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ORIGINAL ARTICLE

Presence of virulence genes, adhesion and invasion of *Arcobacter butzleri*

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Abstract

Aims: The pathogenic potential of *Arcobacter butzleri* isolates was investigated by detecting the presence of putative virulence genes and analysing the adhesive and invasive capabilities in cell cultures of human cell lines.
Methods and Results: The presence of ten putative virulence genes in 52 *A. butzleri* isolates was determined by PCR. The genes *ciaB*, *mviN*, *pldA*, *tlyA*, *cj1349* and *cadF* were detected in all, whilst *irgA* (15%), *iroE* (60%), *hecB* (44%) and *hecA* (13%) were detected only in few *A. butzleri* isolates. On HT-29 cells, four of six isolates adhered to and three of them were able to invade, whilst all six isolates adhered to and invaded Caco-2 cells with higher degrees. The genes *ciaB*, *cadF* and *cj1349* of all six isolates were sequenced, but no considerable changes of the amino acids in putative functional domains were observed.
Conclusion: Selected *A. butzleri* isolates adhere to and invade HT-29 and Caco-2 cells, which emphasize their human pathogenic potential. The efficiency of invasion depends on the eukaryotic cell line and individual bacterial strain used. We could not show any functional correlation between the amino acid sequence of CadF, CiaB or Cj1349 and the adhesive and invasive phenotype.
Significance and Impact of the Study: We have shown that some *A. butzleri* strains invade various cell lines. This underlines their pathogenic potential and hints at their relevance in human disease.

Introduction

Arcobacter spp. are gram-negative, motile and spiral-shaped bacteria belonging to the family of Campylobacteraceae. They are increasingly isolated from a wide range of food of animal origin and water. The highest prevalence in food is reported for poultry meat, followed by pork and beef (Rivas *et al.* 2004; Van Driessche and Houf 2007a,b). *Arcobacter* spp. have been rated a serious hazard to human health by the International Commission on Microbiological Specifications for Foods in 2002 (ICMSF, 2002). At present, 17 *Arcobacter* species have been characterized with *A. butzleri* as the most important and predominant species associated with human disease (Vandenberg *et al.* 2004). *A. butzleri* infections may result in abdominal pain with acute diarrhoea or prolonged watery diarrhoea for up to 2 months (Vandenberg *et al.* 2004). Little is known about the pathogenic mechanisms and putative virulence genes of *A. butzleri*. The genomic sequence of *A. butzleri* RM 4018 revealed the presence of 10 putative virulence genes: *cadF*, *mviN*, *pldA*, *tlyA*, *cj1349*, *hecB*, *irgA*, *hecA*, *ciaB* and *iroE* (Miller *et al.* 2007), but it is still unknown whether these putative virulence factors have similar functions as described for their homologues in other bacterial species. MviN is an essential protein required for peptidoglycan biosynthesis in *E. coli* (Ruiz 2008), but there is no direct evidence for the involvement of MviN in virulence of *Salmonella typhimurium* or *E. coli* (Inoue *et al.* 2008). In *Campylobacter*, *cadF* and *cj1349* encode fibronectin-binding proteins,

which promote the binding of bacteria to intestinal cells (Konkel *et al.* 1999; Flanagan *et al.* 2009), whilst *Campylobacter* invasive antigen B (CiaB) contributes to host cell invasion. HecA is a member of the filamentous hemagglutinin family and involved in attachment, aggregation and epidermal cell killing of *Erwinia chrysanthemi* (Rojas *et al.* 2002). The *hecB* encodes a haemolysin activation protein (Miller *et al.* 2007) and *tlyA* a haemolysin, which is also present in *Mycobacterium tuberculosis* and *Serpulina hyodysenteriae* (Wren *et al.* 1998). The outer membrane phospholipase PldA is associated with lysis of erythrocytes (Grant *et al.* 1997). The iron-regulated outer membrane protein Irg*A* and the periplasmic enzyme IroE are functional components for the iron acquisition in *E. coli* and therefore required for establishing and maintaining infections (Mey *et al.* 2002; Zhu *et al.* 2005; Rashid *et al.* 2006).

