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Mutation of PEB4 alters the outer membrane protein profile of *Campylobacter jejuni*

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Abstract

Campylobacter jejuni is a significant cause of human gastroenteritis worldwide. In an attempt to further define bacterial factors that influence infectivity, Cj0596 was identified as playing a role in *C. jejuni* virulence. Cj0596 is a periplasmic chaperone that is similar to proteins involved in outer membrane protein (OMP) folding in other bacteria. Mutation of *cj0596* caused an alteration in the levels of eight OMPs, compared to wild-type bacteria. Replacement of the *cj0596* mutation with the wild-type *cj0596* gene restored a wild-type OMP profile. The altered OMP profile in the *cj0596* mutant was accompanied by significant changes in several virulence properties, including an increase in the ability to autoagglutinate, increased susceptibility to several antimicrobial agents, and increased biofilm formation. In summary, mutation of *cj0596* alters the *C. jejuni* OMP profile and leads to changes in OMP-related phenotypes involved in *C. jejuni* pathogenesis.

Keywords

Campylobacter jejuni; PPIase; Cj0596

Introduction

Campylobacter jejuni is a microaerophilic Gram-negative bacterium that is a major cause of human gastroenteritis (Miller & Mandrell, 2005). *C. jejuni* gastroenteritis is usually associated with consumption of undercooked poultry that has been contaminated with the bacterium, as a natural reservoir for the bacterium is the cecum of poultry (Miller & Mandrell, 2005). *C. jejuni* colonizes the small and large intestines in humans, causing severe abdominal pain, fever, and watery or bloody diarrhea, in addition to possible bacteremia, other extraintestinal symptoms, and sequelae of infection including Guillain-Barré Syndrome and reactive arthritis (Nachamkin, *et al*., 2000).

C. jejuni surface structures including outer membrane proteins (OMPs) affect virulence in several ways. Flagella are important for *C. jejuni* invasion of host cells in two ways; by motility, and by secretion of proteins including *Campylobacter* invasion antigens (Cia proteins), FlaC, and FspA (Larson, *et al*., 2008). In addition to flagella, several OMPs (CadF, PEB1, JlpA, CapA and OMPs of 32 kDa and 36 kDa) are known to play a role in adherence to host cells (Larson, *et al*., 2008). *C. jejuni* CfrA, ChuA, Cj0178 and Cj0444 are OMPs that allow *C. jejuni* to acquire iron and hemin from the host (Stintzi, *et al*., 2008). Two membrane efflux pumps, CmeABC and CmeDEF, and MOMP are involved in

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transport of a variety of antibiotics, detergents and dyes in *Campylobacter* (Zhang & Plummer, 2008).

The role of peptidyl prolyl cis-trans isomerases (PPIases) in virulence may be related to their ability to fold outer membrane proteins as well as some secreted proteins (Sklar, *et al*., 2007). Cj0596 (the strain 81-176 designation is CJJ81176_0624) is a PPIase that is located in the periplasm, is similar to SurA in *E. coli* and other orthologs in numerous bacteria, and was previously shown to play a role in *C. jejuni* virulence (Asakuray, *et al*., 2007, Rathbun, *et al*., 2009). Other bacteria in which PPIases have been characterized as virulence factors include *Shigella flexneri*, *Legionella pneumophila*, *Chlamydia trachomatis*, and *Neisseria gonorrhoeae* (Cianciotto, *et al*., 1990, Lundemose, *et al*., 1993, Leuzzi, *et al*., 2005, Purdy, *et al*., 2007).

In *E. coli*, SurA is the primary periplasmic chaperone and plays a role in folding the outer membrane proteins LamB, OmpA, OmpF, and OmpC. A *surA* mutant showed reduced piliation resulting from decreased levels of PapC and FimD (Sklar, *et al*., 2007). SurA mutation also results in an increased sensitivity to hydrophobic dyes, detergents, novobiocin, bacitracin, and vancomycin (Sklar, *et al*., 2007).

