Interaction of Arcobacter spp. with human and porcine intestinal epithelial cells

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Abstract

Little is known about the pathogenic mechanisms or potential virulence factors of Arcobacter spp. The aim of the study described here was to obtain more insights in the pathogenicity mechanisms of Arcobacter spp. by testing their ability to adhere to, invade and induce interleukin-8 expression in human Caco-2 and porcine IPI-2I cell lines. Eight Arcobacter strains were tested. Four strains were obtained from a culture collection, and represent the four Arcobacter spp. known to be associated with animals and humans. The other four strains were field isolates from the amniotic fluid of sows and from newborn piglets. All eight Arcobacter strains were able to adhere to both cell lines, and induced interleukin-8 production as early as 2 h after a 1 h incubation period. This production was still increased 6 h postinfection. Differences in the cell association of the eight strains were obvious, with A. cibarius showing the highest adhesion ability. Invasion of intestinal epithelial cells was only observed for A. cryaerophilus strains. No correlation between invasiveness or strong adhesion of the tested strains and the level of interleukin-8 induction was observed.

Introduction

The genus Arcobacter was introduced into the family Campylobacteraceae in 1991 (Vandamme, 2000). Currently, the genus Arcobacter comprises six species. Arcobacter butzleri, A. cryaerophilus and A. skirrowii have been isolated from humans (Vandenbergh et al., 2004; Wybo et al., 2004), nonhuman primates (Anderson et al., 1993), and other animals (chickens, pigs, cattle, horses, and sheep) (Kabeya et al., 2003; Van Driessche et al., 2003). These bacteria have also been recovered worldwide from chicken, pork, and beef (Ohlendorf & Murano, 2002; Atabay et al., 2003; Villarruel-Lopez et al., 2003; Kabeya et al., 2004). In 2005, a new species, A. cibarius, isolated from chicken carcasses, was added to the genus (Houf et al., 2005). Two other species were isolated in the environment: A. nitrofigilis, a free-living nitrogen-fixing organism (McClung et al., 1983), and A. halophilus, isolated from a hypersaline lagoon (Donachie et al., 2005).

Recently, there has been public concern about Arcobacter as a potential zoonotic foodborne and waterborne pathogen (Phillips, 2001; Assanta et al., 2002). Besides the abundance of Arcobacter spp. in meat, A. butzleri has recently been isolated from raw milk samples (Scullion et al., 2006). Associations of A. butzleri, A. skirrowii and A. cryaerophilus with illness in humans and animals have been reported (Vandamme et al., 1992; De Oliveira et al., 1997; Woo et al., 2001; Lau et al., 2002). These three species were detected in stool samples of hospital patients with gastrointestinal disorders and in healthy children (Samie et al., 2002). Arcobacter butzleri was found to be the fourth most common Campylobacter or Campylobacter-like organism isolated from human diarrheic stool specimens (Vandenbergh et al., 2004). The isolation of A. butzleri from a neonate made in utero infection seem likely (On et al., 1995).

However, despite reports of its association with human and animal diseases, knowledge on the pathogenicity of Arcobacter is still very limited. In vivo studies on the invasion and virulence of Arcobacter strains in 1-day-old piglets and in chickens several days of age have been performed (Wesley et al., 1996; Wesley & Baetz, 1999). The results varied with the bacterial strains, as well as with the species and breed of...
the animals. Besides reports on in vitro cytotoxicity (Fernandez et al., 1995; Musmanno et al., 1997; Johnson & Murano, 2002; Villarruel-Lopez et al., 2003; Vandenberg et al., 2005), a few studies of the adhesive and invasive abilities of A. butzleri and A. cryaerophilus have been carried out in the HEP-2, Hela and INT 407 cell lines (Fernandez et al., 1995; Musmanno et al., 1997; Carbone et al., 2003; Vandenberg et al., 2005).

The present study was performed to obtain more information on the pathogenicity of Arcobacter strains associated with human and animals by studying their capacity for adhesion to and invasion of human and pig intestinal cell lines (Caco-2 and IPI-2I, respectively). We also report the ability of these Arcobacter strains to induce expression of the proinflammatory cytokine interleukin-8 (IL-8), which is considered to be a major virulence factor in Helicobacter pylori and Campylobacter spp. (Hickey et al., 1999).

