Published in final edited form as:
Mol Microbiol. 2017 April 1; 104(1): 92–104. doi:10.1111/mmi.13614.

Relaxation of DNA supercoiling leads to increased invasion of epithelial cells and protein secretion by *Campylobacter jejuni*

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Summary

Invasion of intestinal epithelial cells by *Campylobacter jejuni* is a critical step during infection of the intestine by this important human pathogen. In this study we investigated the role played by DNA supercoiling in the regulation of invasion of epithelial cells and the mechanism by which this could be mediated. A significant correlation between more relaxed DNA supercoiling and an increased ability of *C. jejuni* strains to penetrate human epithelial cells was demonstrated. Directly inducing relaxation of DNA supercoiling in *C. jejuni* was shown to significantly increase invasion of epithelial cells. Mutants in the fibronectin binding proteins CadF and FlpA still displayed an increased invasion after treatment with novobiocin suggesting these proteins were not essential for the observed phenotype. However, a large increase in protein secretion from multiple *C. jejuni* strains upon relaxation of DNA supercoiling was demonstrated. This increase in protein secretion was not mediated by outer membrane vesicles and appeared to be dependent on an intact flagellar structure. This study identifies relaxation of DNA supercoiling as playing a key role in enhancing *C. jejuni* pathogenesis during infection of the human intestine and identifies proteins present in a specific invasion associated secretome induced by relaxation of DNA supercoiling.

Introduction

Campylobacter jejuni infection is the leading cause of bacterial gastroenteritis. Although many putative virulence factors have been identified, relatively little is known about the molecular mechanisms responsible for the pathogenesis of infection. An integral step during *C. jejuni* human disease is invasion of intestinal epithelial cells, which does not occur during the commensal infection of chickens. A greater understanding of the mechanisms utilized by *C. jejuni* to invade human epithelial cells and the genetic regulation of this process is thus essential in order to further our understanding of how this organism causes disease and

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reduce the burden of *C. jejuni* gastroenteritis on society (Young *et al.*, 2007; Ó Cróinín and Backert, 2012).

A number of *C. jejuni* virulence factors have been shown to contribute to *C. jejuni* adhesion and invasion *in-vitro* (Ó Cróinín and Backert, 2012). It is clear that production of Peb1 and Peb4 are important for the ability of *C. jejuni* to adhere to the epithelial cell boundary but their influence on *C. jejuni* adhesion/invasion appears to be indirect (Pei *et al.*, 1991; Kervella *et al.*, 1993; Burucoa *et al.*, 1995; Pei *et al.*, 1998; Kale *et al.*, 2011). JlpA is a 42 kDa *C. jejuni* specific adhesin, which is surface exposed. Using strain TGH9011 (ATCC 43431) it was shown that a *jlpA* mutant associates with HEp-2 cells at a lower rate than that of the parent strain (Jin *et al.*, 2001). However, two subsequent studies failed to find significant reductions in adhesion or invasion using *jlpA* mutants in *C. jejuni* strains 81-176 or F38011 (Flanagan *et al.*, 2009; Novik and Galán, 2010), indicating that the importance of *jlpA* may be isolate-specific. CadF '*Campylobacter* adhesion to fibronectin' and FlpA 'fibronectin like protein A' are among the best characterised *C. jejuni* virulence factors and have consistently been shown to contribute to pathogenicity in a variety of studies (Konkel *et al.*, 1997; Moser and Salnikow, 1997; Ziprin *et al.*, 1999). CadF is a 37 kDa outer-membrane protein that can bind to purified fibronectin (Konkel *et al.*, 1997). FlpA was initially identified as a potential virulence factor when 2D proteomic analysis of a fresh human isolate strain JHH1 revealed a two fold up-regulation in a protein encoded by cj1279c compared to a reference strain ATCC 700297 (Cordwell *et al.*, 2008). FlpA also plays a role during colonization of chickens. An F38011 *flpA*- mutant strain associates with chicken epithelial cells at roughly two thirds that of the wild type (Flanagan *et al.*, 2009). Reduced adhesion to INT-407 cells also was shown using an *flpA*- strain and the attenuated interaction was suggested to be due to a reduced ability to bind extracellular fibronectin (Konkel *et al.*, 2010).

Multiple studies have shown that the *C. jejuni* flagellum functions as a secretory organelle. A number of non-flagellar proteins appear to be dependent upon correct flagellar formation for their delivery to the extracellular environment or for translocation to the intracellular environment of cell lines *in-vitro*. These include the Cia (*Campylobacer* invason antigen) proteins CiaB, CiaC, CiaD, CiaI, FlaC and FspA (Konkel *et al.*, 1999; Konkel *et al.*, 2004; Song *et al.*, 2004; Poly *et al.*, 2007; Christensen *et al.*, 2009; Buelow *et al.*, 2011; Samuelson *et al.*, 2013). CiaC and CiaI have also been shown to alter host cell signalling once translocated across the epithelial cell boundary (Buelow *et al.*, 2011; Neal-McKinney and Konkel, 2012). In the absence of a classical T3SS injectosome, the secretion of non-flagellar proteins via the *C. jejuni* flagellum has been implicated as a possible mechanism used by the bacteria to inject effector proteins to mediate bacterial internalization of *in-vitro* cell lines.

Mechanisms of *C. jejuni* gene regulation are poorly defined when compared to other enteric pathogens, although recent strides have been made in the genetic regulation of *C. jejuni* flagellar formation and function (Lertsethtakarn *et al.*, 2011). The regulation of *Campylobacteriales* DNA supercoiling is also poorly defined compared to other pathogens. *Campylobacteriales* do not contain a number of well characterized nucleoid-associated proteins such as FIS, IHF and H-NS that are found in *Escherischia coli* and *Salmonella enterica* (Kelly *et al.*, 2004; Mangan *et al.*, 2006; Mangan *et al.*, 2011). In *Helicobacter*

pylori it has been shown that bacterial growth phase strongly influences DNA supercoiling profiles in a manner similar to that seen in *E. coli* and *S. enterica*. Relaxation of negative DNA supercoiling in *H. pylori* results in a large decrease in expression of important flagellar genes *flaA* and *flgR* as well as the DNA gyrase genes, *gyrA* and *gyrB* (Ye *et al.*, 2007). In a recent study we revealed that relaxation of DNA supercoiling has a similar effect on motility and flagellar gene expression in *C. jejuni* and that supercoiling levels were affected by growth in the presence of mucus (Shortt *et al.*, 2016). This finding implicated a possible role for DNA supercoiling in the regulation of virulence genes in *C. jejuni* during infection of the host.

DNA supercoiling has previously been shown to have a significant influence on the ability of pathogens to invade epithelial cells and survive within macrophages. *Salmonella* and *Shigella* species in particular utilise the regulatory influence of DNA supercoiling for the secretion of proteins to increase their pathogenicity and survival (Dorman *et al.*, 1990; Ó Cróinín *et al.*, 2006). For example in *S. enterica* increased expression of the SPI-I gene, *invA*, occurs during growth of cultures containing high salt, a condition known to cause increased levels of negative DNA supercoiling (Galán and Curtiss, 1990). Intracellular growth of *S. enterica* within the J774A.1 macrophage cell line has also been shown to result in the opposite effect, a reduction of negative DNA supercoiling. This observation was shown to correlate with increased expression of the *ssrA* and *ssaG* promoters, indicative of expression of the SPI-II T3SS (Ó Cróinín *et al.*, 2006).

The aim of this study was to determine whether changes in rates of *C. jejuni* DNA supercoiling results in an altered ability for the bacterium to invade *in-vitro* cell lines as has been previously described for *Salmonella* and *Shigella* species. Furthermore, the effect of DNA supercoiling on known adhesins and secretion systems in *C. jejuni* was studied to identify the mechanism by which any change in the ability of the bacteria to invade epithelial cells could be mediated.

