457644_s_at			BB554288	9.6 (6.7)	16 (18)
1419728_at	chemokine (C-X-C motif) ligand 5	Cxcl5 ^c	NM_009141	10 (20)	13 (4.0)
1420380_at	chemokine (C-C motif) ligand 2	Ccl2 ^d	AF065933	9.2 (4.0)	6.1 (5.4)
1422029_at	chemokine (C-C motif) ligand 20	Ccl20 ^e	AF099052	3.9	28 (12)
1449984_at	chemokine (C-X-C motif) ligand 2	Cxc12 ^f	NM_009140	34 (13)	49 (50)
1419427_at	colony stimulating factor 3 (granulocyte)	Csf3	NM_009971	4.6 (2.3)	8.0 (7.0)
1460220_a_at	colony stimulating factor 1 (macrophage)	Csf1	BM233698	3.7 (2.1)	(2.4)
1455899_x_at	suppressor of cytokine signaling 3	Socs3	BB241535	2.3 (2.2)	14 (13)
1456212_x_at			BB831725	2.0	13 (10)
1450829_at	tumor necrosis factor, alpha-induced protein 3	Tnfaip3	NM_009397	3.1	6.3 (4.2)
1433699_at			BM241351	8.0 (3.6)	18 (14)
<i>Transcription factors/enhancers/regulators</i>					
1420088_at			Al462015	3.4 (2.8)	4.0 (5.2)
1420089_at			Al462015	4.0 (3.0)	2.8 (4.1)
1448306_at	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	Nfkbia	NM_010907	8.9 (4.8)	13 (10)
1449731_s_at			Al462015	8.1 (5.0)	8.8 (10)
1438157_s_at			BB096843	6.5 (4.6)	8.0 (8.7)
1458299_s_at	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	Nfkbie	BB820441	9.3 (6.2)	3.5 (4.4)
1417483_at	expressed sequence AA408868	AA408868	AB026551	4.0 (3.1)	10 (8.9)
1448728_a_at			AB026551	4.2 (2.3)	7.2 (4.6)
1423233_at	CCAAT/enhancer binding protein (C/EBP), delta	Cebpd	BB831146	6.2 (5.6)	12 (10)
1416332_at	cold inducible RNA binding protein	Cirbp	NM_007705	2.1 (2.2)	2.5

Table 2 | (continued)

Probe Set ID	Gene title	Gene symbol	Public ID	F.C. cell culture	F.C. neonates
1418133_at	B-cell leukemia/lymphoma 3	Bcl3	NM_033601	2.8 (2.6)	5.4 (4.5)
1416916_at	E74-like factor 3	Elf3	NM_007921	2.7	3.2 (2.5)
<i>Surface receptor molecules</i>					
1417268_at	CD14 antigen	Cd14	NM_009841	2.1 (2.1)	3.9 (3.6)
1419132_at	toll-like receptor 2	Tlr2	NM_011905	2.1	3.0 (2.6)
1424067_at	intercellular adhesion molecule	Icam1	BC008626	2.2	10 (9.1)
1448162_at	vascular cell adhesion molecule 1	Vcam1	BB250384	3.7 (2.3)	2.1
<i>Miscellaneous upregulated genes</i>					
1427348_at	cDNA sequence BC036563	BC036563	BC006817	3.0 (2.2)	8.1 (2.5)
1427747_a_at	lipocalin 2	Lcn2	X14607	2.4 (2.6)	10 (3.1)
1430295_at	guanine nucleotide binding protein, alpha 13	Gna13	BG094302	(2.0)	2.2
1451924_a_at	endothelin 1	Edn1	D43775	2.1	(2.3)
1455197_at	Rho family GTPase 1	Rnd1	BE852181	3.6	3.9 (5.6)

^ainterferon activating gene-10.

^bGRO1 oncogene (neutrophil specific).

^cGCP-2, granulocyte chemotactic protein-2.

^dMCP-1 (monocyte specific).

^eMIP-3a, macrophage inflammatory protein-3, chemoattractant for T- and B-cells.

^fMIP-2, Gro2.