**Materials and methods**

**Bacterial strains and culture**

The experiments were carried out on four Arcobacter strains obtained from the LMG collection (Laboratory of Microbiology, Gent University, Belgium) – A. butzleri LMG 6620, A. skirrowii LMG 6621, A. cryaerophilus LMG 7537, and A. cibarius LMG 21996 – and four Arcobacter strains isolated on a pig farm in the Netherlands; one of these was A. skirrowii, and the three A. cryaerophilus strains could be genetically distinguished by pulsed-field gel electrophoresis (Ho et al., 2006). A Salmonella enterica strain was used as a control in the adhesion, invasion and IL-8 assays (Van Asten et al., 2000) (Table 1).

To prepare inocula for adhesion and invasion assays, Arcobacter strains from stock cultures were streaked onto blood agar plates (Brain Heart Infusion agar, Oxoid, supplemented with 5% horse blood) and incubated for 48 h at 30°C under microaerophilic conditions (5% O2, 10% CO2, and 85% N2). A single colony of each strain was transferred into Brain Heart Infusion broth (Oxoid) and cultured overnight at 30°C under microaerophilic conditions. The broth cultures were centrifuged (5 min, 6000 g, 4°C) and resuspended in warm (37°C) plain Dulbecco’s modified Eagle’s medium (DMEM; see following section for a description of the medium). Overnight cultures of S. enterica in Luria–Bertani (LB) broth were diluted 1/100 in LB broth and grown at 37°C for 2 h. Bacteria were collected by centrifugation (15 min, 1800 g, at room temperature) and resuspended in warm plain DMEM.

**Cell culture**

Human enterocyte-like Caco-2 cells (ATCC HTB 37) were grown in DMEM (Flow Laboratories, Amstelbd BV, Amsterdam, the Netherlands) supplemented with 1% (v/v) nonessential amino acids, 50 µg mL\(^{-1}\) gentamicin, 10 mM NaHCO\(_3\), 1.7 mM glutamine, 25 mM HEPES, and 20% (v/v) fetal calf serum. The IPI-21 cell line (ECACC 93100622) was established from the ileum of an adult boar (d/d haplotype) and immortalized by transfection with an SV40 plasmid (pSV3-neo) (Kaeffer et al., 1993). IPI-2I cells were grown in the same supplemented DMEM as described above, except for the use of 10% (v/v) fetal calf serum and the addition of 0.024 IU mL\(^{-1}\) bovine insulin (Sigma). Both cell lines were grown at 37°C in a humidified atmosphere of 5% CO\(_2\) and 95% air. The plain DMEM used in the experiments is referred to as supplemented medium without gentamicin and fetal calf serum. Caco-2 and IPI-2I cells were seeded into 12-well plates at 4 × 10\(^4\) and 5 × 10\(^4\) cells cm\(^{-2}\), respectively. Differentiated Caco-2 cells were obtained after 19 days of culture (c. 5 × 10\(^5\) cells cm\(^{-2}\)). A time-course experiment was carried out to determine the confluency stage of IPI-2I cells as described below. At least 1 h before adhesion and invasion assays were started, the monolayers were washed twice with 1 mL of warm plain DMEM. Plates were then incubated in 1 mL of warm plain DMEM per well until the assays of bacterial infection started. All incubation steps of the following assays were carried out at 37°C in a humidified atmosphere of 5% CO\(_2\) and 95% air.

**Characterization of the IPI-2I cell line**

To determine the growth curve of the IPI-2I cells, a culture was seeded in six-well plates (5 × 10\(^4\) cells cm\(^{-2}\)) and followed for 4 weeks. Three times a week, the culture medium was collected and total cell protein was measured with the BCA\textsuperscript{TM}-Protein Assay Kit (Pierce). Cell numbers were counted by microscopy using haemocytometers. IL-8 production was measured in the supernatants (as described below). The experiment was performed in triplicate with one particular passage of the cell line.