Results

C. jejuni DNA supercoiling is correlated with invasion of human cell lines

To measure relative rates of DNA supercoiling, pRY107 was extracted from NCTC11168 and 81-176 grown to mid log phase followed by analysis using chloroquine gel electrophoresis. At 10 μg/ml chloroquine, relaxed topoisomers of pRY107 migrate further than those more negatively supercoiled. Figure 1A demonstrates that strain NCTC11168 maintained pRY107 in a more negatively supercoiled state than plasmid extracted from strain 81-176. This increased rate of negative DNA supercoiling by NCTC11168 correlated with lower numbers of HCT-8 cell internalized bacteria when compared to the more relaxed, and significantly more invasive strain 81-176 after 5 h as observed in Fig. 1B.

To investigate if reduced negative *C. jejuni* supercoiling altered rates of epithelial cell invasion, the poorly invasive, highly negatively supercoiled NCTC11168 was cultured using sub-inhibitory concentrations of the antibiotic novobiocin. Incubation of NCTC11168 with novobiocin to artificially manipulate DNA topology confirmed a dose dependant relaxation of pRY107 supercoiling (Fig. 2A) without affecting viability as previously described (Shortt

et al., 2016). Addition of novobiocin to NCTC11168 cultures also resulted in a large and dose dependent increase in the ability of NCTC11168 to penetrate the HCT-8 (Fig. 2B) and CACO-2 cell lines (Fig. 2C) after 5 h. When invading bacteria were calculated as a percentage of total associated bacteria a similar trend was observed suggesting that greater numbers of associated bacteria were invading as DNA relaxation increased (Supporting Information Fig. S1A and B). This increased invasion of novobiocin treated bacteria with HCT-8 cells was confirmed and visualized using immunofluorescent microscopy (Fig. 2D).

CadF and FlpA proteins are not necessary for increased NCTC11168 invasion following DNA relaxation

To investigate if the observed increase in NCTC11168 invasion following DNA relaxation was mediated by CadF or FlpA, insertional *cadF* and *flpA* mutants were transformed into strain NCTC11168. These strains were then cultured in a range of novobiocin concentrations and used as individual inocula for infection of HCT-8 cells (Fig. 3). As shown in Fig. 3, disruption of either *cadF* or *flpA* resulted in lower levels of invasion than NCTC11168 WT, as has been previously reported (Moser and Salnikow, 1997; Konkel *et al.*, 2010). Figure 3 also shows that neither the CadF nor FlpA proteins are required to mediate an increase in NCTC11168 invasion following DNA relaxation. This trend was also observed when results were expressed as invading bacteria as a percentage of total associated bacteria (Supporting Information Fig. S1C).

DNA relaxation is correlated with increased protein within bacterial supernatants

Protein secretion to both the extracellular environment and translocation across epithelial cell membranes has been proposed as a mechanism by which *C. jejuni* can enhance its ability to cause enteric disease (Ó Cróinín and Backert, 2012). Figure 4A shows that growth of NCTC11168 overnight in the presence and absence of 15 μg/ml novobiocin, resulted in a minimal effect on total cell protein production. In contrast, the addition of 15 μg/ml novobiocin resulted in a large increase in the amount of protein detected within concentrated bacterial supernatants (Fig. 4B). Strikingly, this induction in protein secretion begins at a concentration of 10 μg/ml novobiocin (Supporting Information Fig. S1D), the concentration at which invasion of HCT-8 and CACO-2 cells became statistically significant (Fig. 2).

Increased extracellular protein following DNA relaxation is not mediated by cell lysis or through outer membrane vesicles (OMV's)

In a previous study we have shown that sub inhibitory concentrations of novobiocin had no significant effect on cell growth or viability (Shortt *et al.*, 2016). Growth curve analysis and viability counts under the conditions used in this study again revealed that no evidence of cell lysis could be observed (Fig. 5A and B). Furthermore, supernatant fractions were probed with antibodies raised against the outer membrane protein CadF (Fig. 5C) and the protein could not be detected in the supernatant from novobiocin treated cultures suggesting that increasing quantities of protein within the bacterial supernatant was not due to a general decreased membrane integrity following novobiocin treatment. One potential mechanism for protein export to supernatants involves the production of outer membrane vesicles (OMV's) (Elmi *et al.*, 2012; Jang *et al.*, 2014). Due to our method of concentrating supernatants by centrifugal filtration it was unlikely that we would capture OMVs but to confirm this a

number of tests were carried out. The absence of CadF in supernatants diminished the possibility that OMV's mediated the observed increase in protein secretion based on data from previous studies (Elmi *et al.*, 2012; Jang *et al.* 2014). In addition, supernatant proteins were sensitive to proteinase K treatment (Fig. 5D), further indicating that OMV export was unlikely to mediate the observed novobiocin stimulated protein secretion. Increased protein secretion following DNA relaxation was also observed in the *C. jejuni* chicken isolate C19 and strain 81-176, suggesting this phenotype was not strain specific (Fig. 6A). Furthermore, the more invasive 81-176 which has a more relaxed DNA supercoiling resting level (Fig. 1B) was shown to have a greater number of proteins secreted into the supernatant even in the absence of novobiocin although a further increase was observed after greater relaxation of DNA supercoiling.

Increased extracellular protein following DNA relaxation is dependent on an intact flagellar structure

To investigate if the increase in extracellular protein following novobiocin treatment was dependent upon a functional flagellar export apparatus, isogenic mutations in the flagellar genes *flgI*, *flgE* and *fliD* were tested. Based on previous studies, disruption of *flgI* or *flgE* are predicted to inhibit flagellar protein secretion (Poly *et al.*, 2007; Neal-McKinney and Konkel, 2012; Barrero-Tobon and Hendrixson, 2014). Since strain 81-176 has a more relaxed resting DNA topology it is naturally more sensitive to novobiocin and so a lower concentration of 5 μg/ml was used for induction. This more relaxed strain showed much greater secretion even in the absence of novobiocin strongly supporting the relationship between increased secretion and the more relaxed DNA topology. Treatment with novobiocin again led to an increase in secreted proteins. In contrast, supernatants from 81-176 *flgI*-, 81-176 *flgE*- showed very low amounts of protein and treatment with novobiocin resulted in no detectable increase in protein secretion, Fig. 6B. Cultures of 81-176 *fliD*-showed a higher level of protein in the supernatant, consistent with previous reports (Barrero-Tobon and Hendrixson, 2014), but again no detectable increase upon treatment with novobiocin was observed. This result strengthens the evidence that this is a specific secretome and implicates the flagellar export apparatus as a mechanism utilized to mediate this increased protein secretion following relaxation of *C. jejuni* DNA supercoiling.

LC-MS of bacterial supernatants reveals scale of protein secretion following DNA relaxation

To investigate the composition of protein within NCTC11168 bacterial supernatant both before and after novobiocin treatment, concentrated supernatants were analyzed by LC-MS. In total, 16 proteins were found only in the supernatant of NCTC11168 exclusively prior to DNA relaxation while 308 proteins were found present only in the novobiocin treated supernatant, Fig. 7A. The identities of the top 10 proteins found within the novobiocin treated supernatants are shown in Table 1. Subtraction of this dataset with proteins detected in the supernatant of NCTC11168 prior to novobiocin is also shown in Table 2. These datasets contribute evidence that Peb4, DnaK, a putative periplasmic protease Cj0511, SodB and KatA are all found within NCTC11168 supernatants at a higher rate following DNA relaxation. The identities of proteins found in the supernatant of the naturally more relaxed and invasive strain 81-176 were also compared with NCTC11168 samples. As observed in

Fig. 6A, LC-MS results revealed that a higher number of proteins were present within 81-176 supernatant than that of NCTC11168, of which a large number were also found exclusively within NCTC11168 supernatants following DNA relaxation, 154 in total. Full protein lists have been supplied in Supplementary Data.

Discussion

DNA supercoiling has been shown to impact the regulation of process' involved with pathogenesis of multiple bacterial species. Here we have shown that levels of DNA supercoiling also have a profound effect on the ability of *C. jejuni* to penetrate human cell lines and secrete protein to the extracellular environment.

