Epidemiol. Infect. (1995), **115**, 465–473
Copyright © 1995 Cambridge University Press

Adhesion of *Aeromonas* sp. to cell lines used as models for intestinal adhesion

S. M. KIROV, L. J. HAYWARD AND M. A. NERRIE*

Department of Pathology, University of Tasmania Clinical School, Hobart, Tasmania 7000, Australia

(*Accepted 31 July 1995*)

SUMMARY

Adhesion to HEp-2 cells has been shown to correlate with enteropathogenicity for *Aeromonas* species. Such adhesion is thought to reflect the ability of strains to adhere to human intestinal enterocytes, although HEp-2 cells are not of intestinal origin. In this study strains of *Aeromonas veronii* biotype sobria isolated from various sources were investigated in parallel assays for their ability to adhere to HEp-2 cells and to an intestinal cell line (Caco-2). Quantitative assays showed identical adhesion values were obtained with both cell lines. Adhesion was best when bacteria were grown at 22 °C compared with 37 °C and 7 °C. Some environmental isolates showed greater adhesion when grown at 7 °C than when grown at 37 °C. Filamentous structures on these strains are also optimally expressed under the above conditions (reported elsewhere). Mechanical shearing or trypsin treatment to remove surface structures from several adhesive strains grown at 22 °C decreased adhesion to cell lines by 50–80% providing further indirect evidence that filamentous adhesins may play a role in cell adhesion for this *Aeromonas* species.

INTRODUCTION

Aeromonas species are increasingly implicated as important enteric pathogens in man but the roles of putative virulence factors, such as haemolysin, cytotoxin, enterotoxin and invasive ability, are still speculative. It is difficult to identify strains that may pose a threat to human health among the diversity of strains present in water and foods. Attachment to the intestinal mucosa is likely to be an important virulence determinant for diarrhoea-causing strains, yet little is known about aeromonas intestinal adhesive mechanisms [1]. Knowledge of these may help to identify strains which pose a public health risk.

While there are virtually no reports of aeromonas adhesion to human intestinal tissue or cells, a few studies have described adhesion of aeromonas to cultured cells such as HEp-2 [2–4], mouse Y1 adrenal [5] and INT407 [6]. Such cell lines have proved useful as *in vitro* models of adhesion to intestinal cells for a number of bacterial enteropathogens. Aeromonas adhesion to HEp-2 cells appears to

* Present address: Ludwig Institute for Cancer Research, P.O. Box Royal Melbourne Hospital, Parkville, Victoria 3050, Australia.

correlate with enteropathogenicity [3]. Evidence from a number of investigations identifies *A. veronii* biotype sobria as the most virulent species and strains of this species are universally found to contain the highest proportion of adherent strains and the greatest number of highly adherent strains in the cell adhesion assays above [7–10]. We have found that 50–60% of strains of this species, irrespective of source, adhere in high numbers to HEp-2 cells [3].

HEp-2 cells are derived from human laryngeal epithelial cell carcinoma. It cannot be assumed that these cells possess the same receptors as human enterocytes or that adhesion to them necessarily reflects ability to bind to human enterocytes. Caco-2 cells, derived from a human carcinoma of the colon, exhibit structural and differentiation patterns characteristic of mature enterocytes and are being increasingly used as a substitute for human intestinal cells to study the adhesion of enteric pathogens [11, 12]. The initial aim of this study was to compare the adhesion of aeromonas strains to HEp-2 and Caco-2 cells in parallel assays. Bacteria were also grown at different temperatures to determine conditions under which bacterial adhesins were optimally expressed. Preliminary investigations were undertaken to determine whether filamentous surface structures played a role in mediating adhesion.