The potential virulence of *A. butzleri* isolates on CHO, Vero, HeLa and INT 407 cell lines was investigated by several authors. *A. butzleri* isolates showed *in vitro* cytotoxic effects in CHO cells and Vero cells, but any toxin genes were not identified yet. Adhesion of *A. butzleri* was observed in several cell line models (Musmanno *et al.* 1997; Johnson and Murano 2002; Carbone *et al.* 2003; Ho *et al.* 2007; Gugliandolo *et al.* 2008), but data on its ability to invade are sparse (Vandenberg *et al.* 2005).

The aim of this study was to examine the presence of putative virulence genes in *A. butzleri* strains isolated from different sources, to investigate the capability of *A. butzleri* to adhere to and invade into the cell lines HT-29 and Caco-2 and to analyse the sequence of putative invasive and adhesion genes.

Materials and Methods

Bacterial strains

All strains used in this study are listed in Table 1. *A. butzleri* were isolated from fresh meat (chicken meat, $n = 23$; pork, $n = 12$; mixed minced meat of pork and beef, $n = 2$), from water ($n = 9$) and from humans ($n = 6$). *A. butzleri* CCUG 30485 was included as reference strain. All isolates were grown on Mueller–Hinton blood agar (MHB; Oxoid, Wesel, Germany) or in Brucella Broth (BB; BD, Heidelberg, Germany) at 30°C in a micro-aerobic atmosphere (5 % O_2, 10 % CO_2) generated by the Anoxomat system (Mart, Drachten, the Netherlands). For the adhesion and invasion assays, the *A. butzleri* isolates were precultured overnight in BB at 30°C. The optical density of each preculture was measured at 600 nm and 10 ml BB inoculated obtaining an OD = 0·001. These cultures were further incubated for 24 h at 30°C. A total OD = 1 was centrifuged and resuspended in 450 μl PBS. Each well in the adhesion and invasion assay was inoculated with 50 μl of this bacterial suspension containing approx. 5×10^8 CFU.

Cell culture

The human colon adenocarcinoma cells HT-29 (DSMZ ACC 299) were grown in 25-cm^2 tissue culture flasks in RPMI1640 medium (Biochrom, Berlin, Germany) supplemented with 10% foetal calf serum superior (Biochrom) at 37°C in a 5% CO_2 humidified atmosphere until a confluence of approx. 80% was reached. For adhesion and invasion assays, each well of a 24-well plate was seeded with 2×10^5 HT-29 cells and incubated for 24 h.

The human colon adenocarcinoma cells Caco-2 (DSMZ ACC 169) were grown in 75-cm^2 tissue culture flasks in DMEM medium (Biochrom) supplemented with 10% foetal calf serum superior (Biochrom), 1% nonessential amino acids (Biochrom) and 5 μg ml^{-1} gentamicin (Roth, Karlsruhe, Germany) at 37°C in a 5% CO_2 humidified atmosphere until a confluence of approx. 90% was reached. For adhesion and invasion assays, each well of a 24-well plate was seeded with 5×10^4 Caco-2 cells and incubated for 21 days with media changes every third day but without gentamicin.

DNA extraction

Bacteria were suspended in 500 μl 0·1 × TE buffer (1 mmol l^{-1} Tris/HCl, pH 8·0, 100 μmol l^{-1} EDTA; Roth) and centrifuged for 5 min at 16 000 ***g***. The pellets were resuspended in 250 μl 5% Chelex (Bio-Rad, Munich, Germany) and incubated for 1 h at 56°C followed by 15 min at 95°C. The supernatants obtained after centrifugation were stored at 4°C until used for PCR.

PCR assays

Detection of putative virulence genes was performed by PCR. Primers and PCR protocols for partial amplification of *cadF*, *pldA*, *irgA*, *hecA* and *hecB* were used according to Douidah *et al.* (2012). Primers for partial amplification of *cj1349*, *ciaB*, *mviN*, *tlyA* and *iroE* were designed with Primer3 version 0.4.0 (Rozen and Skaletsky 2000) based on the published sequence of the whole-genome *A. butzleri* RM 4018 (ATCC 49616). All primers used in this study are listed in Table S1. PCRs were carried out in a total volume of 25 μl containing 1 × PCR buffer (Qiagen, Hilden, Germany), 200 μmol l^{-1} of each dNTP (Fermentas, St. Leon-Rot, Germany), 12·5 μmol l^{-1} of each primer, 0·5 U Taq-Polymerase (Qiagen) and 2 μl DNA. An initial denaturation step at 95°C for 4 min was