Observing rates of DNA supercoiling and invasion of HCT-8 cells using strains NCTC11168 and 81-176 implied a potential correlation between reduced rates of negative supercoiling and an increased ability to invade HCT-8 cells. This correlation was further demonstrated by the growth of strain NCTC11168 in the presence of increasing novobiocin concentrations. The ability of NCTC11168 to invade HCT-8 and CACO-2 cell lines was significantly increased following dose dependent DNA relaxation. The relationship between decreased negative supercoiling and increased invasion of epithelial cells is an inverse one to that previously described for *S. enterica*, in which increased rates of negative supercoiling result in an increased ability to invade epithelial cells (Galan *et al.*, 1990) while more relaxed DNA supercoiling is associated with survival in the more stressful environment of the macrophage (Ó Cróinín *et al.*, 2006). In the case of *C. jejuni* we have previously shown that increased negative supercoiling is associated with increased motility (Shortt *et al.*, 2016) while this study suggests that more relaxed DNA supercoiling is associated with the invasive phenotype for this pathogen. This is an intriguing observation as both of these pathogens cause disease in similar niches. One possible explanation would be that the human intestine is a more stressful environment for *C. jejuni* than the chicken intestine leading to a more relaxed DNA supercoiling profile and subsequent increased invasion. Further work would be required to support this hypothesis and to identify the environmental stimuli that lead to relaxation of DNA supercoiling *in vivo*.

Following the observations that reduced negative supercoiling correlates with increased invasion, any potential role the fibronectin binding proteins CadF and FlpA may play to mediate this increase in invasion was investigated. Increased invasion following novobiocin treatment of NCTC11168 *cadF*- or NCTC11168 *flpA*- strains revealed that neither of these proteins are necessary to observe an increase in invasion following novobiocin treatment. Although this was the case, increased invasion only became statistically significant at the highest concentration of novobiocin tested, 15 μg/ml, with disruption of the *flpA* gene resulting in a greater defect in invasion following novobiocin treatment. This may point to a co-operative role for these fibronectin binding proteins with other processes such as flagellar secretion to enhance rates of invasion during conditions of DNA relaxation as has been described by another research group (Eucker and Konkel, 2012). Future research is required to establish the transcriptional influence of reduced negative supercoiling on global *C. jejuni* gene transcription and investigation into its specific influence on genes involved in pathogenesis such as *cadF* and *flpA*.

Strikingly the large increase in protein secretion observed following DNA relaxation occurred at the same concentration in which rates of increased cell line invasion became statistically significant, 10 μg/ml. This increase in protein secretion was also shown to occur when using strains *C. jejuni* C19 and 81-176. Interestingly both baseline levels of protein secretion and the scale by which protein secretion may increase following novobiocin treatment was strain specific. It was also noted that the highly invasive strain 81-176 secreted at a much higher baseline rate than that of strains NCTC11168 and the chicken isolate C19. The specific increase in proteins found within the concentrated supernatant of strains grown in the presence of novobiocin was deemed not to be due to increased rates of OMV release. This was confirmed by probing supernatant samples with antibodies raised against the outer-membrane bound CadF protein. Evidence for this was further strengthened as protein released following novobiocin treatment was not resistant to proteinase K, revealing it appears not to be contained within a lipid bilayer. Multiple studies have now established the *C. jejuni* flagellum as a mechanism for protein secretion (Poly *et al.*, 2007; Neal McKinney and Konkel, 2012; Barrero-Tobon and Hendrixson, 2014). Due to this, strains carrying mutations in flagellar genes which have previously been documented to inhibit the flagellar secretion were analyzed. Novobiocin treatment of strain 81-176 with directed mutations in the flagellar genes *flgI*, *flgE* or *fliD* did not increase protein secretion in the manner observed using the wild type counterpart. This provides strong evidence that the observed increase in protein secretion following reduced negative supercoiling is dependent on a wild type flagellum structure. When taken together with our recent observation that more relaxed DNA supercoiling leads to a reduction in motility without a visible effect on the structure of the flagellum (Shortt *et al.*, 2016) these observations raise the possibility that DNA relaxation provides a key signal to change the function of the flagellar structure from motility to secretion. Previous studies have required induction of secretion from the flagellar structure using environmental stimuli such as bile salts (Rivera-Amill *et al.*, 2001). The secretome identified in this study was induced by novobiocin and thus it would be interesting to test a variety of environmental stimuli to see if these could affect DNA supercoiling and induce secretion.

LC-MS of supernatant fractions from NCTC11168 indicated the extent of increased protein secretion following reduced negative supercoiling. It also revealed that protein secretion induced from NCTC11168 following novobiocin treatment contains many proteins secreted from the more relaxed strain 81-176 in the absence of novobiocin. Intriguingly, peptides found within the supernatant of NCTC11168 treated with 15 μg/ml novobiocin contained multiple chaperone proteins. SodB, KatA and *cj*0511 were also among the proteins secreted from NCTC11168 only after DNA relaxation. The serine peptidase encoded by cj0511 has been identified as being essential for colonization of chickens and has been implicated in the degradation of Occludin and E-cadherin leading to enhanced invasion (Karlyshev *et al.*, 2014; Elmi *et al.*, 2016). The presence of SodB and KatA is also interesting given that reactive oxygen species have recently been implicated as important mucosal defences against pathogens including *C. jejuni* suggesting that secretion of these proteins could be a mechanism of protecting *C. jejuni* from these reactive species (Corcionivoschi *et al.*, 2012; Alvarez *et al.*, 2016). It is also of note that the induction of proteins involved in protection from reactive oxygen species would also be observable during survival of *S. enterica* in the

macrophage, an environment which has also been shown to induce more relaxed DNA supercoiling.

This work reveals a significant influence of DNA supercoiling on the ability of *C. jejuni* to invade human cell lines. It has also begun to define the mechanisms by which DNA relaxation increases rates of cell line invasion. We have established that increased invasion of the HCT-8 and CACO-2 cell lines occurs using NCTC11168 following DNA relaxation and this can occur independently of the CadF or FlpA proteins. We have also described a large increase in protein secretion from multiple *C. jejuni* strains following DNA relaxation, which correlates strongly with the observed increased rates of invasion and appears to be dependent on wild type flagellum formation. In doing this we have identified a novel mechanism by which *C. jejuni* regulates the invasion of epithelial cells and a possible global regulator of virulence in this important human pathogen.

Experimental procedures

Bacterial strains and growth conditions

C. jejuni 81-176 is a well characterized sequenced strain used widely in infection studies, *C. jejuni* NCTC11168 is the type strain of *C. jejuni*. Strain C19 was isolated from a chicken and was acquired from the laboratory of Professor Seamus Fanning. NCTC11168 *cadF*- and *flpA*- were acquired from Professor Steffen Backert and Dr Nick Dorrell respectively. 81-176 *flgI*-, 81-176 *flgE*- and 81-176 *fliD*- as well as the pRY107 plasmid were acquired from Professor Patricia Guerry. All *C. jejuni* strains were cultured on Mueller Hinton (MH) agar (Oxoid) at 37°C under microaerophilic conditions created using Campygen gas packs (Oxoid). *C. jejuni* stock cultures were maintained using MH broth (Oxoid) supplemented with 20% glycerol and stored at −80°C. To revive stocks of *C. jejuni* strains, bacteria were streaked with a single use sterile inoculation loop (Sarstedt) on MH agar and incubated under microaerophilic conditions for 48 h at 37°C. For liquid cultures, *C. jejuni* strains were equalized to specific optical densities in MH broth and incubated under microaerophilic conditions at 37°C, shaking at 200 rpm. NCTC11168 *cadF*-, NCTC11168 *flpA*- or strains transformed with plasmid pRY107 were cultured with the addition of 50 μg/ml kanamycin. 81-176 *flgI*-, 81-176 *flgE*- and 81-176 *fliD*- were cultured with the addition of 10 μg/ml chloramphenicol.