MATERIALS AND METHODS

Bacterial strains

Strains of *A. veronii* biotype sobria (*A. sobria*) ($n = 21$), and one of *A. hydrophila*, were selected from our culture collection. The latter strain was chosen to serve as a negative control in the adhesion assays. The other strains were chosen as representative isolates of *A. veronii* biotype sobria from various sources. Most strains were isolated in Tasmania, Australia. However, a few came from other regions of Australia. Two clinical strains (BC88, BC96) were originally obtained from Dr B. Chang, Perth, Western Australia. Dr K. Majeed, Brisbane, Queensland provided two of the food isolates (AS15, AS02), and two water isolates were from Sydney, New South Wales (S16) and Adelaide, South Australia (AS398). The *A. veronii* biotype sobria strains had been isolated over the last 15 years from diarrhoeal stools ($n = 9$), water ($n = 6$) and food (chicken and lamb) ($n = 6$). The *A. hydrophila* strain (AH26) had been isolated from a diarrhoeal stool and was kindly provided by Dr A. Ho [13]. Strains were maintained in minimal maintenance media (MMM) at room temperature. They had been speciated using the API 20E system (Analytab Products, La Balme-Les Grottes, Montalieu-Vercieu, France) and additional biochemical tests, as previously described [10]. Classification of strains into species was based on the criteria of Popoff [14]. All strains had been tested for their ability to produce enterotoxin, haemolysin and cytotoxin and to grow at 43 °C as described elsewhere [10, 15].

Epithelial cells

HEp-2 cells are routinely maintained in our laboratory. Caco-2 cells were from the American Type Culture Collection (ATCC; HTB37). Cells were grown in Eagle Minimal Essential Medium containing foetal bovine (calf) serum (MEM+FCS)

(Cytosystems, Castle Hill, Australia), 5% for HEp-2 cells and 10% for Caco-2 cells. Semi-confluent monolayers of these cell lines were grown for 24 h and 24–48 h respectively, on 12 mm glass coverslips (Vitromed, Basel, Switzerland) in 24-well plates (Corning, New York, USA). Cell monolayers were washed twice with 1 ml phosphate buffered saline (PBS) (Oxoid, Basingstoke, England), before the addition of bacterial cells.

Adhesion assay

Bacterial strains were grown on tryptone soya agar supplemented with 0·6% yeast extract (TSAY) (Oxoid) and incubated at 37 °C overnight. Several colonies were then inoculated into 10 ml tryptone soya broth containing yeast extract (TSBY). Broth cultures were incubated without agitation at different temperatures (37 °C for 16 h, 22 °C for 2 days, 7 °C for 7 days). After these time intervals the OD_{540} levels of cultures grown at the different temperatures were similar. Bacterial concentration was adjusted to give a final suspension of *c.* 1×10^6 cfu/ml in MEM+1% FCS. For the adhesion assay, 1 ml volumes of these suspensions were added to duplicate coverslip cultures of cell lines. A control adherent strain (BC96) was included in each assay. After incubation (90 min, 37 °C, 5% CO_2), non-adherent bacteria were removed by washing four times with PBS (1–2 ml). The monolayers were fixed with 3:1 methanol:acetic acid (1 ml, 5 min) and stained in the wells with May-Grunwald and Giemsa stains (Gibco, Grand Island, New York). The coverslips were mounted on glass slides, using DePex mounting medium (BDH Gurr, Poole, England).

Adhesion was assessed by bright-field microscopy. For each monolayer, 25 infected cells were chosen at random and the number of cell-associated bacteria counted. The proportion of a further 100 cells per monolayer which were infected was determined. For each coverslip, the mean number of adherent bacteria was calculated. This was multiplied by the proportion of infected cells yielding the average number of bacteria per HEp-2 or Caco-2 cell overall. Experiments with most strains were repeated on 2–9 occasions. Values from coverslip cultures in replicate experiments were pooled and means and standard deviations determined. A strain with a score of 0–1 bacteria per cell was considered 'non-adhesive'. Strains with scores of 1–10 bacteria per cell were defined as 'low adherers', > 10–20 bacteria per cell the strains as 'adherent', > 20 bacteria per cell as 'highly adherent'.

Statistical analysis

Adhesion values were compared by applying analysis of variance (ANOVA) techniques to the mean bacterial counts determined as above.