Previously, we showed that mutation of *cj0596* increased *C. jejuni* motility and ability to invade human intestinal epithelial cells *in vitro*, but decreased growth rate and colonization of the intestinal tracts of mice (Rathbun, *et al*., 2009). Here we investigate the effect of *cj0596* mutation on the *C. jejuni* outer membrane.

Materials and Methods

Bacterial strains and culture conditions

Campylobacter jejuni was routinely maintained with minimal passage on blood agar plates (Remel; Lenexa, KS) at 37°C in sealed culture boxes (Mitsubishi Gas Chemical [MGC], New York, NY) containing a microaerobic atmosphere generated by Pack-Micro Aero (MGC). For the growth of 81-176 and 81-176*cj0596*$^+$, the complemented mutant derived from 81-176 *cj0596* (Rathbun, *et al*., 2009), streptomycin was added to 30 μg/ml. For the growth of 81-176*cj0596*, an isogenic mutant derived from 81-176, chloramphenicol (30 μg/ml) was added to select for the mutation. Liquid cultures of all three strains were grown in Mueller-Hinton (MH) broth and cultured in the same environments described above.

Outer Membrane Profile Comparison

To determine whether the *C. jejuni* OMP composition was altered by mutation of *cj0596*, OMPs were isolated from 81-176, 81-176*cj0596*, and 81-176*cj0596*$^+$ by a modified Sarkosyl extraction method (Hobb, *et al*., 2009) and 15 μg of protein was run on a single dimension 12% SDS-PAGE gel, which was stained with Coomassie Blue (Hobb, *et al*., 2009). Densitometry measurements were performed on digital images of the gel using ImageJ software (Abramoff, *et al*., 2004), by defining small, identical rectangular boundaries around protein bands to be compared and determining the relative number of pixels in each as described (http://rsb.info.nih.gov/ij/index.html). Bands of interest were cut from the gel and the proteins identified using MALDI-ToF-ToF tandem mass spectrometry (MCG Mass Spectrometry Core Facility), using a combination of MS spectra (tryptic fingerprint) and collision-mediated fragmentation MS/MS of peaks isolated from first-dimension MS. Identifications were made using GPS Explorer software (Applied Biosystems) and MASCOT searches of an NCBI database of all known proteobacterial proteins. Two biological replicates were performed, with essentially identical results.

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Autoagglutination Assay

The autoagglutination abilities of 81-176, 81-176*cj0596*, and 81-176*cj0596*$^+$ were assessed by growing cells overnight in MH broth, then diluting the following morning in MH broth to OD_{600}=1.0. The suspensions were aliquoted into borosilicate glass tubes (2 ml per tube) and allowed to sit undisturbed in room air at 25°C. The OD_{600} of each cell suspension was measured every 30 min for 2 h, with a decrease in OD_{600} reflecting autoagglutination (Misawa & Blaser, 2000). Three biological replicates were performed, each in triplicate, with essentially identical results.

Biofilm Formation Assay

The abilities of 81-176, 81-176*cj0596*, and 81-176*cj0596*$^+$ to form biofilms were assessed as previously described (Candon, *et al*., 2007). Briefly, cells were grown overnight in MH broth, then diluted the following morning in MH broth to OD_{600}=0.05. Sterile 96-well polystyrene plates were inoculated with 100 μl *C. jejuni* cells per well (6 wells per strain) and incubated at 37°C under microaerobic conditions without agitation. After 72 h, biofilm formation was assessed by staining the plates with crystal violet (CV) solution (1% CV in 95% ethanol) and incubating at room temperature for 15 min, washing twice with distilled water to remove unbound CV, and dissolving the bound CV by adding 1.5 ml DMSO and incubating at room temperature for 48 h. The OD_{570} was then measured, with OD_{570} reflecting biofilm formation.