Table 1 Bacterial isolates used in this study

Strain	Source	Supplier	Strain designation
CCUG 30485	Human	CCUG	H1
FR1	Human	NRZ *Helicobacter*	H2
FR2	Human	NRZ *Helicobacter*	H3
FR3	Human	NRZ *Helicobacter*	
FR4	Human	NRZ *Helicobacter*	
FR5	Human	NRZ *Helicobacter*	
Ab 47	Water	LGL Oberschleißheim	
Ab 48	Water	LGL Oberschleißheim	
Ab 49	Water	LGL Oberschleißheim	
Ab 50	Water	LGL Oberschleißheim	
Ab 51	Water	LGL Oberschleißheim	
Ab 52	Water	LGL Oberschleißheim	
Ab 53	Water	LGL Oberschleißheim	
Ab 54	Water	LGL Oberschleißheim	
Ab 55	Chicken meat	LGL Oberschleißheim	
Ab 56	Chicken meat	LGL Oberschleißheim	
Ab 57	Chicken meat	LGL Oberschleißheim	
Ab 60	Chicken meat	LGL Oberschleißheim	
Ab 61	Chicken meat	LGL Oberschleißheim	
Ab 62	Chicken meat	LGL Oberschleißheim	
Ab 63	Chicken meat	LGL Oberschleißheim	
Ab 64	Chicken meat	LGL Oberschleißheim	
Ab 65	Chicken meat	LGL Oberschleißheim	
Ab 66	Chicken meat	LGL Oberschleißheim	
Ab 68	Chicken meat	LGL Oberschleißheim	
Ab 69	Chicken meat	LGL Oberschleißheim	
Ab 70	Mixed minced meat*	LGL Oberschleißheim	
Ab 73	Mixed minced meat*	LGL Oberschleißheim	
Ab 74	Chicken meat	LGL Oberschleißheim	
Ab 75	Pork	LGL Oberschleißheim	
Ab 84	Water	LGL Oberschleißheim	
Ab 86	Chicken meat	LGL Oberschleißheim	
Ab 87	Chicken meat	LGL Oberschleißheim	
Ab 88	Chicken meat	LGL Oberschleißheim	C1
Ab 89	Chicken meat	LGL Oberschleißheim	C2
Ab 91	Chicken meat	LGL Oberschleißheim	
Ab 92	Chicken meat	LGL Oberschleißheim	
Ab 93	Chicken meat	LGL Oberschleißheim	
Ab 94	Chicken meat	LGL Oberschleißheim	C3
Ab 96	Chicken meat	LGL Oberschleißheim	
Ab 97	Pork	LGL Oberschleißheim	
Ab 98	Pork	LGL Oberschleißheim	
Ab 99	Pork	LGL Oberschleißheim	
Ab 100	Pork	LGL Oberschleißheim	
Ab 101	Pork	LGL Oberschleißheim	
Ab 102	Pork	LGL Oberschleißheim	
Ab 103	Pork	LGL Oberschleißheim	
Ab 104	Pork	LGL Oberschleißheim	
Ab 106	Pork	LGL Oberschleißheim	
Ab 109	Pork	LGL Oberschleißheim	
Ab 110	Pork	LGL Oberschleißheim	
Ab 111	Chicken meat	LGL Oberschleißheim	

CCUG, Culture Collection University of Göteborg, Sweden; NRZ *Helicobacter*, National Reference Centre for *Helicobacter pylori*; University Medical Center Freiburg, Germany; LGL Oberschleißheim, Bavarian Health and Food Safety Authority, Oberschleißheim, Germany.
*Pork and beef.

followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and elongation at 72°C for 30 s and a final elongation step for 6 min at 72°C.

To investigate whether adhesive and invasive phenotypes depend on alterations in the amino acid sequence of CadF, Cj1349 or CiaB, the genes of these proteins were sequenced and *in silico*-translated. The complete genes *cj1349*, *ciaB* and *cadF* were amplified in a total volume of 50 μl containing 1× PCR buffer, 200 μmol l^{-1} of each dNTP, 12·5 μmol l^{-1} of each primer, 0·5 U Taq-Polymerase and 5 μl DNA. An initial denaturation step at 95°C for 5 min was followed by 36 cycles of denaturation (94°C, 60 s), primer annealing (the temperatures are indicated in Table S1) for 60 s and elongation at 72°C for 60 s followed by final elongation for 5 min at 72°C.