Chloroquine gel electrophoresis

To visualize the supercoiling profile of plasmid DNA, chloroquine gel electrophoresis was carried out on plasmids isolated from strains grown to mid log phase in the presence and absence of novobiocin. All chloroquine gels comprised of 0.8% agarose made with 2X Tris borate EDTA (TBE) buffer. Addition of 10 μg/ml chloroquine salt solution to both the gel and running buffer allows relaxed circular DNA to migrate further while supercoiled circular DNA is retarded in the gel remaining higher. All chloroquine gels were run for 24 h at 100 V. At this point the gel was washed approximately 10 times with water for 30-minute periods to remove the chloroquine salt. Ethidium bromide solution at a concentration of 10 μg/ml was then added for two hours to visualize plasmid DNA

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Treatment of *C. jejuni* strains with novobiocin to relax DNA

To achieve relaxation of DNA, the aminocoumarin antibiotic novobiocin was used at sub-inhibitory concentrations to *C. jejuni* growth. For overnight cultures of *C. jejuni* containing novobiocin, strains were equalized at an O.D.600 of 0.02 and incubated under microaerophilic conditions in MH broth containing indicated concentrations of novobiocin for 18 h at 37°C, shaking at 200 rpm. To demonstrate the effect of novobiocin on DNA supercoiling in *C. jejuni*, chloroquine gel analysis was carried out on mid log phase cultures. For this, *C. jejuni* strains were equalized at an O.D.600 of 0.2 and incubated for 4 h under microaerophilic conditions at 37°C, shaking at 200 rpm. At this point novobiocin was added to the culture and incubated for a further 3 h.

Culture of *in-vitro* cell lines

The human ileocecal adenocarcinoma HCT-8 and CACO-2 cell lines were purchased from the ATCC (CC-L244, HTB-37). HCT-8 cells were grown using RPMI 1640 (Gibco) medium supplemented with 10% FBS, 1% sodium pyruvate (Sigma) and 1% Glutamax (Gibco). CACO-2 cells were grown using DMEM (life technologies) supplemented with 10% FBS and 1% non-essential amino acids. Cells were grown in 75 cm^2 tissue culture flasks and incubated at 37°C with 5% CO_2 in a humidified atmosphere. Cells were grown until roughly 80% confluence was observed after being seeded with 2×10^5 cells.

Total association and gentamycin protection assays

HCT-8 cells were seeded at 2×10^5 cells on 12 well plates (Corning) for 48 h until 80% confluence was observed. The multiplicity of infection for all total association or invasion assays was bacteria:cells, 100:1. *C. jejuni* strains to be used for total association and gentamycin protection assays were prepared as follows. Strains were equalized at an O.D. 600 of 0.02 in MH broth with or without relevant concentration of novobiocin for 18 h at 37°C, shaking at 200 rpm under microaerophilic conditions. For total association assays infected cells were incubated at 37°C in a microaerophilic conditions for 5 h in the presence or absence of novobiocin. Wells were then washed three times with PBS and cells were lysed with 0.1% Triton-X-100 in PBS for 15 min. After this, serial dilutions of the cell lysate were carried out using MH broth and plated on MH agar. To determine the number of internalized bacteria in gentamycin protection assays, infected HCT-8 or CACO-2 cells were incubated at 37°C under microaerophilic conditions for 3 h in the presence or absence of novobiocin. After 3 h, the media overlying the infected HCT-8 or CACO-2 cells was changed to complete RPMI/DMEM media containing 400 μg/ml gentamycin sulphate (Lonza) and infected HCT-8 cells were incubated at 37°C under microaerophilic conditions for a further 2 h. After this, cells were washed 3 times with PBS and lysed with 0.1% Triton X-100 in PBS for 15 min. Serial dilutions of the cell lysates were carried out using MH broth and plated on MH agar. Dilutions of mutant *C. jejuni* strains were carried out on MH agar plates containing 50 μg/ml kanamycin. All MH plates were incubated for 72 h under microaerophilic conditions using Campygen gas packs (Oxoid) at 37°C before colony counting took place.

Statistical analysis

Tests for statistical analysis in total association and invasion assays were carried out using the Student's t-test (two-tailed distribution). Statistically different values between samples possessed *P*-values <0.05. All total association and invasion experiments were performed in duplicate, with at least three biological replicates. The average number of bacteria counted post total association or invasion assay was counted and error bars represent the standard deviation for each sample condition.

Immunofluorescent microscopy—All immunofluorescent microscopy was done using HCT-8 cells grown on microscope coverslips within 6 well tissue culture plates (Corning) seeded with HCT-8 cells at 2×10^5 and grown for 2 days at 37°C in a humidified atmosphere. Infection of the cells was carried out as explained for infection assays. Once bacteria had been incubated with the cells, the wells were washed in triplicate with PBS and cells were fixed with 2% formaldehyde for 10 min. Cells were again washed in triplicate and permeabilized with 0.025% Triton-X-100 for 10 min. Once washed for a third time, a blocking solution of PBS containing 10% goat serum and 1% BSA was applied and left overnight. The following day cells were incubated with a primary antibody (Anti-*C. jejuni* 1:400) for 30 min and again washed in triplicate with PBS. A secondary antibody was then added for a further 30 min (AlexaFluor 488 conjugate Anti-Rabbit, Thermo Scientific 1:500). DAPI stain also was added to visualize nuclei of HCT-8 cells (1:5,000). Finally the coverslips were washed once more in triplicate with PBS and mounted on microscope slides using fluorescent mounting medium (Dako) prior to being examined using a fluorescent microscope.

Immunoblotting—Ten percent SDS polyacrylamide gels were transferred to nitrocellulose membranes at 100 V for one hour. All blocking steps were done overnight at 4°C shaking at 30 rpm using 3% fat free skimmed milk (Marvel). Anti-CadF primary antibodies for whole cell and supernatant samples were used at 1:10,000. This antibody was provided by Prof. Steffan Backert. Secondary antibody anti-rabbit IgG conjugated to HRP was used at 1:10,000.

Isolation of secreted protein from *C. jejuni* liquid cultures

C. jejuni cultures for the isolation of secreted proteins were inoculated at 0.02 O.D. in 10 ml in duplicate under microaerophilic conditions, shaking at 200 rpm. Duplicates for each sample were combined to compose 20 ml cultures equalized to an O.D.600 of 0.6. Each 20 ml culture was centrifuged at 4000 × g for 20 min, the supernatant was transferred to a fresh tube and the centrifuge step was repeated. Supernatants were then passed through a syringe with a 0.22 μm filter to remove any remaining whole bacteria. Once this was carried out supernatants were transferred to Amicon Ultra centrifugal filter units with a nominal molecular weight limit of 30 K (Millipore) and centrifuged at 4000 × g for 20 min. Resulting concentrated supernatant was aliquoted and stored at −20°C for future use.

Two-dimensional polyacrylamide gel electrophoresis

Total cell protein was collected from *C. jejuni* overnight liquid cultures in the presence/absence of novobiocin at concentrations indicated. Bacteria were pelleted, re-suspended in

PBS and protein concentration was calculated. Total proteins of concentrated supernatants were precipitated using methanol/chloroform (Wessel and Flugge, 1984), dried and stored for future use at −20°C. Precipitated protein samples were re-suspended for 16 h in rehydration buffer (7M urea, 2M thiourea and 1% ABS-15). DTTand pH 4-7 IPG buffer (GE) were then added to a final concentration of 30 mM and 0.5% respectively. 1 μl of 1% bromophenol blue was also added and sample was made up to 126 μl for 7 cm immobiline drystrip (GE) rehydration. Once strips were rehydrated, isoelectric focusing was carried out under the following conditions: 300 V for 30 min, a gradient to 1,000 V for 30 min, a gradient to 5,000 V for 90 min and 5,000 V for a further 25 min. Following isoelectric focusing, strips were washed for 10 min in equilibration buffer (6 M Urea, 75 mM Tris-HCL, pH 8.8, 29.3% Glycerol, 2% (w/v) SDS, 0.002% Bromophenol blue) containing 1% DTT shaking at 60 rpm. A second wash of each strip using equilibration buffer containing 2.5% iodoacetamide was then carried out. Strips were then placed on top of 10% polyacrylamide gels and sealed into place using an agarose seal solution (0.5% agarose, 0.002% bromophenol blue, made to 100 ml with 1xSDS electrophoresis buffer). Gels were run at 160 V for approximately 90 min and stained using Sypro ruby protein stain (Life Technologies) as per the manufacturer's instructions.