Antimicrobial agent sensitivity assays

The sensitivities of *C. jejuni* 81-176, 81-176*cj0596*, and 81-176*cj0596*$^+$ to a variety of antimicrobial compounds were determined using disk diffusion assays. Bacterial suspensions (OD_{600}=1.0) were streaked on MH plates and 7 mm filter paper disks impregnated with an antimicrobial agent were placed on the plate. Plates were incubated at 37°C under microaerobic conditions for 48 h and the zones of inhibition were measured. The antimicrobial agents tested were ampicillin (6.3 ng/disk), polymyxin B (25 ng/disk), vancomycin (500 ng/disk), gentamicin (3.1 ng/disk), ethidium bromide (3.1 ng/disk), and CV (7.8 ng/disk).

Results

Mutation of *cj0596* alters the outer membrane composition of *C. jejuni*

The effect of *cj0596* mutation on the *C. jejuni* outer membrane was studied by purifying the outer membrane fractions from 81-176, 81-176*cj0596,* and 81-176*cj0596*$^+$, and comparing the OMP profiles using SDS-PAGE. Several proteins showed altered abundance in the *cj0596* mutant compared to the wild-type (Fig. 1); those bands were excised and the proteins identified by using MALDI-ToF/ToF mass spectrometry (MS/MS data shown in Fig. S1). The proteins that were more abundant in the mutant were FlgE (Cj0043, flagellar hook protein; 2.5-fold), OMP85 (Cj0129, a predicted component of the OMP insertion machinery; 3.0-fold), FlaA (Cj1339, flagellar filament protein; 1.8-fold), CmeC (Cj0365, the outer membrane component of the CmeABC efflux pump; 1.9-fold), and Ef-Tu (Cj0470, a translation elongation factor; 2.4-fold). The proteins that were less abundant in the mutant were 50 kDa OMP (Cj1170, minor porin; 1.5-fold), major outer membrane protein (Cj1259, MOMP; 1.5-fold), and CadF (Cj1478, fibronectin binding protein; 1.3-fold). All proteins that showed altered abundance in the mutant returned to wild-type or near wild-type levels in the complemented mutant.

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Autoagglutination ability of *C. jejuni* is enhanced by mutation of *cj0596*

To examine the role of Cj0596 in autoagglutination, the abilities of *C. jejuni* 81-176, 81-176*cj0596,* and 81-176*cj0596*$^+$ to autoagglutinate were compared. The 81-176*cj0596* mutant strain autoagglutinated to a greater extent than the wild-type and complemented mutant (Fig 2). Under these conditions, the wild-type and complemented mutant showed no decrease in OD_{600} at 2 h, whereas the OD_{600} of the mutant was decreased to 38.7% of the initial OD_{600} (the experiment was performed in triplicate, $p<0.001$).

cj0596 mutation increases *Campylobacter* biofilm formation

The role of Cj0596 in biofilm formation was assessed by comparing the biofilm forming capacities of *C. jejuni* 81-176, 81-176*cj0596,* and 81-176*cj0596*$^+$. The OD_{570} measurements for the wild-type and complemented mutant (0.53 and 0.41, respectively) differed significantly ($p<0.01$) from the OD_{570} measured for the mutant (1.50), indicating that the *cj0596* mutant showed a much greater ability to form biofilms than either the wild-type or complemented mutant (Fig. 3).

Mutation of *cj0596* increases the susceptibility of *C. jejuni* to antimicrobial agents

A disk diffusion method was used to determine whether deletion of *cj0596* affected the susceptibilities of *C. jejuni* 81-176, 81-176*cj0596,* and 81-176*cj0596*$^+$ to a variety of antimicrobial agents. The *cj0596* mutant was significantly more susceptible (the experiment was performed in triplicate, $p<0.001$) to ampicillin (6.3 ng/disk). ethidium bromide (3.1 ng/disk), and vancomycin (500 ng/disk) (Fig 4); whereas no significant increase in susceptibility to CV (7.8 ng/disk), polymyxin B (25 ng/disk), or gentamicin (3.1 ng/disk) was observed (data not shown).