| Table 1. Bacterial strains used in this study |
|-----------------|-----------------|
| Strains         | Origin of isolates                  |
| A. butzleri LMG 6620 | Blood sample of a 65-year-old woman with cellulites |
| A. skirrowii LMG 6621 | Feces of a lamb with history of persistent diarrhea |
| A. cryaerophilus LMG 7537 | Aborted ovine fetus                  |
| A. cibarius LMG 21996 | Chicken carcass                      |
| A. skirrowii 125    | Field strain from sow amniotic fluid |
| A. cryaerophilus 141 | Field strain from newborn piglet     |
| A. cryaerophilus 112 | Field strain from sow amniotic fluid |
| A. cryaerophilus 1038 | Field strain from newborn piglet     |
| S. enterica        | Chicken, serotype Enteritidis 90-13-706 (ID-DLO, Lelystad, the Netherlands) |
Adherence and invasion assays

The assays were performed essentially as described by Kusters et al. (1993). Briefly, the two cell lines were infected with 1 mL of culture of each bacterial strain for 1 h. On the basis of a cell-associated saturation study with C. jejuni and Caco-2 cells (Russell & Blake, 1994), 1–3 × 10⁷ CFUs of Arcobacter strains were added per well for both adhesion and invasion assays. Inocula of the S. enterica strain were c. 10⁶ CFUs well⁻¹ (Kusters et al., 1993; Van Asten et al., 2000). To determine the number of cell-associated Arcobacter organisms (adhering and invading), the monolayers were washed three times with plain DMEM and lysed in 10% Triton-X in phosphate-buffered saline at room temperature for 5 min, in order to release the bacteria. The suspensions were serially diluted 10-fold, and 100 μL of each dilution was plated on BHI agar supplemented with 5% horse blood. The plates were incubated for 48 h at 30 °C under microaerophilic conditions. Lysates of cells infected by S. enterica were plated onto LB agar and incubated for 24 h at 37 °C. The numbers of cell-associated bacteria were calculated as total (adhering and intracellular) bacterial CFUs recovered per 10 Caco-2 or IPI-2I cells.

In the invasion assay, the monolayers in the wells were washed once with 1 mL of warm plain DMEM after 1 h of incubation with bacteria, and then incubated for 2 h with 1 mL of 300 μg mL⁻¹ colistin in warm plain DMEM to kill extracellular bacteria. Cells were washed three times with plain DMEM, and finally lysed in 1% Triton X-100. The number of intracellular bacteria was determined by plating as described above. Cell association and invasion assays were performed in triplicate per passage on two different cell passages.

Enzyme-linked immunosorbent assay for IL-8

Monolayers in 12-well plates were infected with bacteria and incubated for 1 h as described above. The plates were washed once with 1 mL of warm plain DMEM. One microliter of 300 μg mL⁻¹ colistin in warm plain DMEM was added, and the plates were incubated for 2 h. In order to monitor the kinetics of IL-8 induction vs. incubation time, the monolayers in the wells were incubated for 2, 6 or 24 h with A. butzleri LMG 6620, A. skirrowii LMG 6621, A. cryaerophilus LMG 7537 and S. enterica, respectively. The medium was then collected for testing of IL-8 production. The cytokine concentration was determined using the IL-8 Cytosets™ antibody pair kit containing matched, prefiltered and fully optimized capture and detection antibodies, and recombinant standard and streptavidin–horseradish peroxidase’s (Biosource Europe SA, Nivelles, Belgium). The assay was performed following the manufacturer’s instructions.

Scanning electron microscopy (SEM)

Both cell lines were seeded on plastic slides in 12-well tissue culture plates. Differentiated cells were incubated with the various Arcobacter strains for 1 h. The cells were then washed three times with plain DMEM and fixed with Karnovsky’s glutaraldehyde fixative for more than a week. After being washed with 0.1 mL of cacodylate buffer (pH 7.4), the samples were postfixed with 2% OsO4 in 0.1 mL of cacodylate buffer (pH 7.4) for 2 h. The specimens were dehydrated in serial solutions of 50%, 70%, 80%, 96% and 100% acetone, and then critical-point-dried using a Bal-Tec CPD 030 system. Finally, the slides were coated with platinum to a thickness of about 10 nm, and examined by SEM (Phillips XL 30 SFEG).