Treatment of secreted protein samples with proteinase K

Proteinase K was added to concentrated supernatant samples to a final concentration of 100 μg/ml in PBS. Samples were then incubated in a water bath at 37°C for 1 h. PMSF was then added to each sample at a final concentration of 5 mM and left for 10 min at room temperature. Reducing SDS-PAGE sample buffer was then added to each sample and boiled for 5 min prior to loading on a 12.5% polyacrylamide SDS-PAGE gel.

Liquid chromatography-mass spectrometry

Bacterial supernatant samples analysed by LC-MS were concentrated and precipitated as described above. The samples were denatured and reduced using 8 M urea, 50 mM ammonia bicarbonate and 10 mM dithiothreitol, alkylated using 15 mM iodoacetamide and digested with 1 ug of trypsin (sequencing grade Trypsin, Promega) at 37°C for 18 h on the thermomixer at 350 rpm. Prior to LC-MS/MS analysis possible contaminants were removed using C18 Ziptips (Millipore). The samples were run on a Thermo Scientific Q Exactive mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Tryptic peptides were resuspended in 0.1% formic acid. Each sample was loaded onto a fused silica emitter (75 μm ID, pulled using a laser puller (Sutter Instruments P2000), packed with Reprocil Pur C18 (1.9 μm) reverse phase media and was separated by an increasing acetonitrile gradient over 40 or 60 min at a flow rate of 250 nL/min. The mass spectrometer was operated in positive ion mode with a capillary temperature of 320°C, and with a potential of 2,300 V applied to the frit. All data was acquired with the mass spectrometer operating in automatic data dependent switching mode. A high resolution (70,000) MS scan (300–1600 m/z) was performed using the Q Exactive to select the 12 most intense ions prior to MS/MS analysis using HCD. Proteins identified were present within two biological replicates of novobiocin treatment. Using the peaks 7 software, data filtration was set to ≥2 unique peptides per protein and a false discovery rate of 1%. The parent mass tolerance was 10 ppm and the fragment mass error tolerance was 0.03 Da. The raw data was

de novo sequenced and searched against the *C. jejuni* NCTC11168 Uniprot unreviewed database (http://www.uniprot.org/taxonomy/1385724) using the search engine PEAKS Studio 7 (Bioinformatics Solutions), for peptides cleaved with trypsin. Each peptide used for protein identification met specific Peaks parameters, i.e. only peptide scores that corresponded to a false discovery rate (FDR) of ≤1% were accepted from the Peaks PTM database search.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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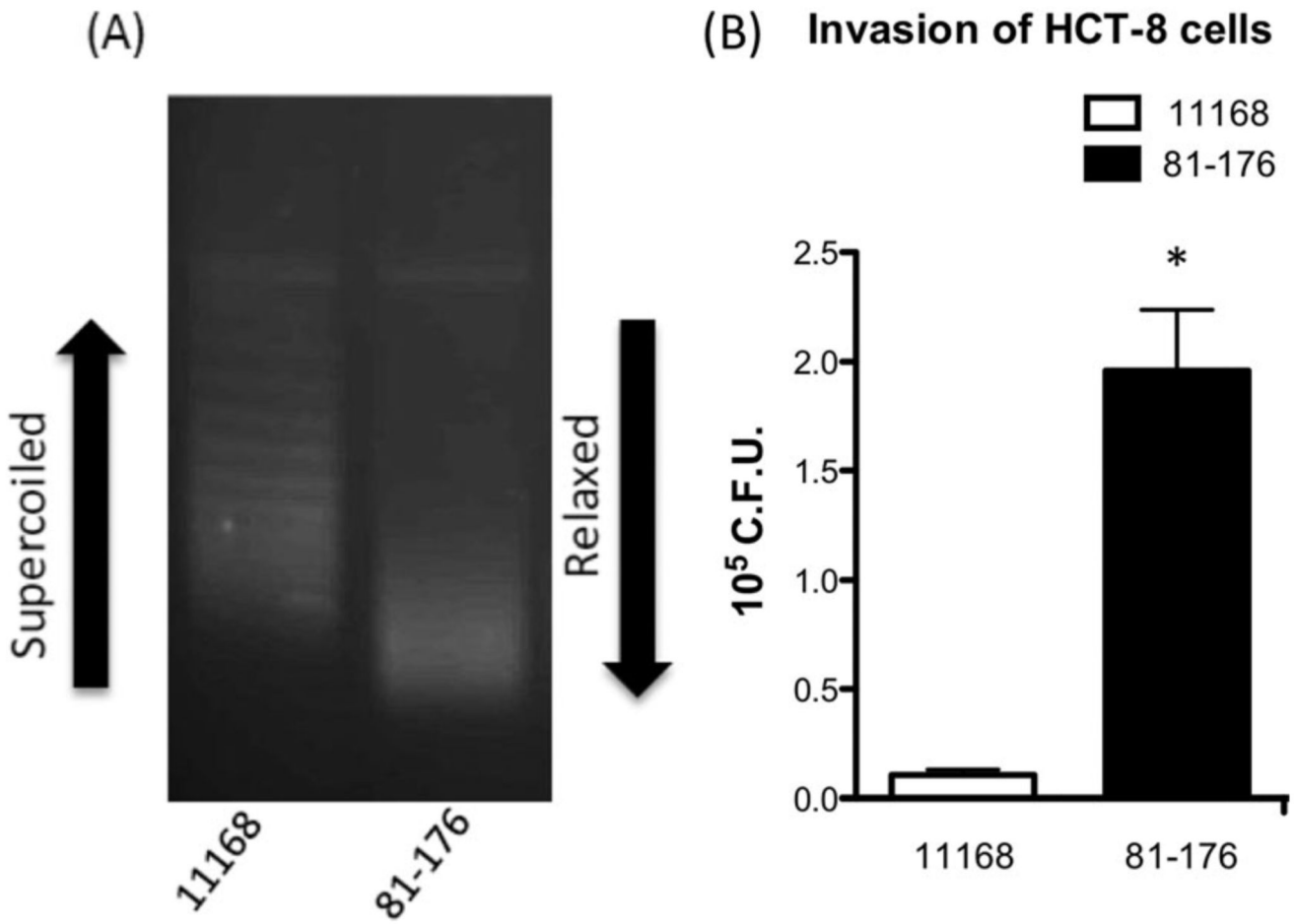

Fig. 1.

Correlation exists between DNA supercoiling profiles and invasion of epithelial cells.

A. Chloroquine gel analysis revealing resting supercoiling profiles of reporter plasmid pRY107 in strains NCTC11168 and 81-176 after overnight growth.

B. The topologically more relaxed strain 81-176 shows significantly greater numbers of invasion of HCT-8 cells after 5 h than the more negatively supercoiled strain NCTC11168. * denotes a P value < 0.05.