Gene expression upregulated in neonates and intestinal cell culture

Table 2 lists the 26 genes whose expression was significantly changed in both the neonates and cell cultures. Seven of these genes (Ccl2, Csf3, Socs3, Tnfaip3, Nfkb1a, Cepbd, and Icam1) were also reported by Galindo *et al.* (2003) to be up-regulated in murine macrophages exposed to the purified *Aeromonas* cytotoxic enterotoxin Act. A majority of these 26 upregulated genes have been associated with the innate immune response, for example, cytokines and transcription factors that act to regulate cytokine gene expression. In addition, four surface receptor molecule genes (Icam1, Vcam1, CD14, Tlr2) were also up-regulated.

NF- κ B is a transcription factor known to be a central regulator of the innate immune response to entero-invasive

bacteria (Elewaut *et al.* 1999). The Nfkb1a gene encodes one of three inhibitors which maintain NF- κ B in an inactive state. If cells are stimulated by exposure to bacterial lipopolysaccharide (LPS), the complex bound to NF- κ B is phosphorylated and subsequently degraded by proteases, allowing NF- κ B to translocate to the nucleus. There the NF- κ B binds to a number of promoters inducing the transcription of a variety of genes including those coding for several cytokines and chemokines. Nfkb1a is also upregulated as a mechanism to shut down the cytokine production before extensive cellular damage occurs.

CD14 and toll-like receptor 2 (Tlr2) are both known mediators of bacterial induced cellular signaling. Soluble CD14 (sCD14) has been shown to be up-regulated in LPS-stimulated human intestinal cells (Funda *et al.* 2001) and is

thought to activate membrane CD14 (mCD14) positive cells (e.g., monocytes and macrophages). Toll-like receptor 4 (Tlr4) is the toll receptor known to bind LPS and activate the NF- κ B pathway. Recent research has demonstrated that the Tlr4-LPS complex is localized in the Golgi apparatus (Hornef *et al.* 2002; Hornef *et al.* 2003). However, Tlr2 also mediates trans-membrane LPS signaling via the NF- κ B pathway and is enhanced by CD14 (Yang *et al.* 1998). Tlr2 detects structural variants of LPS. In addition, LPS-binding protein (LBP), a required participant in the LPS/CD14 complex leading to NF- κ B nuclear translocation, is produced by epithelial cells (Bals & Hiemstra 2004). This provides an explanation of Tlr2 being upregulated rather than Tlr4. Tlr4, the Toll-like receptor involved in the activation of LPS and localized in the Golgi apparatus, is not activated by *A. hydrophila*. In the membrane, Vcam1 causes recruitment of leucocytes to sites of infection and was induced in immortalized murine small intestinal cells by LPS (Li *et al.* 1997). Also, Vcam-1 is a product of NF- κ B induced genes and upregulated in a number of inflammatory conditions of the gut (Jobin & Sartor 2000).

Socs3 and Tnfaip3 are genes involved with cell signaling. For example, suppressor of cytokine signaling 3 (Socs3) takes part in inhibiting the production of pro-inflammatory signals and favors the expression of anti-inflammatory molecules (Berlato *et al.* 2002). Tumor necrosis factor alpha-induced protein 3 (Tnfaip3) is associated with negative feedback in cellular systems. Tnfaip3 inhibits NF- κ B activation and suggests a role for this gene in limiting inflammation by terminating NF- κ B responses (Lee *et al.* 2000). In addition, Tnfaip3 has an anti-apoptotic effect by potently inhibiting NF- κ B activation induced by tumor necrosis factor receptor 1 (TNFR1) (He & Ting 2002).

The gene Icam1, part of the immunoglobulin superfamily, is expressed on epithelial cell surfaces and is upregulated in response to bacterial infections. For example, Icam-1 was up-regulated in human colonic epithelial cells within 4–9 hours of a bacterial infection and produced an increase in adherence of neutrophils to epithelial cells (Huang *et al.* 1996).

Studies are underway comparing isogenic avirulent mutants of virulent *A. hydrophila* strains, as well as virulent and avirulent *A. caviae* and *A. veronii* (biotype *sobria*)

isolates. Future studies focused on additional *Aeromonas* species and other pathogenic genera will determine if the measurement of specific gene expression patterns might be useful in defining potential virulence of environmental bacteria.