Statistical analysis

One-way ANOVA and Tukey’s multiple comparison tests were used to analyze differences in cell association and IL-8 induction among Arcobacter strains (using Minitab version 12.32). Repeated-measures analysis was performed for the monitoring of IL-8 induction vs. incubation time (spss version 10.0.5). A confidence level of 95% was defined for these analyses.

Results

Growth curve of the IPI-2I cell line

The growth and the confluency of the IPI-2I cells were determined over a 28-day period of culturing. Confluent monolayers were reached on day 5, as judged by microscopic examination. Both the cell number and the total cell protein of the IPI-2I cells increased initially but leveled off after about 7 days of culture (c. 10⁷ cells well⁻¹ were obtained with a total protein content of c. 1 mg well⁻¹). On the basis of these results, IPI-2I cells were grown for 5 days to obtain confluent monolayers of c. 0.75–1 × 10⁶ cells cm⁻² to be used in the adherence and invasion assays.

Cell association and invasion

All eight Arcobacter strains demonstrated the ability to adhere to Caco-2 and IPI-2I cells (Table 2). Scanning electron micrographs of Arcobacter organisms adhering to the epithelial cells are shown in Fig. 1. In general, the association of each strain with both cell lines was comparable. Tukey’s pairwise comparison indicated a significantly stronger association of the A. cibarius strain with both Caco-2 and IPI-2I cells than that of the other Arcobacter strains (more than 10 bacteria per Caco-2 cell). The capacity of A. skirrowii strains to adhere to both cell lines appeared to be lower than that of the other strains, although a statistically
### Table 2. Interaction of *Arcobacter* strains with Caco-2 and IPI-2I cells

<table>
<thead>
<tr>
<th>Strains</th>
<th>Cell association (CFUs per 10 Caco-2 or IPI-2I cells)</th>
<th>Invasion (CFUs per 10⁷ Caco-2 or IPI-2I cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Caco-2</td>
<td>IPI-2I</td>
</tr>
<tr>
<td><em>A. butzleri</em> LMG 6620</td>
<td>49 ± 15</td>
<td>38 ± 20</td>
</tr>
<tr>
<td><em>A. skirrowii</em> LMG 6621</td>
<td>18 ± 12</td>
<td>8 ± 5*</td>
</tr>
<tr>
<td><em>A. cryaerophilus</em> LMG 7537</td>
<td>53 ± 15</td>
<td>56 ± 12</td>
</tr>
<tr>
<td><em>A. cibarius</em> LMG 21996</td>
<td>123 ± 26**</td>
<td>78 ± 49*</td>
</tr>
<tr>
<td><em>A. skirrowii</em> 125</td>
<td>18 ± 10</td>
<td>11 ± 10*</td>
</tr>
<tr>
<td><em>A. cryaerophilus</em> 141</td>
<td>28 ± 26</td>
<td>32 ± 29</td>
</tr>
<tr>
<td><em>A. cryaerophilus</em> 112</td>
<td>18 ± 13</td>
<td>42 ± 38</td>
</tr>
<tr>
<td><em>A. cryaerophilus</em> 1038</td>
<td>42 ± 37</td>
<td>49 ± 42</td>
</tr>
<tr>
<td><em>S. enterica</em></td>
<td>68 ± 25</td>
<td>54 ± 5</td>
</tr>
</tbody>
</table>

¹The numbers are presented as mean ± SD of bacterial CFUs.
²*Arcobacter cryaerophilus* 1038 showed ability to invade Caco-2 cells in the first assay.
³*Arcobacter cryaerophilus* 1038 did not invade Caco-2 cells in the repeated assay.
**Tukey’s test demonstrated a significantly higher level of cell association of the *Arcobacter cibarius* strain with Caco-2 than seen with the other *Arcobacter* strains (P < 0.001).
*Significant differences were observed between *Arcobacter cibarius* LMG 21996 and the two *Arcobacter skirrowii* strains (P < 0.01).