After gel electrophoresis, PCR products were visualized with GR Green (Labgene, St. Ingbert, Germany) under UV light. PCR products were purified by GeneJet PCR-Purification Kit (Fermentas) and sequenced (GATC Biotech, Konstanz, Germany) with primers shown in Table S1. Sequences were trimmed and cds-translated with standard genetic code by EditSeq (DNASTAR Lasergene version 7, Madison, WI, USA). Alignment of the amino acid (aa) sequences was performed with Multalin version 5.4.1 (Corpet 1988).

Adhesion and invasion assay

The adhesion and invasion assays with HT-29 and Caco-2 cells were performed with six randomly selected *A. butzleri* isolates (three chicken meat isolates, C1–C3; three human isolates, H1–H3). HT-29 cells and Caco-2 cells were infected with approx. 5×10^8 bacteria. This corresponds to an MOI 1000 for HT-29 cells. The MOI for Caco-2 cells could not be calculated as cells were only enumerated before seeding. For adhesion assay, infected monolayers were incubated for 1 h at 37°C and rinsed three times with phosphate-buffered saline to remove unbound *A. butzleri*. To count adherent *A. butzleri*,

HT-29 and Caco-2 cells were lysed with 500 μl 1 % SDS for 10 min.

To determine the invasive abilities of *A. butzleri*, monolayers were infected for 3 h, and extracellular bacteria were killed by 2-h incubation at 37°C with 300 μg ml^{-1} gentamicin (Roth). Cell monolayers were rinsed three times with PBS and lysed with 500 μl 1 % SDS for 10 min.

As control, the *Campylobacter* (*Camp.*) *jejuni* 81-176 strain was included in the adhesion and invasion assays. For *Camp. jejuni* 81-176, cells were lysed with 1 % Triton X-100 (Roth). *Camp. jejuni* 81-176 was cultivated as described for *A. butzleri* except at 37°C.

Total numbers of adherent or invasive bacteria were determined by plating serial dilutions of respective lysates on MHB agar, which were incubated for 48 h at 30°C. Each experiment was performed in triplicates, and average cell numbers were calculated out of three infected wells in each approach. Adhesion index and invasion index are calculated as ratio of adhering and invading bacteria of the inoculum, respectively.

Statistical analysis

P-values were calculated by nonparametric Mann–Whitney *U*-test (GraphPad Prism version 5, La Jolla, CA, USA). Results were considered significant at $P < 0{\cdot}05$. Data were shown as median with interquartile range (IQR).

Results

Presence of putative virulence genes in *Arcobacter butzleri*

The presence of ten putative virulence genes in the 52 investigated *A. butzleri* strains is summarized in Table 2. Thirteen per cent (7 of 52) of the strains possessed all ten genes, whilst 31% of isolates carried only six genes (*iroE*, *hecB*, *irgA* and *hecA* were not detected). In 25% of the isolates, *hecB*, *irgA* and *hecA* were missing; in 10 %, *iroE*, *irgA* and *hecA* were absent; in 17 %, *irgA* and *hecA* were missing; and in 4 %, only *hecA* was missing (Fig. S1).

All investigated *A. butzleri* isolates carried the genes *mviN*, *cadF*, *cj1349*, *ciaB*, *tlyA* and *pldA* (Table 2). The gene *iroE* was detected in 50% of the human (3 of 6), pork (6 of 12) and minced meat (1 of 2) isolates and in 65–67% of the chicken meat (10 of 23) and water (6 of 9) isolates. None of the two isolates from minced meat encoded *hecB*, but 33 % of the human isolates, 42–43% of the pork and chicken meat isolates and 67% of the water isolates. The genes *irgA* and *hecA* were detectable in 17% of the human isolates, 9% of chicken meat isolates, 44% of water isolates, but in none of the minced meat isolates. In isolates recovered from pork meat, only *irgA* (17%) but not *hecA* was detected.

