Transcription of \( \sigma^{54} \)-dependent but not \( \sigma^{28} \)-dependent flagellar genes in *Campylobacter jejuni* is associated with formation of the flagellar secretory apparatus

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Introduction

Construction of the flagellar organelle of a bacterium is an exquisitely ordered process involving the temporal expression of approximately 45 genes. Genes encoding structural subunits of the flagellum are transcribed in the order in which they are incorporated in the flagellum, starting from the cell envelope and extending extracellularly to the tip of the flagellum (Macnab, 1996). In the classic paradigm of the flagellar transcriptional cascade in *Salmonella* species, flagellar genes can be divided into three classes based on their order of transcription (Kutsukake et al., 1990; Karlinsey et al., 2000). Briefly, class 1 genes include genes for the major transcriptional regulators *flhDC* that are required for expression of the remaining flagellar genes. Class 2 genes include genes encoding the flagellar secretory apparatus, the flagellar basal body and hook structures and *flaA*, encoding the alternative \( \sigma \) factor, \( \sigma^{28} \). Class 3 genes, transcription of which requires \( \sigma^{28} \), include genes encoding the flagellin subunits that are incorporated last into the growing flagellum. Feedback loops are incorporated into the regulatory system to ensure that the flagellar genes are transcribed in appropriate order. For example, flagellin subunits are not transcribed until a complete basal body and hook structure is formed. To accomplish this, a class 2 flagellar gene product, FlgM, represses \( \sigma^{28} \) activity, thereby preventing transcription of the flagellin genes (Gillen and Hughes, 1991). After a complete basal body and hook are formed, the FlgM anti-\( \sigma \) factor is secreted through this nascent structure to the extracellular environment, thereby reducing intracellular FlgM concentrations and relieving repression of \( \sigma^{28} \), which allows for transcription of the class 3 genes including the flagellin genes (Hughes et al., 1993).

This flagellar transcriptional cascade is generally conserved in many different bacteria, including *Helicobacter pylori*, *Vibrio cholerae* and *Pseudomonas aeruginosa*, although these bacteria use both \( \sigma^{24} \) and \( \sigma^{28} \) to control transcription of flagellar genes (Totten et al., 1990; Starnbach and Lory, 1992; Klose and Mekalanos, 1998a,b; Spohn and Scarlato, 1999; Colland et al., 2001; Prouty et al., 2001; Josenhans et al., 2002; Jyot et al., 2002). In...
these organisms, both $\sigma^{28}$- and $\sigma^{54}$-dependent transcription of flagellar genes is incorporated into the flagellar transcriptional cascade to achieve proper temporal regulation of the flagellar genes. For instance, $\sigma^{54}$ in conjunction with $\sigma^{54}$-dependent transcriptional activators has been proposed or shown to be involved in transcription of many of the class 2 genes, whereas $\sigma^{28}$ is involved in transcription of the class 3 genes including those encoding the flagellins (Starnbach and Lory, 1992; Arora et al., 1998; Spohn and Scarlato, 1999; Prouty et al., 2001; Jyot et al., 2002). V. cholerae presents an alternative to this regulatory cascade as $\sigma^{28}$ is required for transcription of the major flagellin, whereas $\sigma^{28}$ is involved in the transcription of the remaining minor flagellins (Prouty et al., 2001). In Caulobacter crescentus, regulation of flagellar genes only involves $\sigma^{54}$, as $\sigma^{28}$ is apparently absent in the bacterium (Wu and Newton, 1997). Activation of $\sigma^{54}$-dependent transcription of the flagellar hook, basal body and flagellin genes in C. crescentus is linked to the formation of the flagellar secretory apparatus and the MS ring–switch complex (Ramakrishnan et al., 1994; Wu and Newton, 1997).

Campylobacter jejuni, a common commensal organism of the gastrointestinal tracts of many birds and a frequent cause of gastroenteritis in humans, elaborates a single flagellum at one or both poles of the bacterium. Two flagellin genes, flaA and flaB, are present in tandem on the chromosome of C. jejuni and are approximately 95% identical to each other (Nuijten et al., 1990). Both FlaA and FlaB appear to be incorporated into the flagellum. Transcription of flagellar genes in C. jejuni involves both $\sigma^{54}$ and $\sigma^{28}$; the former is required for transcription of genes encoding a putative hook protein, hook-associated protein and the minor flagellin FlaB, and the latter is involved in transcribing the gene encoding the major flagellin FlaA (Hendrixson et al., 2001). Even though either the flagellum or motility is required for colonization (Nachamin et al., 1993; Wassenaar et al., 1993), disease in humans (Black et al., 1988) and in vitro invasion (Wassenaar et al., 1991; Grant et al., 1993; Yao et al., 1994), little is known about the flagellar transcriptional cascade in C. jejuni. An NtrC homologue, FlgR, is required for flagella biogenesis and motility (Jagannathan et al., 2001), but the specific role of this protein in the flagellar transcriptional cascade in C. jejuni has yet to be determined.