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**Fig. 1.** Scanning electron micrographs of cell-associated *Arcobacter*. (a, b) *Arcobacter cryaerophilus* LMG 7537 on Caco-2. (c) *Arcobacter skirrowii* LMG 6621 on Caco-2. (d) *Arcobacter skirrowii* LMG 6621 on IPI-2I. Scale bar = 0.5 μm (a) or 1 μm (b, c, d).
significant difference was only detected in comparison with the *A. cibarius* strain.

Of the eight *Arcobacter* strains, only *A. cryaerophilus* LMG 7537 invaded both human and pig epithelial cells (Table 2). *Arcobacter cryaerophilus* 1038 was the only porcine strain that exhibited an ability to invade Caco-2 cells, although it did not invade IPI-2I cells. When the assay was repeated, this capacity was lost.

**IL-8 production**

All *Arcobacter* strains tested induced IL-8 production in both Caco-2 and IPI-2I cells (Fig. 2). Two hours postinfection, the upregulation of IL-8 production by Caco-2 induced by *Arcobacter* strains was lower than that induced by the control *Salmonella* strain (Fig. 2a). *Arcobacter butzleri* LMG 6620 isolated from a female patient caused significantly higher induction of IL-8 in Caco-2 cells than the other *Arcobacter* strains. Pairwise comparison showed that, among the LMG strains, *A. cibarius* induced Caco-2 to produce the lowest levels of IL-8. The porcine isolates were seen to upregulate IL-8 secretion at higher levels in IPI-2I than the LMG strains, especially *A. cryaerophilus* 141, for which the IL-8 measures were statistically higher than those of *A. butzleri* LMG 6620, *A. skirrowii* LMG 6621, and *A. cryaerophilus* LMG 7537. It should be noted that cytokine expression by IPI-2I cells varied remarkably between the different experiments, and even among the triple incubations of one experiment (as demonstrated by the high SD).

A kinetic study of IL-8 secretion by Caco-2 and IPI-2I cells upon bacterial stimulation was done with the three LMG strains by measuring IL-8 production at 2, 6 and 24 h after a 1-h incubation with bacteria. The analysis indicated significant increases in IL-8 measures for both cell lines at these time points. Accumulation of IL-8 in the medium of infected Caco-2 cells increased for up to 24 h after bacterial challenge, whereas IL-8 production by uninfected cells could hardly be detected (Fig. 3a). At 2 h postinfection, IL-8 production by Caco-2 cells induced by *A. skirrowii* LMG 6621 was lower than that induced by *A. butzleri* LMG 6620 and *A. cryaerophilus* LMG 7537. Nevertheless, the IL-8 concentrations in the 6- and 24-h samples of *A. skirrowii* were higher than those for the two other strains. However, the difference was not statistically significant. During the first 6 h postexposure, the IL-8 contents in the medium of infected IPI-2I cells rose remarkably, whereas those of the untreated cells remained at low levels (Fig. 3b). However, at 24 h postexposure, IL-8 accumulation in all samples, including those from uninfected cells, was still rising.

Therefore, a kinetic study of IL-8 secretion by IPI-2 cells over time without bacterial infection was performed (in triplicate of one cell passage). It was shown that without bacterial infection, IPI-2I cells expressed IL-8, and the cytokine content in 2–3-day-old medium ranged from 1.22 to 1.57 ng per 10⁶ cells.

**Discussion**

As pigs are hosts and important reservoirs for a number of zoonotic pathogens, including *Arcobacter*, we intended in this study to further characterize porcine IPI-2I intestinal epithelial cells and to investigate their use as a model in the study of swine-associated pathogens. Although the cells possess microvilli, they are poorly differentiated (Fig. 1d); this is related to the lack of villin expression (Kaeffer et al., 1993). In comparison to the well-established human Caco-2 cell line, IL-8 production by porcine IPI-2I cells in response to bacterial infection varied among cells in different wells seeded from the same cell culture of one particular passage. According to Kaeffer et al. (1993), who established this cell line, IPI-2I cells are genetically unstable, and this can lead to phenotypic variation and heterogeneity. Importantly, IL-8 production was observed after prolonged incubation of uninfected cells. Apparently, the cells were under some kind of stress that may have come from the growth conditions or some component(s)
in the growth medium, although the medium was prepared as indicated by the supplier (Kaeffer et al., 1993). Therefore, in order for the IPI-2I cell line to be used as a model in the study of porcine-associated intestinal pathogens, more research is needed to optimize this cell line for this purpose.