Discussion

C. jejuni is an important human pathogen, but many details of the mechanisms involved in infection are unknown. Previously, we and others found that mutation of *cj0596* affects several virulence-related phenotypes (Asakura, *et al*., 2007, Rathbun, *et al*., 2009). In our previous study, we found that a *cj0596* mutant showed an increase in motility and invasion of INT407 intestinal epithelial cells, but also showed a modestly lower growth rate and decreased ability to colonize mice (Rathbun, *et al*., 2009). Furthermore, proteomics experiments comparing the whole-cell protein profiles of *C. jejuni* strains 81-176, 81-176*cj0596*, and 81-176*cj0596*$^+$ also indicated that the changes in virulence properties were accompanied by changes in the *C. jejuni* whole-cell proteome. While our prior studies may have identified some proteins involved in the observed mutant phenotypes, we considered that analysis of the OMPs expressed by these strains may be informative given that Cj0596 is a periplasmic PPIase involved in folding integral OMPs.

OMPs play a large role in *C. jejuni* pathogenesis. It should be noted that the analysis of OMP profiles can be inherently challenging if the expression of an abundant protein (including a porin such as MOMP, see below) is altered, due to the relatively small total number of proteins in the outer membrane. While this can complicate the analysis somewhat, careful loading of the samples such that the abundance of the majority of the proteins is unchanged still allows important comparisons to be made. Consequently, in the present study, the 81-176 *cj0596* mutant showed altered levels of eight OMPs, five of which were more abundant in the *cj0596* mutant and three proteins which were less abundant in the mutant. The five proteins that were more abundant in the *cj0596* mutant were FlaA (Cj1339), FlgE (Cj0043), OMP85 family protein (Cj0129), CmeC (Cj0365), and Ef-Tu (Cj0470). Upregulation of FlaA, the major subunit of the *C. jejuni* flagellum, was seen previously in a proteomic study done on whole cells (Rathbun, *et al*., 2009). FlgE is the

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flagellar hook protein, which is required for flagellar structure, flagellin secretion, and motility. The increased levels of both FlgE and FlaA is consistent with the increase in motility and invasion of INT407 cells seen in the *cj0596* mutant (Rathbun, *et al*., 2009), possibly by increasing the functionality and/or efficiency of the flagella. Alternatively, the greater abundance of these flagellar proteins may reflect that a larger proportion of the *C. jejuni* population is expressing the phase-variable motile phenotype (Hendrixson, 2006).

Cj0129 is a member of the highly conserved Omp85 protein family that is involved in insertion of OMPs into the outer membrane of bacteria, as well as of mitochondria and chloroplasts (Schleiff & Soll, 2005). The upregulation of Cj0129 in the *cj0596* mutant may be a response to the altered levels of other OMPs and/or an increase in incorrectly folded proteins in the periplasm. CmeC is the outer membrane component of the CmeABC efflux pump, which is responsible for the intrinsic resistance of *Campylobacter* to a variety of antimicrobial agents (see also below)(Lin, *et al*., 2002). Although Ef-Tu is primarily cytoplasmic and involved in protein translation, it has been identified in the outer membrane of several bacterial species, including *E. coli* and *Mycoplasma pneumoniae* (Berrier, *et al*., 2000, Pancholi & Chhatwal, 2003, Prokhorova, *et al*., 2006, Kolberg, *et al*., 2008). In our previous *C. jejuni* whole-cell proteomic study, Ef-Tu was less abundant overall in the *cj0596* mutant (Rathbun, *et al*., 2009). It is possible that this reflects increased localization of EF-Tu to the outer membrane fraction, despite an overall decrease in cellular EF-Tu.