CONCLUSIONS

This study has identified a group of genes whose expression is up- or downregulated in response to exposure to viable and virulent *A. hydrophila* bacteria. The results show that a subset of upregulated genes in a mouse intestinal cell line responds in a similar fashion to intestinal cells in a whole animal model. This is an important finding given that the use of whole animals in determining bacterial virulence is laborious and not suited to the screening of large numbers of isolates. Few similarities were seen in the downregulated genes in the two models. Downregulated genes did not exhibit large fold changes in either model and therefore consistent differences are more difficult to distinguish statistically. Gene chip technology provides the basis for a more rapid process of identifying virulent *A. hydrophila* isolated from drinking water.

NOTICE

The U.S. Environmental Protection Agency (EPA) through its Office of Research and Development, partially funded and collaborated in the research described here. It has been subjected to the Agency's peer review and has been approved as an EPA publication. Mention of trade names or commercial products does not constitute endorsement or recommendation by the EPA for use.

REFERENCES

- Bals, R. & Hiemstra, P. S. 2004 Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. *Eur. Respir. J.* **23**, 327–333.
- Bens, M., Bogdanova, A., Cluzeaud, F., Miquerol, L., Kerneis, S., Kraehenbuhl, J. P., Kahn, A., Pringault, E. & Vandewalle, A. 1996 Transimmortalized mouse intestinal cells (m-ICc12) that maintain a crypt phenotype. *Amer. J. Phys.* **270**, C1666–C1674.