Removal of bacterial surface structures

One half (5 ml) of the broth cultures (TSBY, 22 °C, 2 days) of selected strains were subjected to mechanical blending at high speed (4 × 45 s cycles, speed 10) in an Omni-mixer (FSE, Co, USA). Between each cycle, the cultures were cooled for 15 s intervals in an ice bath. Bacteria were recovered by centrifugation, washed once with PBS, diluted and assayed for adhesion to cell lines. In separate experiments, the effect of trypsin treatment on adhesive ability was also

Table 1. *Properties of bacterial strains tested in adhesion assays*

Strain no.*	Source	Isolation	Ent†	Hly‡	Cyt§	Growth 43 °C‖
37	Human diarrhoeal stool	1984	+	512	−	3
18	Human diarrhoeal stool	1980	+	1024	−	1
164	Human diarrhoeal stool	1993	+	512	+	
163	Human diarrhoeal stool	1992	+	0	+	2
BC96	Human diarrhoeal stool	Before 1985	+	64	+	2
110	Human diarrhoeal stool	1985	+	> 2048	+	3
BC126	Human diarrhoeal stool	Before 1985	+	1024	+	2
BC88	Human diarrhoeal stool	1983	+	512	+	2
AH26	Human diarrhoeal stool	Before 1990	±	8	+	2
135	Poultry carcass rinse	1988	+	512	+	2
148	Poultry carcass rinse	1988	+	256	+	4
128	Poultry carcass rinse	1988	+	0	+	2
132	Poultry carcass rinse	1988	+	64	+	2
AS02	Lamb meat	1987	+	256	+	4
AS15	Lamb meat	1987	+	0	+	4
60	Water	1984	+	16	+	3
49	Water	1984	+	256	+	0
AS398	Water	1991	+	1024	+	4
S16	Water	1991	+	1024	+	2
34	Water	1984	+	128	±	3
50	Water	1984	+	64	+	2

* All strains were *A. veronii* biotype sobria, except AH26 which was an *A. hydrophila* [13].
† Enterotoxin, detected by suckling mouse assay; '±' borderline positive [10].
‡ Haemolysin titre *vs.* rabbit erythrocytes [10].
§ Cytotoxin, detected in Vero cell assay; '+': ⩾ 50% of Vero cells affected [10].
‖ Growth on TSAY for 36 h; 0: no growth, 4: heavy growth [10].

determined for selected strains. Bacteria grown as above (5 ml of culture) were incubated with trypsin (Boerhinger Mannheim, Germany) at a final concentration of 1 mg/ml for 30 min at 37 °C. Residual trypsin was removed by washing the bacteria once in 10 ml PBS and the resuspended bacteria adjusted to the concentration used in the adhesion assay. Adhesion by the treated bacteria (sheared or trypsin-treated) was assayed in parallel with untreated bacteria from the same culture. Loss of pili was confirmed by transmission electron microscopy (EM) and bacterial integrity confirmed by EM and culture on TSAY.

Electron microscopy

Bacteria and preparations of filamentous structures obtained from bacterial strains by mechanical shearing were examined under a Philips 410 electron microscope at 80 kV following staining with uranyl acetate (1%) for 30 s to 1 min as described elsewhere [16].

RESULTS

Properties of bacterial strains

Details of the source, date of isolation and virulence properties of the strains tested in the adhesion assays are summarized in Table 1. The *A. veronii* biotype sobria strains all produced two or more exotoxins and most grew at 43 °C.

Table 2. *Comparison of adhesion of aeromonas strains to HEp-2 and Caco-2 cell lines*