Two studies have been reported on the adhesive ability of *A. butzleri* strains isolated from environmental (sea water and river water) samples (Musmanno et al., 1997; Carbone et al., 2003). In one, only one of 18 strains adhered to Hela and INT 407 cells (Musmanno et al., 1997). In the other, only one-third of the strains (six of 17) tested were found to be adhesive to HEp-2 and Hela cells (Carbone et al., 2003). However, all 12 *A. butzleri* strains isolated from human stool specimens were able to adhere to HEp-2 cells (Vandenbergen et al., 2005). Similarly, all eight strains of the four *Arcobacter* species tested in the present study showed the ability to adhere to both human Caco-2 and porcine IPI-2I cells. Probably, the different results obtained in studies of the adhesive ability of *Arcobacter* strains may be partly attributed to the origin of the strains, i.e. human- or animal-associated strains vs. environmental isolates. The different cell lines used and the assays employed could also influence the outcome. In the present study, a well-established adhesion and invasion protocol (Kusters et al., 1993; Van Asten et al., 2000) was used on Caco-2 and IPI-2I cells. With this protocol, live adhering bacteria are counted, using an adhesive and invasive wild-type *S. enterica* strain as a control.

In the study of *A. butzleri* strains isolated from river water (Musmanno et al., 1997), none of the 18 tested isolates invaded Hela and INT 407 cells. Nevertheless, four of 12 *A. butzleri* strains isolated from human stools were able to invade HEp-2 cells (Vandenbergen et al., 2005). Of eight *Arcobacter* strains tested in the present study, only two strains of *A. cyaerophilus* showed the ability to invade the human Caco-2 cell line. The ability of *A. cyaerophilus* strains isolated from an aborted bovine fetus and from swine feces to invade HEp-2 cells was demonstrated by Fernandez et al. (1995). Evidently, *A. cyaerophilus* strains are more invasive than those of other *Arcobacter* spp., which allows them to penetrate the porcine intestinal tissue and placenta and to invade fetuses (Ho et al., 2006). However, upon repeated testing, the invasiveness of the porcine *A. cyaerophilus* strain 1038 was lost. Apparently, *in vitro* subculture of the strain had resulted in a change in virulence; this has also been reported for other pathogenic bacteria, including *Helicobacter* and *Campylobacter* (Norris et al., 1995; On, 1998; Kim et al., 2002; Gaynor et al., 2004).

Upregulation of IL-8 expression by Caco-2 and IPI-2I cells was seen by 2 h after infection with all of the tested *Arcobacter* strains. However, the upregulation in Caco-2 cells was less than that induced by the control *Salmonella* strain. The results showed no correlation between cell invasion or level of adhesion and IL-8 induction by the tested *Arcobacter* strains. Thus different species and strains of *Arcobacter* may possess different virulence mechanisms (Villarruel-Lopez et al., 2003). This is not unlikely, as the tested strains were isolated under different circumstances and from different host species. The *A. cyaerophilus* strains used in the present study were isolated from an aborted fetus (*A. cyaerophilus* LMG 7537), amniotic fluid samples and newborn piglets, for which a vertical transmission route has been demonstrated (Ho et al., 2006). Therefore, the ability to invade epithelial cells would be a prerequisite for these *Arcobacter* strains. On the other hand, for strains isolated from a case of persistent diarrhea, e.g. *A. skirrowii* LMG 6621, intestinal association and the ability to trigger local inflammation but not invasion would be more appropriate for their successful survival. It is difficult to draw conclusions on the correlation between invasion ability, level of cell association and level of IL-8 induction of the tested *Arcobacter* strains.

Finally, our study clearly demonstrated that all of the tested strains of the four human- and animal-associated *Arcobacter* species possess the ability to adhere to and to induce IL-8 production by human Caco-2 and porcine IPI-2I intestinal epithelial cells. Two *A. cyaerophilus* strains, isolated from an aborted ovine fetus and a newborn piglet, were able to invade both cell lines.

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