Three proteins with decreased abundance in the *cj0596* mutant were 50 kDa OMP (Cj1170), MOMP (Cj1259), and CadF (Cj1478). The 50kDa OMP belongs to the OmpA family and forms a cation selective pore (Bolla, *et al*., 2000). It is upregulated in *C. jejuni* during *in vivo* growth (Stintzi, *et al*., 2005). Because it is upregulated during *in vitro* growth, the decreased abundance of the 50 kDa OMP in the *cj0596* mutant may play a role in the decrease in maximum culture density described previously (Rathbun, *et al*., 2009). In addition to its porin activity, MOMP is involved in the structural organization of the outer membrane and in adherence to cultured cells (Schroder & Moser, 1997). The change in MOMP abundance in the outer membrane therefore could be responsible for, or reflective of, overall changes in outer membrane architecture. CadF promotes the binding of *C. jejuni* to fibronectin on host cells and is required for maximal adherence and invasion of INT407 cells and colonization of the chicken cecum (Larson, *et al*., 2008). As fibronectin is found in the basement membrane of the mouse intestine, it is possible that the decreased amount of CadF found in the *cj0596* mutant may play a part in the mouse colonization defect previously seen in the mutant (Rathbun, *et al*., 2009). It is also possible that the changes in the abundance of each these OMPs is related to the modest growth defect of the cj0596 mutant, although clearly both the altered OMP profile and growth defect result from mutation of cj0596 as WT characteristics for both are restored in the complemented mutant.

While the levels of eight OMPs were altered in the *cj0596* mutant, the mutation may affect other OMPs whose abundances are not altered. Such proteins may be improperly folded and non-functional, as was shown to occur with the aberrant presentation of *Shigella flexneri* IcsA in a *surA* mutant (Purdy, *et al*., 2007). Any poorly folded, non-functional proteins may affect the various phenotypes seen in the *cj0596* mutant, but would not have been detected by SDS-PAGE.

Accompanying the altered *cj0596* mutant OMP profile were changes in surface-related characteristics. Autoagglutination is a known virulence factor associated with pili or other OMPs in bacteria including enteropathogenic *E. coli* and *Vibrio cholerae* (Chiang, *et al*., 1995, Knutton, *et al*., 1999). In *C. jejuni*, autoagglutination requires glycosylated flagella (Guerry, 2007). The *cj0596* mutant autoagglutinated more quickly than the wild-type and complemented mutant. Because deletion of *cj0596* alters the *C. jejuni* OMP profile,

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including the expression of flagellar proteins, the increased ability of the *cj0596* mutant to autoagglutinate is consistent with increased expression of flagella.

Biofilms are groups of cells enclosed in a matrix of extracellular polymeric substances. Biofilm formation is important in bacterial pathogenesis as the ability of bacteria to form biofilms may allow them to better survive both in the environment and during infection. *C. jejuni* forms biofilms (Svensson, *et al*., 2009), and a recent proteomic analysis identified Cj0596 as being more highly expressed in biofilm-forming cells compared to planktonic cells (Kalmokoff, *et al*., 2006). Previously, a *C. jejuni* NCTC 11168 *cj0596* mutant was found to show a decreased ability to form biofilms when compared to the wild-type (Asakura, *et al*., 2007). However, our assays showed that an 81-176 *cj0596* mutant formed significantly more biofilms. It is possible that the difference between the two studies lies in interstrain differences (81-176 vs. NCTC 11168). However, it is also possible that the biofilm phenotype in the previous study was due to an unlinked mutation, as the *cj0596* mutation was not complemented in that work. The increased ability of the *cj0596* mutant to form biofilms may be due to altered outer membrane architecture, or may be partially due to the increased motility of this mutant.