		Bacteria per cell*			
		37 °C		7 °C	
Source	Strain no.	HEp-2†	Caco-2†	HEp-2‡	Caco-2‡
A. veronii biotype sobria					
Human diarrhoeal stool	37	8·9±1·8	4·9±0·5	6·3±0·5	7·3±0·4
	18	10·3±0·9	9·1±0·4	1·7±0·4	4·6±3·7
	164	12·0±1·8	14·2±0·7	13·1±1·8	16·0±0·6
	163	11·6±1·3	16·2±0·6	—	—
Food	135	11·2±4·9	5·5±0·7	8·5±1·3	3·7±0·4
	148	28·0±6·2	20·9±2·6	6·0±2·9	2·3±0·3
	AS02	1·2±0·7	2·5±0·2	8·3±1·7	11·6±2·5
Water	60	0·8±0·3	0·2±0·1	10·4±0·7	11·6±1·5
	49	5·1±0·4	2·2±0·1	2·6±2·7	5·3±1·0
	AS398	9·6±0·7	3·1±0·5	11·4±1·5	12·8±0·3
	S16	1·9±0·1	1·2±0·3	1·0±0·2	2·3±0·6
A. hydrophila	AH26	0·6±0·2	0·9±0·5	—	—

* Mean ± standard deviation of duplicate coverslips from one experiment. Bacteria were grown in TSBY at 37 °C for 16–18 h and at 7 °C for 7 days.

† Adhesion following bacterial growth at 37 °C was independent of the cell line, $P = 0{\cdot}778$ by ANOVA.

‡ Adhesion following bacterial growth at 7 °C was independent of the cell line, $P = 0{\cdot}417$ by ANOVA.

Adhesion vs. *cell line*

Results of adhesion by bacterial strains grown at different temperatures and tested in parallel adhesion assays to HEp-2 and Caco-2 cells are shown in Tables 2 and 3. It was found that adhesion was independent of the cell line used for all bacterial growth temperature (Tables 2, 3).

Effect of bacterial growth temperature on aeromonas adhesion

It was observed that some environmental strains (strains 60, AS02) showed better adhesion when grown at 7 °C compared to 37 °C (Table 2). Adhesive ability, however, was best overall when bacteria were grown at 22 °C (Table 3). The 37 °C data presented in the Tables was for cultures grown for 16–18 h. Six strains were also tested for adhesion following growth at 37 °C for 3 h according to the method described by Carrello and colleagues [2]. Under these conditions the strains were more adhesive than when grown for 16 h ($P < 0{\cdot}001$) (data not shown). They were still significantly less adherent, however, than when grown at 22 °C ($P < 0{\cdot}001$).

Effect of removal of surface structures on adhesive ability

Table 4 compares the adhesive ability of selected strains grown at 22 °C before and after mechanical blending to shear off surface structures. After blending, bacteria remained intact with no visible filamentous surface appendages. They were viable on culture. Electron microscopy of concentrates of the blended

Table 3. *Effect of bacterial growth temperature on* Aeromonas veronii *biotype sobria adhesion to cell lines*

		Bacteria per cell*					
		HEp-2‡			Caco-2‡		
Source	Strain no.	37 °C	22 °C†	7 °C	37 °C	22 °C†	7 °C
Human diarrhoeal stool	BC96	13·6±1·0 (n = 4)	22·8±4·8 (n = 9)	8·8±3·7 (n = 4)	16·7±4·5 (n = 6)	20·2±5·7 (n = 2)	7·8±2·4 (n = 4)
	110	12·0±1·5 (n = 2)	9·5±6·5 (n = 8)	8·5±0·6 (n = 1)	6·3±1·3 (n = 1)	18·9±0·1 (n = 1)	7·9±3·6 (n = 1)
	BC126	3·1±0·1 (n = 1)	30·3±10·3 (n = 3)	13·6±0·0 (n = 1)	9·0±2·1 (n = 1)	22·0±2·8 (n = 1)	11·5±2·1 (n = 1)
Food	128	10·8±0·6 (n = 1)	26·3±5·2 (n = 4)	—	14·4±0·2 (n = 1)	18·4±7·5 (n = 1)	—
	132	12·3±2·6 (n = 2)	22·5±5·3 (n = 9)	8·1±2·3 (n = 2)	10·8±1·4 (n = 3)	21·4±0·7 (n = 1)	3·5±2·1 (n = 2)
	AS15	7·4±2·3 (n = 2)	14·0±1·2 (n = 2)	9·5±3·0 (n = 3)	10·9±3·4 (n = 2)	17·0±2·9 (n = 1)	5·5±3·9 (n = 2)
Water	34	6·0±4·6 (n = 2)	18·5±8·6 (n = 9)	13·3±3·8 (n = 2)	12·2±3·1 (n = 2)	18·8±0·2 (n = 1)	12·5±1·5 (n = 1)
	50	8·4±2·2 (n = 1)	14·8±2·2 (n = 1)	10·7±0·7 (n = 1)	—	—	—