- Berlato, C., Cassatella, M. A., Kinjyo, I., Gatto, L., Yoshimura, A. & Bazzoni, F. 2002 Involvement of suppressor of cytokine signaling-3 as a mediator of the inhibitory effects of IL-10 on liposaccharide-induced macrophage activation. *J. Immun.* **168**, 6404–6411.
- Burke, V., Robinson, J., Gracey, M., Peterson, D. & Partridge, K. 1984 Isolation of *Aeromonas hydrophila* from a metropolitan water supply: seasonal correlation with clinical isolates. *Appl. Environ. Microbiol.* **48**, 361–366.
- Cohen, P., Bouaboula, M., Bellis, M., Baron, V., Jblilo, O., Poinot-Chazel, C., Galiègue, S., Hadibi, E.-H. & Casellas, P. 2000 Monitoring cellular response to *Listeria monocytogenes* with oligonucleotide arrays. *J. Biol. Chem.* **275**, 11181–11190.
- Cummings, C. A. & Relman, D. A. 2000 Using DNA Microarrays to Study Host-Microbe Interactions. *Emerg. Infect. Dis.* **6**, 513–525.
- Elewaut, D., DiDonato, J. A., Kim, J. M., Truong, F., Eckmann, L. & Kagnoff, M. F. 1999 NF- κ B is a central regulator of the intestinal epithelial cell innate immune response induced by infection with enteroinvasive bacteria. *J. Immun.* **163**, 1457–1466.
- Funda, D. P., Tučková, L., Farré, M. A., Iwase, T., Moro, I. & Tlaskalová-Hogenová, H. 2001 CD14 is expressed and released as soluble CD14 by human intestinal epithelial cells in vitro: lipopolysaccharide activation of epithelial cells revisited. *Infect. Immun.* **69**, 3772–3781.
- Galindo, C. L., Sha, J., Ribardo, D. A., Fadl, A. A., Pillai, L. & Chopra, A. K. 2003 Identification of *Aeromonas hydrophila* cytotoxic enterotoxin-induced genes in macrophages using microarrays. *J. Biol. Chem.* **278**, 40198–40212.
- Gavriel, A. A., Landre, J. P. B. & Lamb, A. J. 1998 Incidence of mesophilic *Aeromonas* within a public drinking water supply in north-east Scotland. *J. Appl. Bacteriol.* **84**, 383–392.
- He, K.-L. & Ting, A. T. 2002 A20 inhibits tumor necrosis factor (TNF) alpha-induced apoptosis by disrupting recruitment of TRADD and RIP to the TNF receptor 1 complex in Jurkat T cells. *Mol. Cell. Biol.* **22**, 6034–6045.
- Hornef, M. W., Frisan, T., Vandewalle, A., Normark, S. & Richter-Dahlfors, A. 2002 Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. *J. Exp. Med.* **195**, 559–570.
- Hornef, M. W., Normark, B. H., Vandewalle, A. & Normark, S. 2003 Intracellular recognition of lipopolysaccharide by Toll-like receptor 4 in intestinal epithelial cells. *J. Exp. Med.* **198**, 1225–1235.
- Huang, G. T.-J., Eckmann, L., Savidge, T. C. & Kagnoff, M. F. 1996 Infection of human intestinal epithelial cells with invasive bacteria upregulates apical intercellular adhesion molecule-1 (ICAM-1) expression and neutrophil adhesion. *J. Clin. Invest.* **98**, 572–583.
- Irizarry, R. A., Bolstad, B. M., Collin, F., Cope, L. M., Hobbs, B. & Speed, T. P. 2003 Summaries of Affymetrix GeneChip probe level data. *Nuc. Acids Res.* **31**, e15.
- Jobin, C. & Sartor, R. B. 2000 The I κ B/NF- κ B system: a key determinant of mucosal inflammation and protection. *Am. J. Physiol. Cell Physiol.* **278**, C451–C462.
- Lee, E. G., Boone, D. L., Chai, S., Libby, S. L., Chien, M., Lodolce, J. P. & Ma, A. 2000 Failure to regulate TNF-induced NF- κ B and cell death responses in A20-deficient mice. *Science* **289**, 2350–2354.
- Legnani, P., Leoni, E., Soppelsa, F. & Burigo, R. 1998 The occurrence of *Aeromonas* species in drinking water supplies of an area of the Dolomite Mountains, Italy. *J. Appl. Microbiol.* **85**, 271–276.
- Li, X. C., Jevnikar, A. M. & Grant, D. R. 1997 Expression of functional ICAM-1 and VCAM-1 adhesion molecules by an immortalized epithelial cell clone derived from the small intestine. *Cell. Immun.* **175**, 58–66.
- Luangsay, S., Kasper, L. H., Rachinel, N., Minns, L. A., Mennechet, F. J. D., Vandewalle, A. & Buzoni-Gatel, D. 2003 CCR5 mediates specific migration of *Toxoplasma gondii*-primed CD8⁺ lymphocytes to inflammatory intestinal epithelial cells. *Gastroenterology* **125**, 491–500.
- Peng, K.-C., Cluzeaud, F., Bens, M., Van Huyen, J.-P. D., Wioland, M. A., Lacave, R. & Vandewalle, A. 1999 Tissue and cell disruption of the multidrug resistance-associated protein (MRP) in mouse intestine and kidney. *J. Histochem. Cytochem.* **47**, 747–767.
- Picard, B. & Goulet, P. 1987 Seasonal prevalence of nosocomial *Aeromonas hydrophila* infection related to *Aeromonas* in hospital water. *J. Hosp. Infect.* **10**, 152–155.
- Saviozzi, S. & Calogero, R. A. 2003 Microarray probe expression measures, data normalization and statistical validation. *Comp. Func. Genom.* **4**, 442–446.
- Smith, H. R. & Cheasty, T. 1998 Diarrhoeal Disease Due to *Escherichia coli* and *Aeromonas*. In: *Microbiology and Microbial Infections* (ed. Hausler, W. J. & Sussman, M.), pp. 513–529. Oxford University Press, New York.
- Tusher, V. G., Tibshirani, R. & Chu, G. 2001 Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Nat. Acad. Sci.* **98**, 5116–5121.
- Yang, R.-B., Mark, M. R., Gray, A., Huang, A., Xie, M. H., Zhang, M., Goddard, A., Wood, W. I., Gurney, A. L. & Godowski, P. J. 1998 Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signaling. *Nature* **395**, 284–288.
- Wong, C. Y. F., Mayrhofer, G., Heuzenroeder, M. W., Atkinson, H. M., Quinn, D. M. & Flower, R. L. P. 1996 Measurement of virulence of aeromonads using a suckling mouse model of infection. *FEMS Immunol. Med. Microbiol.* **15**, 233–241.