Alteration of the outer membrane can render a bacterium more susceptible to antimicrobial agents, particularly those that are hydrophobic, through increased permeability or alteration of drug efflux pumps. Efflux pumps in *C. jejuni* include CmeABC (predominant) and CmeDEF (secondary), which confer resistance to fluoroquinolones, other antibiotics, dyes, detergents, disinfectants, and bile (Lin, *et al*., 2002, Akiba, *et al*., 2006). An *E. coli surA* mutant was more sensitive to bacitracin, vancomycin, and bile salts due to an alteration in the outer membrane (Lazar & Kolter, 1996). Similarly, the *cj0596* mutant was more susceptible to ethidium bromide, vancomycin, and ampicillin (Fig. 4) whereas no effect was seen with crystal violet, polymyxin B, or gentamicin (data not shown). Of the compounds tested in this study, ampicillin, ethidium bromide, gentamicin, and polymyxin B were previously tested in an 81-176 *cmeB* mutant (Lin, *et al*., 2002). The *cmeB* mutant was more susceptible to ampicillin (32-fold), ethidium bromide (8-fold), and gentamicin (2-fold); polymyxin B showed no change in susceptibility. The change in susceptibilities may be due to an alteration of antimicrobial targets in the membrane, an overall increase in membrane permeability, or a change in efflux pump structure due to changes in the stoichiometry of its subunits.

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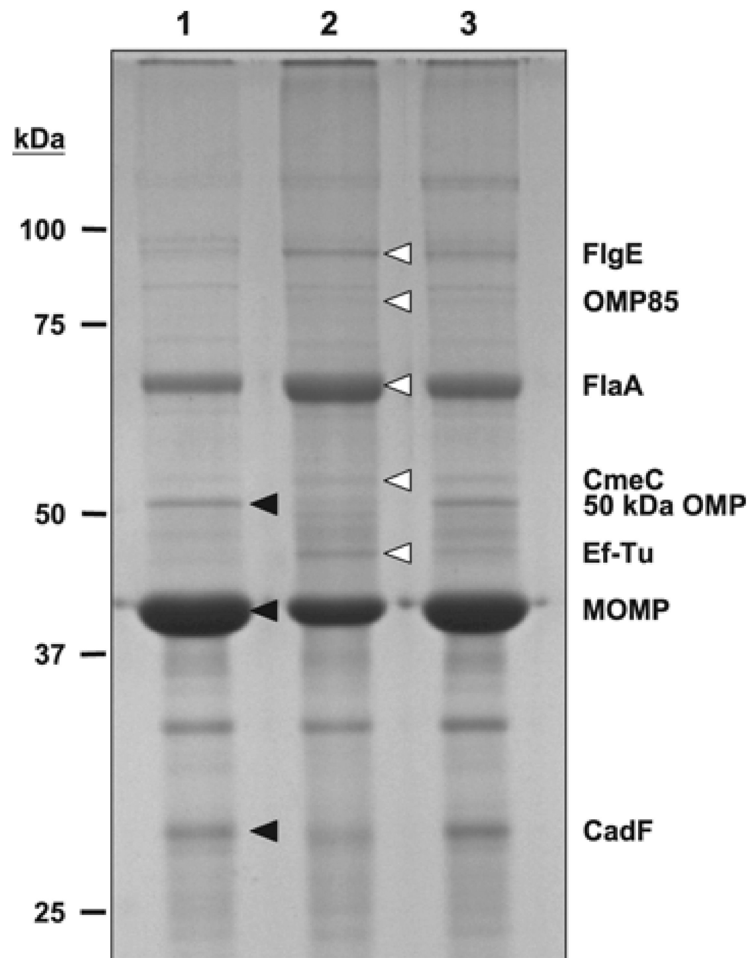

Figure 1.

SDS-PAGE gel (12%) showing differences in OMP profiles of 81-176 (lane 1), 81-176*cj0596* (lane 2), 81-176*cj0596*$^{+}$ (lane 3). Proteins with altered expression are indicated with arrowheads. Proteins with greater expression in the *cj0596* mutant: FlgE, OMP85, FlaA, CmeC, Ef-Tu. Proteins with lesser expression in the *cj0596* mutant: 50 kDa OMP, MOMP, CadF.