* Mean ± standard deviation of duplicate coverslips from one or more experiments (n = number of times adhesion assay was performed). Bacteria were grown in TSBY at 37 °C for 16–18 h, at 22 °C for 2 days and at 7 °C for 7 days.

† Greater adhesion with bacteria grown at 22 °C on both cell lines, $P < 0{\cdot}001$ by ANOVA.

‡ Adhesion was independent of the cell line used at the three temperatures, $P = 0{\cdot}659$ by ANOVA.

Table 4. *Effect of mechanical blending on* Aeromonas veronii *biotype sobria adhesion.*

		Bacteria per HEp-2 cell*		
Source	Strain no.	Before blending	After blending	Decrease in adhesion (%)†
Human diarrhoeal stool	BC96	35·2, > 30 (n = 1)	14·8±1·5 (n = 1)	~ 60
	110	5·4±2·7 (n = 3)	4·0±2·1 (n = 3)	30
	BC88	14·3±2·9 (n = 1)	3·1±0·6 (n = 1)	80‡
	163	12·1±3·1 (n = 2)	3·9±0·6 (n = 2)	70
Food	128	27·4±2·4 (n = 2)	13·1±6·5 (n = 2)	50
	132	27·4±7·4 (n = 2)	12·1±6·2 (n = 3)	60

* Mean ± standard deviation.

† Except for strain 110, percentage reductions in bacteria are significant, $P < 0{\cdot}001$ by ANOVA.

‡ Identical results were obtained on Caco-2 cells for this strain.

supernatant showed pili of varied morphology, including thin, flexible pili, bundles of pili, flagella and outer membrane vesicles. Removal of these structures decreased adhesive ability by 50–80% for the adherent strains BC96, BC88, 128, 132 and 163. Trypsin treatment similarly decreased adhesion (> 70%) for these strains (results not shown).

DISCUSSION

This study has shown that strains of *A. veronii* biotype sobria show similar adhesion characteristics to both HEp-2 and Caco-2 cells. Although it is possible that bacteria could be binding to different receptors on the two cell lines, this seems unlikely given the very similar adhesive values obtained with a variety of different bacterial strains under the different conditions tested. Our results provide further evidence that adhesion to HEp-2 cells will be useful as a screening model in investigations of intestinal adhesion mechanisms by the species, *A. veronii* biotype sobria. Validation of the cell models still requires comparative studies of aeromonas adhesion to human intestinal tissue.

Adhesion to both cell lines was best when bacterial strains of this species were grown at 22 °C. Several environmental strains showed better adhesion when grown at 7 °C than when grown at 37 °C. Optimal expression of other aeromonas virulence determinants has also been found to occur at *c.* 20 °C [17, 18]. This may have implications for foodborne aeromonas infection as foods stored at room temperature or below may provide the conditions for aeromonads to be most virulent.

The mechanism(s) of adhesion by *A. veronii* biotype sobria to cell lines remain to be elucidated. Nishikawa and colleagues have recently investigated several aeromonas isolates for adhesion and invasion of the Caco-2 cell line [19]. They concluded pili were unlikely to play an important role in Caco-2 cell adhesion as they observed few filamentous structures on their adherent strains and that bacteria were closely bound to the cell surface. However, their study only included two '*A. sobria*' strains which were grown at 30 °C.