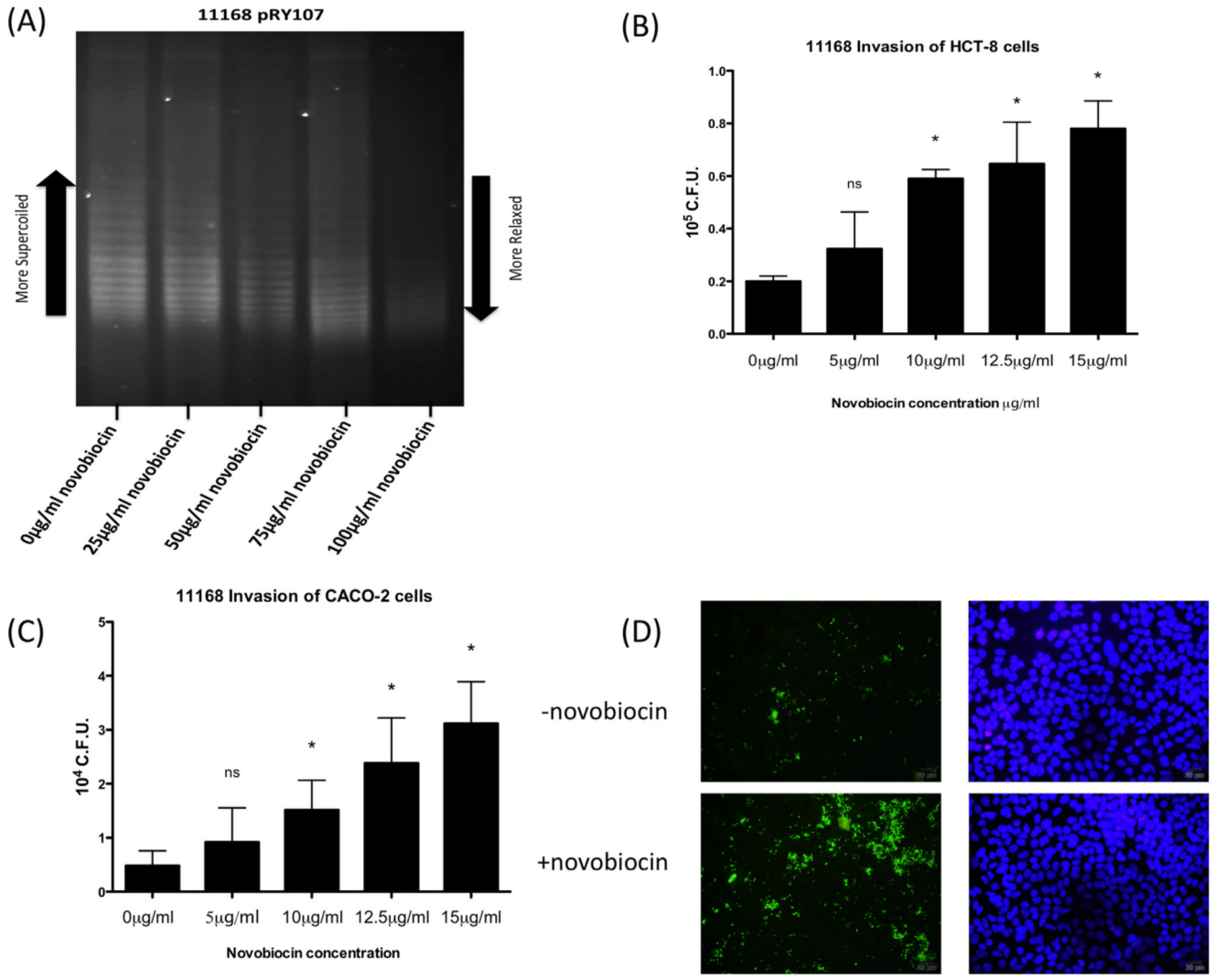

Fig. 2.

A. Treatment of NCTC11168 with the DNA gyrase inhibitor novobiocin results in dose dependent DNA relaxation as observed in mid log bacteria. Treatment of NCTC11168 overnight cultures with sub-inhibitory concentrations of novobiocin results in a dose dependent increase in bacterial invasion of the HCT-8 (B) and Caco2 (C) cell lines after 5 h. * denotes a *P* value <0.05 compared to the untreated strain. (D) Immunofluorescence microscopy confirms a higher rate of HCT-8 cell interaction following culture of NCTC11168 in the presence of novobiocin.

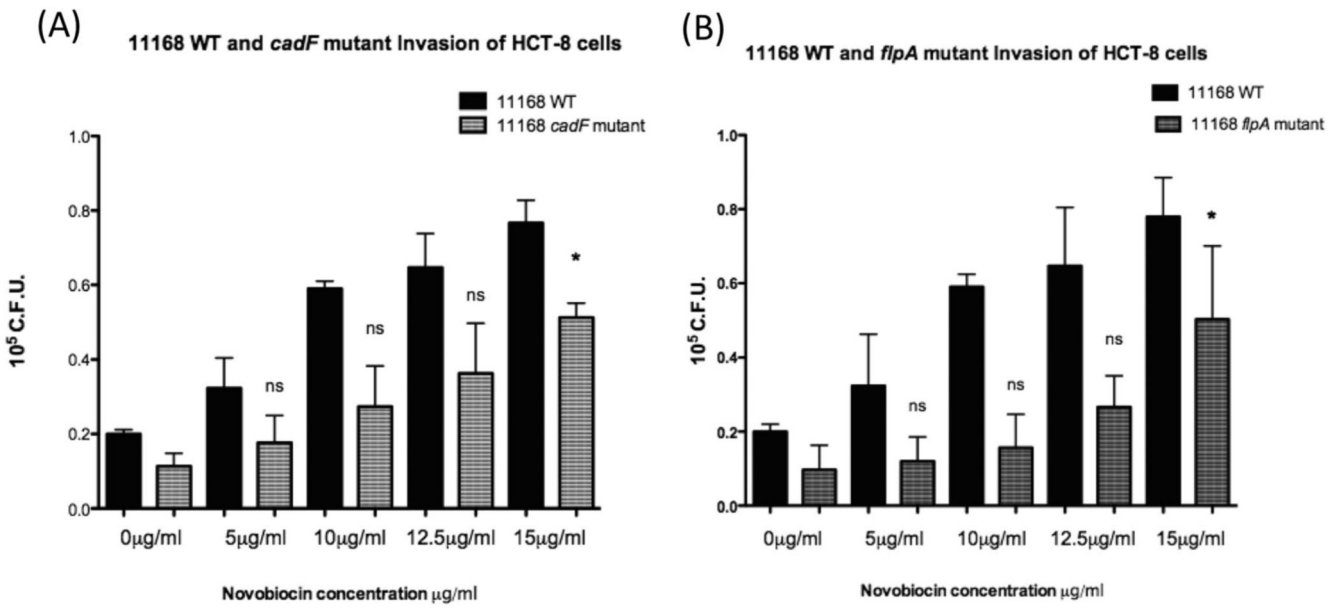

Fig. 3.

Disruption of either *cadF* or *flpA* genes does not prevent increased invasion of NCTC11168 following growth in the presence of novobiocin. Invasion of the HCT-8 cell line using NCTC11168 with a disrupted (A) *cadF* or (B) *flpA* gene resulted in increased invasion following DNA relaxation but to a lesser extent observed than the wild type for both mutants. * denotes a *P* value <0.05 compared to the untreated strain.