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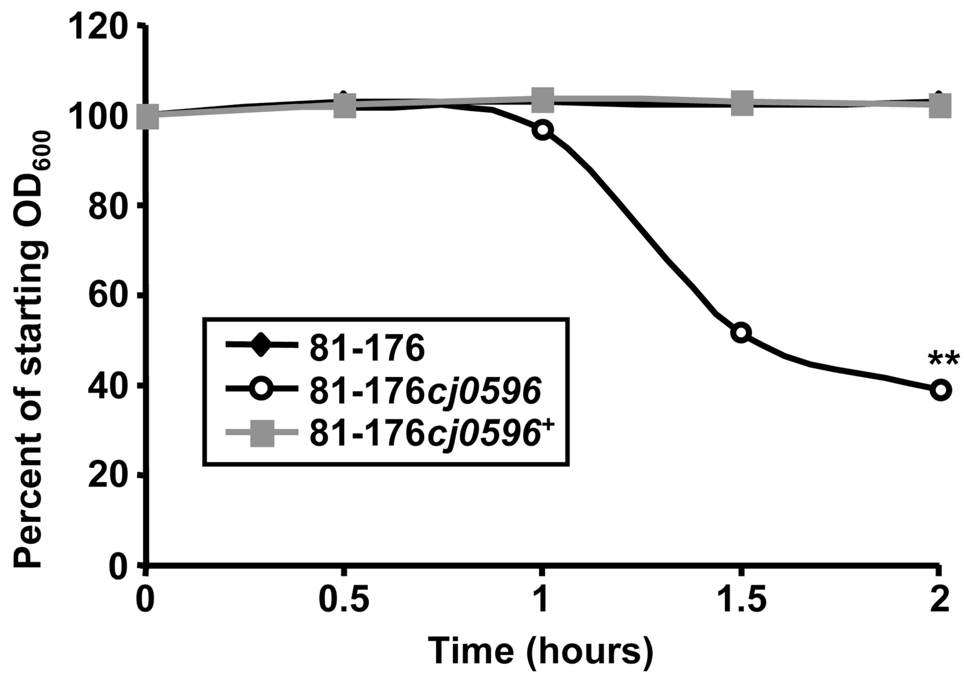

Figure 2.

Autoagglutination abilities of *C. jejuni* strains. Culture suspensions (OD_{600}=1.0) of strains 81-176 (black line), 81-176*cj0596* (dashed line) and 81-176*cj0596*$^{+}$ (gray line) were allowed to sit undisturbed at 25°C and OD_{600} was measured every 30 min for 2 h. Statistical significance (p <0.001) is represented by two asterisks. Error bars are present but are smaller than the symbols designating data points.

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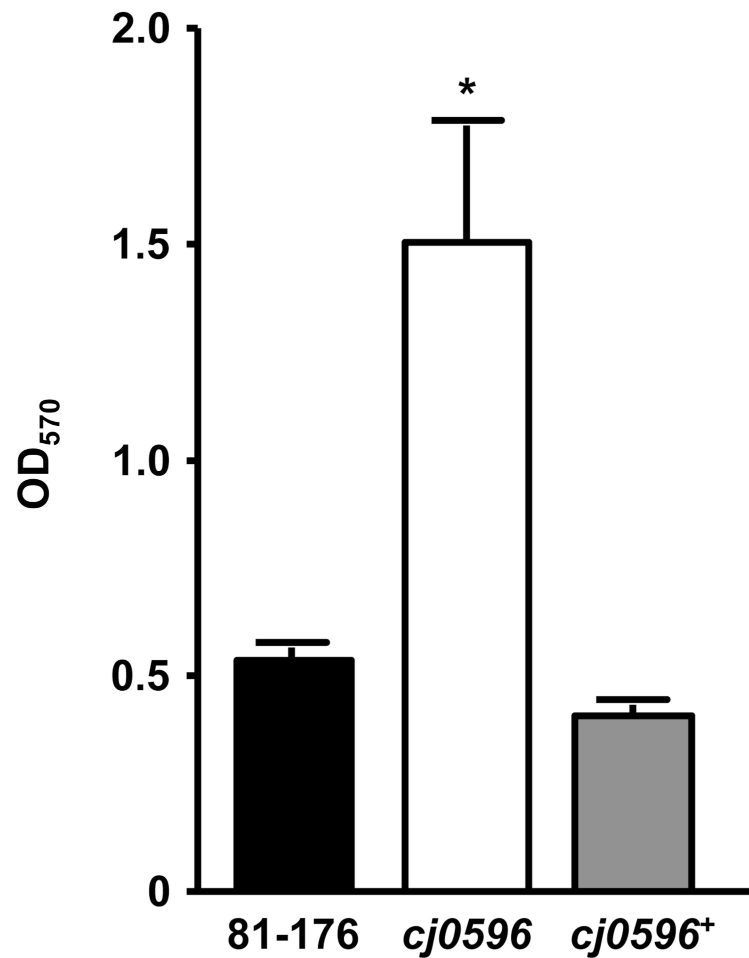