We have found that pili and other filamentous structures such as bundle-forming pili (BFP) and filamentous networks (FN) are optimally expressed at 22 °C or below [20]. This, and the fact that removal of surface structures by shearing or trypsin treatment resulted in a significant decrease in adhesive ability for several highly adherent strains, provides some indirect evidence that filamentous structures may be involved in adhesion. Carrello and colleagues also found similar percentage decreases in binding to HEp-2 cells by three strains of this species after treatments to remove pili [2]. Pili of long, flexible morphology have been characterized by Hokama and colleagues as intestinal colonization factors for three strains of *A. veronii* biotype sobria [21–23]. Thin, flexible pili are, however, scant (< 50 per cell on > 50% of bacteria in the culture) for most adherent strains of this species isolated from human diarrhoeal faeces and chicken carcasses, even when bacteria are grown at 22 °C. Moreover, BFP and FN were not seen on all adherent strains. Therefore, non-filamentous adhesins may also be involved.

Our sheared preparations contained some outer membrane vesicles. Several reports have implicated lipopolysaccharide (LPS) in adhesion of aeromonas to

HEp-2 cells [25, 26]. LPS has also been correlated with enteropathogenicity [18, 24]. Carbohydrate-reactive OMPs (CROMPS), which may be porins for fucose, have also been postulated as adhesins and may be intestinal adhesive factors [27, 28], but do not appear to mediate binding to cell lines. The *A. hydrophila* strain (strain A6) from which these OMPs were purified was not adhesive in the above cell line assays [3, 16]. Indeed, we have found that most *A. hydrophila* strains, even those isolated from diarrhoeal faeces as sole potential pathogen (eg. strain AH26, this study), are much less adherent in cell line assays than strains of *A. veronii* biotype sobria [3]. Clinical strains of *A. caviae* are generally (> 30% of strains) adherent [3, Dickson, Kirov and Sanderson, unpublished observations].

Further studies are required to determine the nature of aeromonas adhesins for these cell lines and their significance in pathogenicity. This study has established that these adhesins are optimally expressed on strains of *A. veronii* biotype sobria grown under environmental conditions (liquid medium and temperatures of 22 °C or below).

ACKNOWLEDGEMENTS

This work was supported by a grant from the Australian National Health and Medical Research Council. We thank Dr C. Thomas, University of Adelaide, South Australia for his assistance in obtaining the Caco-2 cell line and Dr K. Sanderson for his help with the preparation of the Tables for this manuscript.

REFERENCES

1. Kirov SM. Adhesion and piliation of *Aeromonas* spp. Med Microbiol Lett 1993; **2**: 274–80.
2. Carrello A, Silburn KA, Budden JR, Chang BJ. Adhesion of clinical and environmental *Aeromonas* isolates to HEp-2 cells. J Med Microbiol 1988; **26**: 19–27.
3. Grey PA, Kirov SM. Adherence to HEp-2 cells and enteropathogenic potential of *Aeromonas* spp. Epidemiol Infect 1993; **110**: 279–87.
4. Neves MS, Nunes MP, Milhomen AM. *Aeromonas* species exhibit aggregative adherence to HEp-2 cells. J Clin Microbiol 1994; **32**: 1130–1.
5. Clark RB, Knoop FC, Padgitt PJ, Hu DH, Wong JD, Janda MJ. Attachment of mesophilic aeromonads to cultured mammalian cells. Curr Microbiol 1989; **19**: 97–102.
6. Nishikawa Y, Kimura T, Kishi T. Mannose-resistant adhesion of motile *Aeromonas* to INT407 cells and the differences among isolates from humans, food and water. Epidemiol Infect 1991; **107**: 171–9.
7. Daily OP, Joseph SW, Coolbaugh JC et al. Association of *Aeromonas sobria* with human infection. J Clin Microbiol 1981; **13**: 769–77.
8. Janda JM, Oshiro LS, Abbott SL, Duffey PS. Virulence markers of mesophilic aeromonads. Association of the autoagglutination phenomenon with mouse pathogenicity and the presence of a peripheral cell-associated layer. Infect Immun 1987; **55**: 3070–7.
9. Janda JM, Reitano M, Bottone EJ. Biotyping of *Aeromonas* isolates as a correlate to delineating a species-associated disease spectrum. J Clin Microbiol 1984; **19**: 44–7.
10. Kirov SM, Rees B, Wellock RC, Goldsmid JM, Van Galen AD. Virulence characteristics of *Aeromonas* spp. in relation to source and biotype. J Clin Microbiol 1986; **24**: 827–34.
11. Pinto M, Robine-Leon S, Appay M-D et al. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol Cell 1983; **47**: 323–30.
12. Russel RG, Blake DC. Cell association and invasion of Caco-2 cells by *Campylobacter jejuni*. Infect Immun 1994; **62**: 3773–9.
13. Ho ASY, Mietzner TA, Smith AJ, Schoolnik GK. The pili of *Aeromonas hydrophila*: identification of an environmentally regulated 'mini-pilin'. J Exp Med 1990; **172**: 795–806.