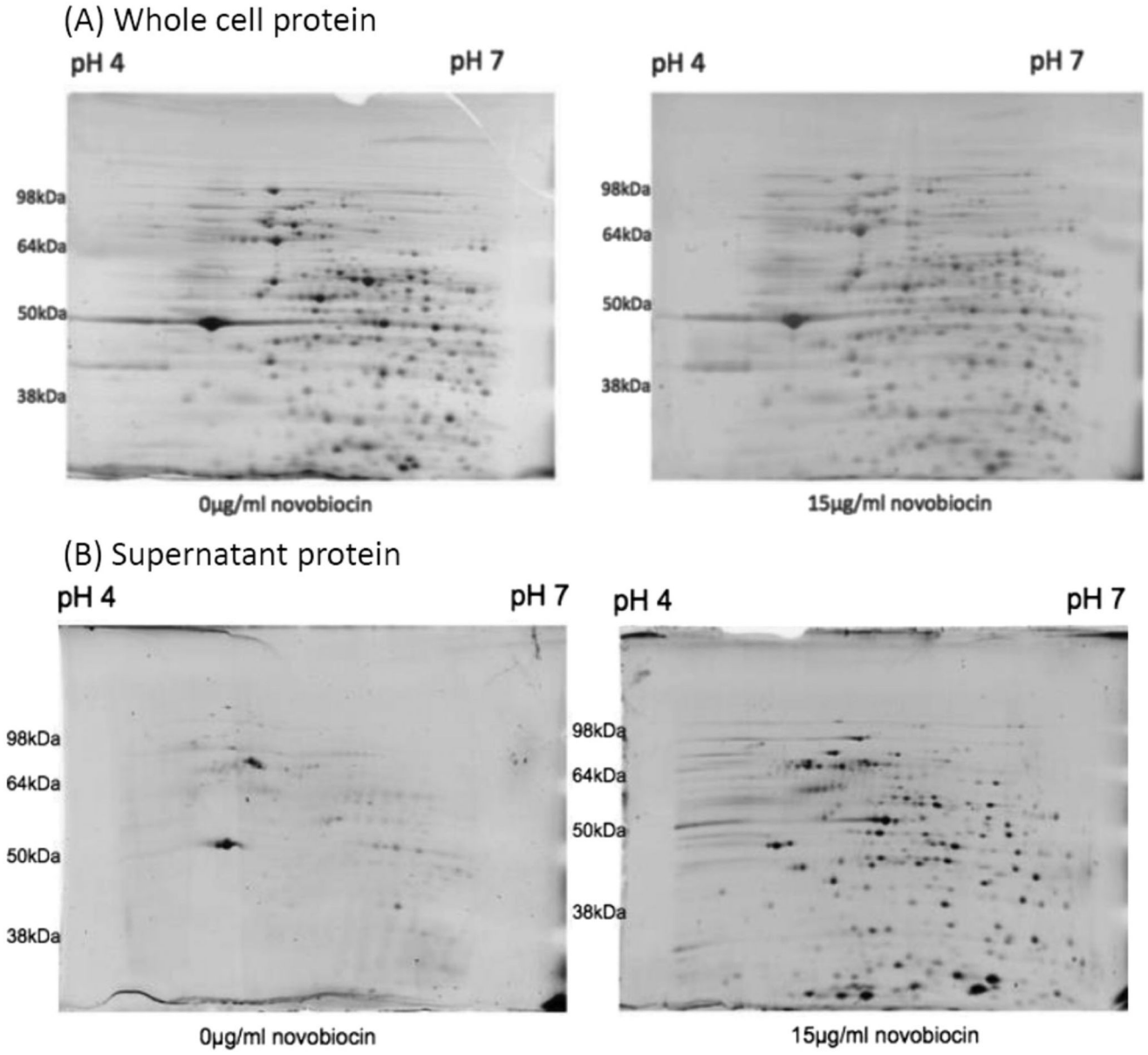

Fig. 4.

Reduced negative DNA supercoiling induced by 15 ug/ml novobiocin does not lead to large changes in (A) whole cell protein production but leads to a large increase in (B) protein found within concentrated bacterial supernatants.

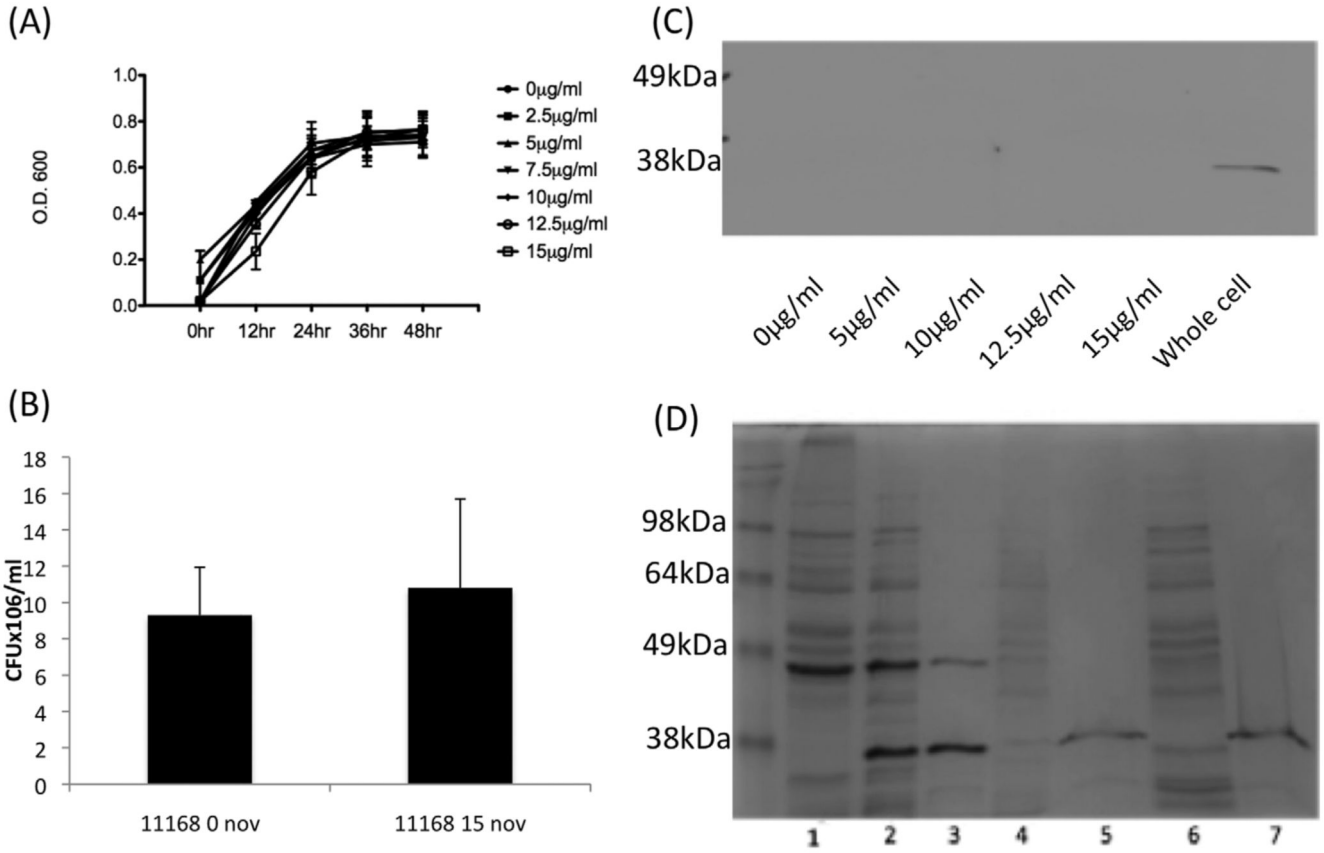

Fig. 5.

The observed increased secretion is not mediated by cell lysis or outer membrane vesicles.

A. NCTC11168 grown in the presence of subinhibitory concentrations of novobiocin shows no significant effect on growth.

B. NCTC11168 grown overnight in the presence of novobiocin shows no significant difference in numbers of colony forming units.

C. NCTC11168 supernatant from bacteria grown in the presence of novobiocin does not react with antibodies raised against the outer membrane protein CadF.

D. NCTC11168 protein secreted when grown in the presence of novobiocin is not resistant to proteinase K treatment. 1-Whole cell lysate, 2-Whole cell lysate, Prot. K treated, 3-Whole cell lysate boiled, Prot. K treated, 4-Uninduced secreted fraction, 5-Uninduced secreted fraction, Prot. K treated, 6-Induced fraction, 7-Induced fraction, Prot. K treated.

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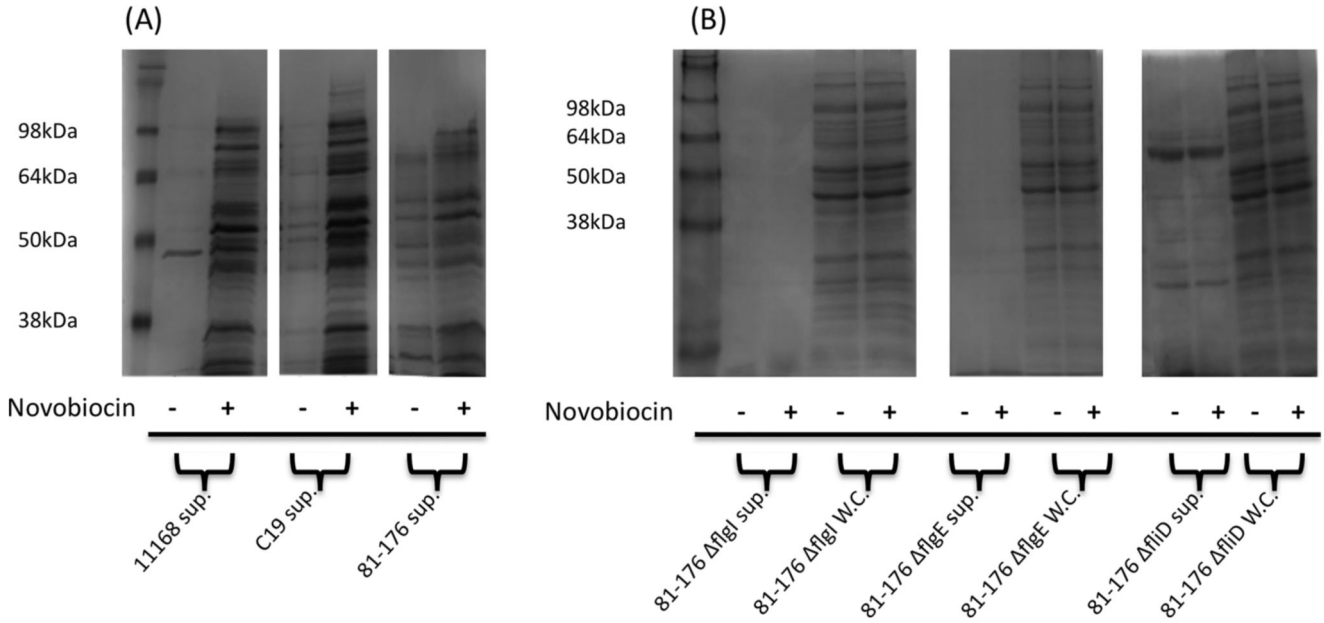

Fig. 6.