Adhesive and invasive abilities of *Arcobacter butzleri*

Three *A. butzleri* isolates recovered from chicken meat (C1-3) and three from humans (H1-3) were tested for their adhesive and invasive properties in HT-29 cells (Fig. 1). Two human and two chicken meat *A. butzleri* isolates adhered to HT-29 cells (Fig. 1a). Highest adherence was observed with the isolate C2 ($1{\cdot}14 \times 10^{0}$ fold [$3{\cdot}02 \times 10^{0}$] of the inoculum), followed by H1 ($5{\cdot}4 \times 10^{-1}$ fold [$5{\cdot}76 \times 10^{-1}$]), H3 ($1{\cdot}49 \times 10^{-1}$ fold [$2{\cdot}12 \times 10^{0}$]) and C1 ($1{\cdot}3 \times 10^{-1}$ fold [$9{\cdot}22 \times 10^{-1}$]). Both chicken isolates showed invasion in HT-29 cells (C1 with $4{\cdot}26 \times 10^{-5}$ fold [$1{\cdot}54 \times 10^{-4}$] and C2 with $5{\cdot}05 \times 10^{-5}$ fold [$2{\cdot}73 \times 10^{-4}$] of the inoculum; Fig. 1b). Of the two adherent human isolates, only the human isolate H1 was invasive ($2{\cdot}86 \times 10^{-5}$ fold [$3{\cdot}8 \times 10^{-5}$]). The chicken isolate C3 showed adhesion and invasion in one of three approaches only, but to a far lesser extent ($1{\cdot}0 \times 10^{-5}$ fold [$2{\cdot}65 \times 10^{-5}$] and $5{\cdot}0 \times 10^{-7}$ fold [$9{\cdot}76 \times 10^{-7}$]). The differences between the adhesive and nonadhesive or between invasive and noninvasive *A. butzleri* isolates, respectively, were statistically significant. As control, the *Camp. jejuni* 81-176 strain was investigated. This strain showed $5{\cdot}64 \times 10^{-3}$ fold [3×10^{-3}] adhesion and $2{\cdot}93 \times 10^{-4}$ fold [$2{\cdot}7 \times 10^{-4}$] invasion on HT-29 cells. This result shows differences in the abilities of *A. butzleri* strains to adhere to and to invade on HT-29 cells, independent of their origin.

All isolates investigated were adhesive on Caco-2 cells (Fig. 2a). Highest adherence was observed for the human isolate H1 ($3{\cdot}2 \times 10^{0}$ fold [$1{\cdot}91 \times 10^{0}$]) followed by the chicken isolates C2 and C1 ($1{\cdot}37 \times 10^{0}$ fold [$2{\cdot}18 \times 10^{0}$]

Table 2 Presence of putative virulence genes in *Arcobacter butzleri* isolates

Source	*n*	*mviN*	*cadF*	*cj1349*	*ciaB*	*hecA*	*hecB*	*iroE*	*irgA*	*tlyA*	*pldA*
Human	6	100	100	100	100	17	33	50	17	100	100
Chicken meat	23	100	100	100	100	9	43	65	9	100	100
Pork	12	100	100	100	100	0	42	50	17	100	100
Minced meat	2	100	100	100	100	0	0	50	0	100	100
Water	9	100	100	100	100	44	67	67	44	100	100

The presence of putative virulence genes is represented as percentage of analysed isolates for each source of origin.

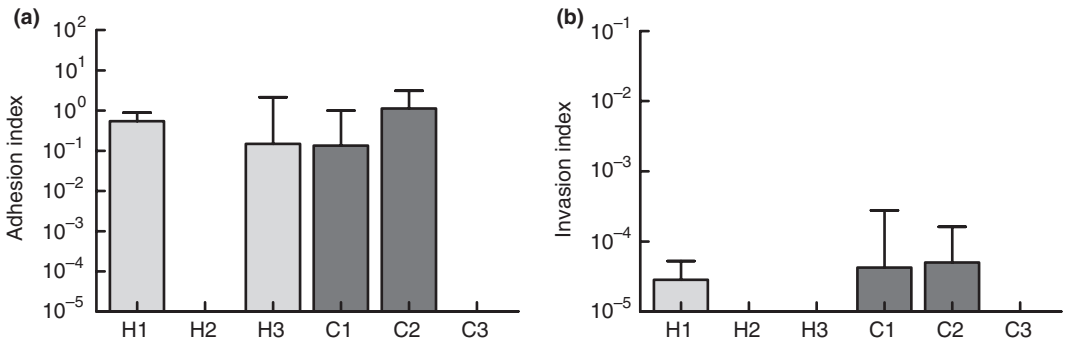

Figure 1 Adhesion index and invasion index of *Arcobacter butzleri* with the intestinal cell line HT-29. Adhesion of six *A. butzleri* isolates to HT-29 cells was detected after 1 h of incubation. To investigate the invasion ability, HT-29 cells were incubated for 3 h followed by 2-h gentamicin treatment, with six *A. butzleri* isolates. Adhesion index (a) and invasion (b) index are calculated as ratio of the inoculum. Expressed are the medians + interquartile range (IQR) (n = 3). H = human isolates, C = chicken meat isolates.