14. Popoff M. Genus III. *Aeromonas*. In Krieg NR, Holt JG, eds. Bergey's manual of systematic bacteriology, vol. 1. Baltimore: Williams & Wilkins Co, 1984; 545–8.
15. Kirov SM, Brodribb F. Exotoxin production by *Aeromonas* spp. in foods. Lett Appl Microbiol 1993; **17**: 208–11.
16. Kamperman L, Kirov SM. Pili and the interaction of *Aeromonas* species with human peripheral blood polymorphonuclear cells. FEMS Immunol Med Microbiol 1993; **7**: 187–96.
17. Mateos D, Anguita J, Naharro G, Paniagua C. Influence of growth temperature on the production of extracellular virulence factors and pathogenicity of environmental and human strains of *Aeromonas hydrophila*. J Appl Bacteriol 1993; **74**: 111–8.
18. Merino S, Camprubí S, Tomás JM. Effect of growth temperature on outer membrane components and virulence of *Aeromonas hydrophila* strains of serotype O:34. Infect Immun 1992; **60**: 4343–9.
19. Nishikawa Y, Hase A, Ogawasara J, Scotland SM, Smith HR, Kimura T. Adhesion and invasion of human colon carcinoma Caco-2 cells by *Aeromonas* strains. J Med Microbiol 1994; **40**: 55–61.
20. Kirov SM, Jacobs I, Hayward LJ, Hapin RH. Electron microscopic examination of factors influencing the expression of filamentous surface structures on clinical and environmental isolates of *Aeromonas veronii* biotype sobria. Microbiol Immunol 1995; **39**: 329–38.
21. Hokama A, Iwanaga M. Purification and characterization of *Aeromonas sobria* pili, a possible colonization factor. Infect Immun 1991; **59**: 3478–83.
22. Hokama A, Iwanaga M. Purification and characterization of *Aeromonas sobria* Ae24 pili: a possible new colonization factor. Microb Pathog 1992; **13**: 325–34.
23. Iwanaga M, Hokama A. Characterization of *Aeromonas sobria* TAP13 pili: a possible new colonization factor. J Gen Microbiol 1992; **138**: 1913–9.
24. Maruvada R, Das P, Ghosh AN, Pal SC, Balakrish Nair G. Electrophoretic mobility of and immunoblot analysis of the outer membrane proteins of *Aeromonas hydrophila*, *A. sobria* and *A. caviae*. Microbios 1992; **71**: 105–13.
25. Francki KT, Chang BJ. Variable expression of O-antigen and the role of lipopolysaccharide as an adhesin in *Aeromonas sobria*. FEMS Immunol Med Microbiol 1994; **122**: 97–101.
26. Nandapalan N, Chang BJ. Production and characterization of monoclonal antibodies to *Aeromonas sobria* surface antigens. FEMS Microbiol Immunol 1989; **47**: 515–24.
27. Quinn DM, Atkinson HM, Bretag AH, Tester M, Trust TJ, Wong CYE, Flower RLP. Carbohydrate-reactive, pore-forming outer membrane proteins of *Aeromonas hydrophila*. Infect Immun 1994; **62**: 4054–8.
28. Quinn DM, Wong CYE, Atkinson HM, Flower RLP. Isolation of carbohydrate-reactive outer membrane proteins of *Aeromonas hydrophila*. Infect Immun 1993; **61**: 371–7.