DNA relaxation leads to increased protein found within the supernatants of multiple *C. jejuni* strains and novobiocin induced secretion is inhibited by mutations in the flagellar genes *flgI*, *flgE* and *fliD*.

A. Coomassie staining of concentrated bacterial supernatants from *C. jejuni* strains 11168, C19 and 81-176 grown in the absence and presence of novobiocin.

B. No increase in protein within supernatants or whole cell lysates (WC) of strain 81-176 containing mutations in genes *flgI*, *flgE* or *fliD* is observed following DNA relaxation.

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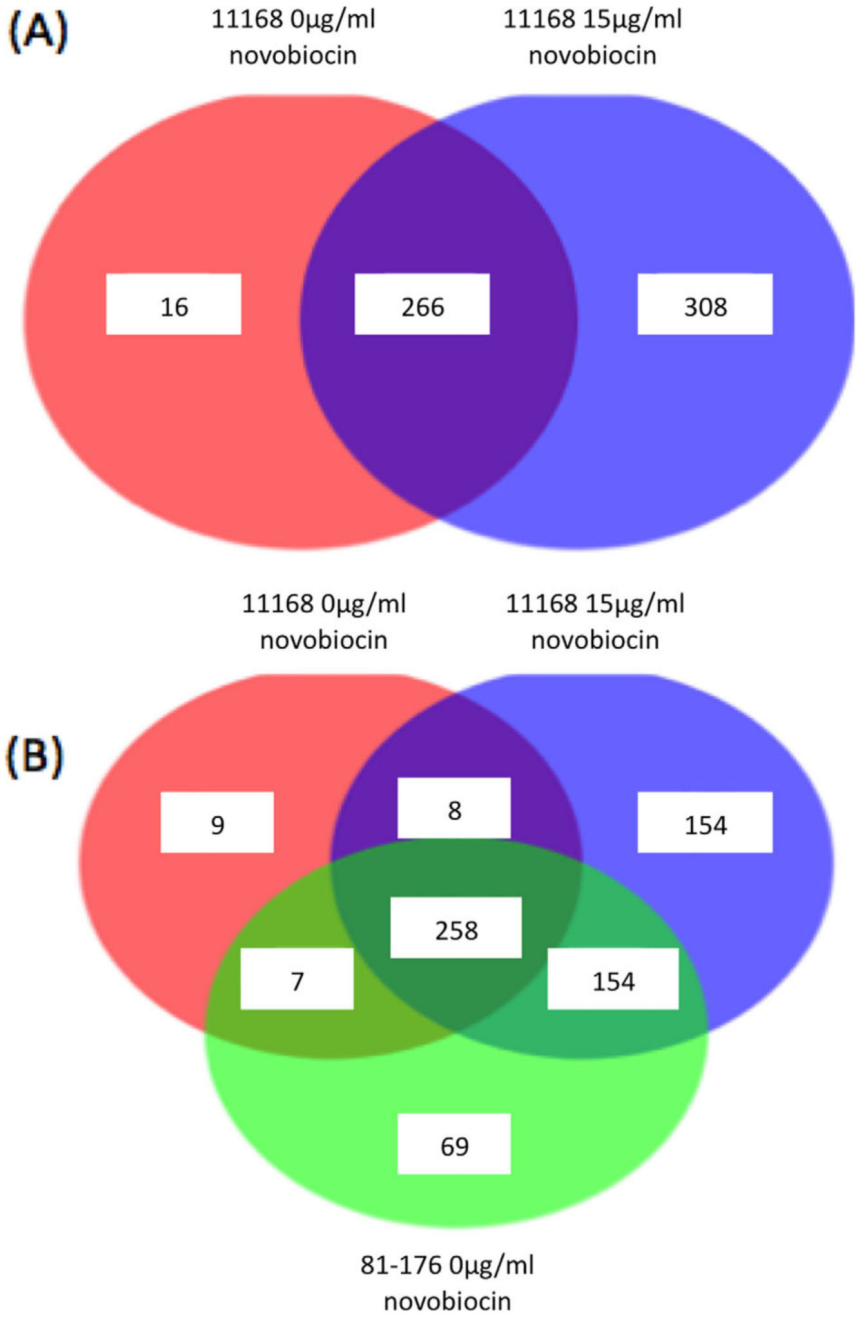

Fig. 7.

LC-MS of bacterial supernatants grown in the absence and presence of novobiocin.

A. Numbers of proteins found within the supernatant of strain NCTC11168 grown in the absence and presence of novobiocin reveals the extent of increased protein secretion following DNA relaxation.

B. Many of the proteins found within the supernatant of NCTC11168 following DNA relaxation are also secreted by the less negatively supercoiled strain 81-176 in the absence of novobiovin.

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Table 1

The identities of the top 10 proteins found within the concentrated supernatant of NCTC11168 treated with Novobiocin.

Accession	Gene	Name	Product	% Coverage	#Peptides	#Unique	Mass (Da)
Q9PPD9	Cj0780	napA	nitrate reductase catalytic subunit	79	94	94	104,944
QOPAS1	Cj0596	peb4cbf2	major antigenic peptide PEB-cell binding factor	89	76	75	30,518
046108	Cj0193c	tig	trigger factor	87	75	74	50,968
QOPAYS	Cj0531	icd	isocitrate dehydrogenase	75	69	68	82,331
Q9PI16	Cj0493	fusA	elongation factor G	86	67	67	76,719
QOPA55	Cj0835c	acnB	bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	62	61	60	92,793
069298	Cj0759	dnaK	molecular chaperone DnaK	79	58	58	67,419
069303	Cj0470	tuf	elongation factor Tu	99	50	50	43,594
QOPBP1	Cj0264c	-	molybdopterin containing oxidoreductase	62	47	47	93,278
QOP8A3	Cj1516	-	oxidoreductase	67	46	46	59,077

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Table 2

The identities of the top 10 proteins found within the concentrated supernatant of NCTC11168 treated with Novobiocin but absent in the concentrated supernatants of untreated NCTC11168.

Accession	Gene	Name	Product	%10 Coverage	#Peptides	#Unique	Mass (Da)
QOPCA1	Cj0024	nrdA	ribonucleotide-diphosphate reductase subunit alpha	35	35	35	90,052
QOPC5O	Cj0087	aspA	aspartate ammonia-lyase	54	32	32	51,766
QOPC85	Cj0041	fliK	flagellar hook-length control protein	39	24	23	67,841
QOPBR4	Cj0240c	iscS	cysteine desulfurase	60	22	22	43,053
Q0PB04	Cj0511	-	protease	49	21	20	48,876
Q59296	Cj1385	katA	catalase	47	22	22	58,416
Q9PMKS5	Cj1455	prf13	peptide chain release factor 2	54	21	21	41,068
QOPBW9	Cj0169	sodB	superoxide dismutase	70	20	20	24,813
QOPBZ1	Cj0146c	trxB	thioredoxin reductase	61	20	20	33,118
Q9PNCO	Cj1175c	argS	arginyl-tRNA synthetase	36	20	19	60,190