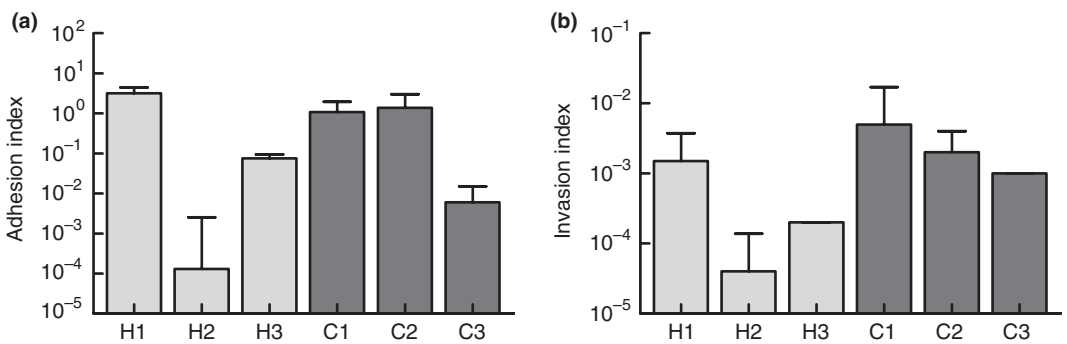

Figure 2 Adhesion index and invasion index of *Arcobacter butzleri* with the intestinal cell line Caco-2. Adhesion of six *A. butzleri* isolates to Caco-2 cells was detected after 1 h of incubation. To investigate the invasion ability, Caco-2 cells were incubated for 3 h followed by 2-h gentamicin treatment, with six *A. butzleri* isolates. Adhesion index (a) and invasion (b) index are calculated as ratio of the inoculum. Expressed are the medians + interquartile range (IQR) (n = 3). H = human isolates, C = chicken meat isolates.

and $1{\cdot}07 \times 10^{0}$ fold [$1{\cdot}35 \times 10^{0}$]). The isolates H3, C3 and H2 showed lower adherence with $7{\cdot}55 \times 10^{-2}$ fold [3×10^{-2}], $5{\cdot}71 \times 10^{-3}$ fold [$1{\cdot}2 \times 10^{-3}$] and $1{\cdot}25 \times 10^{-4}$ fold [$2{\cdot}45 \times 10^{-3}$]. These values were all significantly different ($P < 0{\cdot}05$), except for C2 and C1. Further, all isolates were invasive on Caco-2 cells (Fig. 2b). The isolates H2, H3 and C3 showed lesser invasion ($1{\cdot}88 \times 10^{-5}$ fold [6×10^{-5}], $1{\cdot}43 \times 10^{-4}$ fold [1×10^{-4}] and $6{\cdot}47 \times 10^{-4}$ fold [7×10^{-3}]) than the isolates C2 and H1 ($1{\cdot}52 \times 10^{-3}$ and $1{\cdot}47 \times 10^{-3}$ fold [2×10^{-3} and $2{\cdot}5 \times 10^{-3}$]) and C1 ($5{\cdot}0 \times 10^{-3}$ fold [$1{\cdot}5 \times 10^{-2}$]). On Caco-2 cells, $5{\cdot}41 \times 10^{-3}$ fold [$2{\cdot}5 \times 10^{-3}$] of the control strain *Camp. jejuni* 81-176 adhered to and $5{\cdot}88 \times 10^{-3}$ fold [$1{\cdot}5 \times 10^{-2}$] invaded.