Figure 3.

Biofilm formation of *C. jejuni* strains. Culture suspensions (OD_{600}=0.05) of strains 81-176 (black), 81-176*cj0596* (white) and 81-176*cj0596*$^{+}$ (gray) were incubated statically in 96-well polystyrene plates for 72 h at 37°C, then stained with CV. Biofilm formation was quantified by measuring OD_{570}. Statistical significance (p <0.05) is represented by an asterisk.

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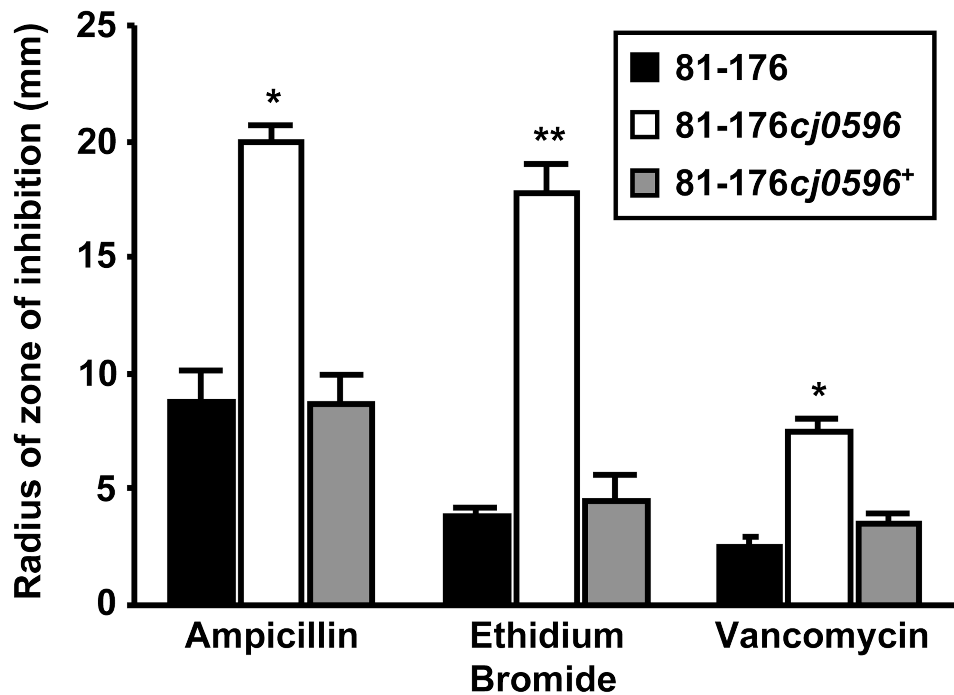

Figure 4.

Susceptibilities of *C. jejuni* strains to antimicrobial agents. The disk diffusion method was used to compare the susceptibilities of strains 81-176 (black), 81-176*cj0596* (white) and 81-176*cj0596*$^{+}$ (gray) to ampicillin (6.3 ng/disk), ethidium bromide (3.1 ng/disk), and vancomycin (500 ng/disk). Susceptibilities are expressed as the radius of the zone of inhibition by each compound, and statistical significance is represented by one asterisk ($p < 0.05$) or two asterisks ($p < 0.001$).