Comparison of CadF, CiaB and Cj1349 amino acid (aa) sequences

To elucidate whether the different adhesive and invasive phenotypes depend on alterations in CadF, Cj1349 or CiaB, the genes of these proteins were sequenced and *in silico*-translated employing the standard genetic code in the six investigated *A. butzleri* isolates. The Cj1349 aa sequences of the H2, H3, C1, C2 and C3 isolates showed only 0·9–1·6% substitutions compared with the reference strain *A. butzleri* CCUG 30485 (H1), but all isolates showed a conserved motif D/E-X-W/Y-X-H (Fig. S2A; positions 367-371). This motif is part of a conserved domain of the DUF814 super family, occurring in proteins annotated as fibrinogen-/fibronectin-binding proteins, and might be functionally important (box in Fig. S2A). When comparing the CadF sequences of isolates investigated with the reference strain *A. butzleri* CCUG 30485, aa alterations varied from 1·5 to 3·25%. These alterations were often located at the same position (Fig. S2B). The fibronectin-binding site of *Camp. jejuni* (FRLS) described by Konkel *et al.* (2005) is substituted to YNLA in *A. butzleri*, but this site is conserved within the analysed isolates (box in Fig. S2B). Pronounced variations could be observed within the CiaB sequences (Fig. S2C). The aa sequences of H2 and H3 had the same lengths (630 aa) as the reference strain *A. butzleri* CCUG 30485,

whilst CiaB in C2 is composed of 633 aa, in C3 of 623 aa and in C1 of 523 aa. The CiaB sequences of C1 and C2 showed a three-aa insert (AKS) in between positions 332 and 333. However, all isolates showed aa substitutions of 3–5 % compared with the reference strain. But they all shared the conserved zinc-binding domain TIGHEYGHIL at positions 441-450 (Onozato *et al.* 2009). In the isolates C1 and C3, isoleucine is substituted by valine I442V (box in Fig. S2C).

Discussion

Arcobacter butzleri is considered a potential foodborne pathogen (summarized by Collado and Figueras 2011), but little is known about the pathogenic mechanisms and putative virulence factors. Investigations on the pathogenicity of *A. butzleri* have been primarily based on the knowledge on *Campylobacter* (Musmanno *et al.* 1997; Johnson and Murano 2002). However, a study by Miller *et al.* (2007) indicated that the proteome of *A. butzleri* shares greater phylogenetic similarities with members of the *Helicobacteraceae* like *Sulfurimonas denitrificans* and *Wolinella succinogenes* and those of the deep-sea vent Epsilonproteobacteria *Sulfurovum* and *Nitratiruptor* than with *Campylobacter*.

In this study, we investigated the presence and distribution of putative virulence genes in *A. butzleri* isolates recovered from different sources ($n = 52$, including the reference strain *A. butzleri* CCUG 30485). Only the genes *mviN*, *cadF*, *cj1349*, *ciaB*, *pldA* and *tlyA*, which share homologies to genes in *Campylobacter*, were identified in all *A. butzleri* isolates. This is in agreement with Douidah *et al.* (2012) who detected these genes in all 182 investigated *A. butzleri* isolates originating from different sources. Furthermore, these authors identified the presence of nine virulence genes (*iroE* was not investigated) in 15 % of analysed strains, which is comparable to our results (13 %). In contrast to Douidah *et al.* (2012), we could confirm neither the similar occurrence of *hecA* in human, pork and chicken meat isolates nor the higher prevalence of *hecB* in human isolates compared with pork and chicken meat isolates. Furthermore, we could not detect a lower occurrence of *irgA* in pork isolates compared with human and chicken meat isolates. However, these conflicting results might be due to the lower number of isolates included in our study.

Previous studies demonstrated the capacity of *A. butzleri* to adhere to different cell lines such as HeLa, Hep-2, INT 407, Caco-2 and IPI-2I (Musmanno *et al.* 1997; Ho *et al.* 2007). Only few studies examined the ability of *A. butzleri* to invade colon and larynx cells (Musmanno *et al.* 1997; Vandenberg *et al.* 2005; Ho *et al.* 2007). Amongst these studies, only Vandenberg *et al.* (2005) described an ability of *A. butzleri* to invade cell lines. These authors showed that 33% of the strains (4/12) were invasive in the human larynx carcinoma cell line Hep-2.

We also investigated the adhesive and invasive properties of three human and three chicken meat *A. butzleri* isolates on HT-29 and Caco-2 cells. Whilst two human (H1 and H3) and two chicken meat (C1 and C2) isolates adhered to HT-29 cells, only one human (H1, CCUG 30485) but both chicken meat isolates also invaded. On Caco-2 cells, all investigated isolates adhered to and invaded. The two isolates H2 and C3, which showed no adhesion on HT-29 cells, showed also significantly lower adhesion on Caco-2 cells compared with the four isolates H1, H3, C1 and C3. Coincident with this, the three isolates H2, H3 and C3, noninvasive on HT-29 cells, showed lesser invasion in the Caco-2 cells. Therefore, it seems that the adhesive and invasive phenotype of *A. butzleri* depends on the isolate as well as the cell line used.

That *A. butzleri* isolates investigated by others did not show invasive potential on the cell lines HeLa, INT 407, Caco-2 and IPI-2 could be explained by the different isolates, different cell lines or protocols used (Musmanno *et al.* 1997; Ho *et al.* 2007).

So far, it is still unknown whether *A. butzleri* adhere to and invade *in vivo* and which part of the human intestine might be affected. Based on the invasion data obtained by our assays, it can be speculated that *A. butzleri* invade poorly differentiated cryptic cells (HT-29: invasion index from $2{\cdot}86 \times 10^{-5}$ to $5{\cdot}05 \times 10^{-5}$ fold) with lower efficiency than the better differentiated colonocytes (Caco-2: invasion index from $1{\cdot}88 \times 10^{-5}$ fold to $5{\cdot}0 \times 10^{-3}$ fold). In addition, Bücker *et al.* (2009) demonstrated that *A. butzleri* CCUG 30485 is able to induce barrier dysfunctions in HT-29/B6 cells, thereby facilitating translocation of bacteria and probably inducing diarrhoea.

To elucidate which virulence genes might be responsible for adhesion and invasion, *A. butzleri* isolates with different virulence gene patterns were used for *in vitro* cell culture assays. The isolates H1 and C1 possessed all ten putative virulence genes, whilst in the isolates H2 and C2 the genes *irg*A, *hec*A and *hec*B and in H3 and C3 additionally *iro*E were missing. Based on these groupings, we could not detect any correlation between virulence gene patterns and adhesive or invasive capabilities. Because only two isolates per virulence gene pattern group were used, further strains need to be tested to confirm our observations. As all isolates possessed the adhesion genes *cad*F and *cj1349* and the invasion gene *cia*B but had different adhesive and invasive phenotypes, we compared the amino acid sequences of these putative virulence factors. Although the amino acid sequences of the three genes showed alterations from 0·9 to 5 % when compared with the reference strain *A. butzleri* CCUG

30485, the putative functional domains were conserved for all three genes in the six isolates investigated.

The CadF fibronectin-binding site of *Camp. jejuni* (FRLS) revealed substitution of similar amino acids within *A. butzleri* (YNLA). F/Y are both hydrophobic and aromatic, R/N are both polar, and S/A are both small and less hydrophobic amino acids. Therefore, this domain might represent also the fibronectin-binding site in *A. butzleri*. Similarly, both I and V (substitution in the zinc-binding domain of CiaB) belong to hydrophobic and aliphatic amino acids (Livingstone and Barton 1993). In addition, the C-terminus of CiaB does not seem to be important for efficient invasion of *A. butzleri* as isolate C1 showed high invasiveness in HT-29 and Caco-2 cells although the last 100 C-terminal amino acids of CiaB are missing. Also the insertion of the three-aa AKS in CiaB of the isolates C1 and C2 does not seem to be functionally relevant. In addition to these two strains showing invasion in HT-29 cells, strain H1 showed invasion on HT-29 cells, even though the three amino acids were absent in this isolate. The reasons for these differences in the amino acid sequence are not known. However, no correlation between adhesive and/or invasive phenotypes and the amino acid sequence of Cj1349, CiaB and CadF factors was shown.

We have shown here that *A. butzleri* isolates have the capability to adhere to and to invade cells as classical gastrointestinal pathogens do. However, further *in vitro* and *in vivo* studies are required to clarify the role of these virulence factors in the pathogenesis of *A. butzleri* infections.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Primers used in this study.

Figure S1 Occurrence of putative virulence genes in *Arcobacter butzleri* isolated from environment, food and human.

Figure S2 Alignment of Cj1349, CadF and CiaB sequence.