In this study, we performed experiments to elucidate the flagellar transcriptional cascade in C. jejuni. To do so, we adapted the astA gene, encoding the enzyme arylsulphatase, as a new reporter gene for C. jejuni that allowed us to monitor transcription of flagellar genes. We performed transposon mutagenesis with C. jejuni harbouring a transcriptional fusion of the $\sigma^{54}$-dependent flagellar operon flgDE2 to astA and identified 10 chromosomal genes required for transcription of $\sigma^{54}$-dependent flagellar genes, including genes encoding proteins at the beginning of the flagellar transcriptional cascade in other organisms, a gene encoding a putative sensor kinase and flgR, encoding the NtrC homologue noted above (Jagannathan et al., 2001). All the mutants were deficient for $\sigma^{54}$-dependent transcription of flagellar genes but, unlike what has been described for other flagellar systems, these mutants still expressed the major flagellin gene flaA, which uses $\sigma^{28}$ for transcription. Further analysis revealed a homologue of FlgM in C. jejuni that acts to inhibit $\sigma^{28}$ activity in other flagellar systems. In contrast, the FlgM homologue in C. jejuni appears to be only weakly active as an anti-$\sigma$ factor for $\sigma^{54}$-dependent transcription of flaA. Thus, our study reveals unique features of the C. jejuni flagellar transcriptional cascade compared with other bacteria.

Results

Development of a reporter system for analysis of flgDE2 gene expression

We wanted to analyse control of genes expressed relatively late in the hierarchy of flagellar assembly, and also to focus on those controlled by $\sigma^{54}$, which we and others have shown to be important in flagellar gene regulation in C. jejuni (Hendrixson et al., 2001; Jagannathan et al., 2001). We considered that this approach would lead us to identify potential regulators of $\sigma^{54}$ activity necessary for both flagellar development and other aspects of C. jejuni pathogenicity, given that flagellar motility or flagella per se appear to be important for host association by this organism (Black et al., 1988; Wassenaar et al., 1991; 1993; Grant et al., 1993; Nachamin et al., 1993; Yao et al., 1994). We anticipated that our approach would identify two classes of genes: one encoding transcriptional regulators that function with $\sigma^{54}$ and another encoding flagellar proteins whose proper expression might be required in order for late genes to be expressed.

We chose to focus on an operon at Cj0042–Cj0043, which we identified previously as regulated by $\sigma^{54}$ and in which Cj0042 (flgD) had been shown to be essential for wild-type motility (Hendrixson et al., 2001). FlgD is predicted to be a hook assembly protein, presumably required later in the assembly pathway (Parkhill et al., 2000). Cj0043 is annotated as flgE, encoding a probable hook protein (Parkhill et al., 2000), but deletion of this gene had no effect on motility or flagellar assembly (Fig. 1A, B and D). Another gene, Cj1729c, is annotated as flgE2 (Parkhill et al., 2000), but another group has already shown it to encode a hook protein and termed the gene flgE (Lüneberg et al., 1998). In contrast to deletion of Cj0043, disruption of Cj1729c had a dramatic effect on motility and flagellar assembly (Fig. 1A–C). We propose to reverse the annotation of Cj1729c and Cj0043 and will
Flagellar transcriptional cascade in C. jejuni

was then electroporated into DRH461 and DRH453, and as a reporter the analysis. These considerations, we chose to use proved its dependence on flgDE2 downstream of the pDRH351 harbouring kanamycin resistance; see (1998). The pDRH351 harbouring kanamycin resistance; see (1998). The flgDE2 operon met our criteria for further study in that we have already determined experimentally that it is regulated by σ54 and that flgD encodes a flagellar protein predicted to function late in flagellar assembly. Although the flgE gene (Cj1729c) does harbour a putative σ54 binding site upstream, we have not yet experimentally proved its dependence on σ54 for transcription. Given these considerations, we chose to use flgDE2 for further analysis.

To study σ54-regulated flgDE2 transcription, we adapted as a reporter the C. jejuni gene astA (Yao and Guerry, 1996), which encodes arylsulphatase. This enzyme cleaves sulphate groups from aryl compounds, and its activity can be detected by the addition of the chromogenic substrate XS to MH agar, resulting in a blue colony phenotype (Yao and Guerry, 1996), which encodes arylsulphatase. This enzyme cleaves sulphate groups from aryl compounds, and its activity can be detected by the addition of the chromogenic substrate XS to MH agar, resulting in a blue colony phenotype (Yao and Guerry, 1996). In addition, a quantitative assay allows monitoring levels of the enzyme in whole-cell lysates spectrophotometrically by measuring the release of nitrophenol from nitrophenylsulphate (Henderson and Milazzo, 1979), similar to the assay traditionally used to quantify β-galactosidase activity with the substrate o-nitrophenylgalactoside.

We deleted astA from C. jejuni strain 81-176 SmR (DRH212) and 81-176 ΔrpoN (DRH321; Hendrixson et al., 2001), creating DRH461 and DRH453 respectively. DRH461 and DRH453 showed nearly undetectable aryl-sulphatase activity compared with the parental strains (Fig. 2). To construct a flgDE2::astA operon fusion, we isolated an insertion of the nemo transposon (which contains a promoterless astA gene linked to aphA-3 encoding kanamycin resistance; see Experimental procedures) in pDRH351 harbouring flgDE2, thereby creating pDRH532. The nemo insertion in this plasmid is located 515 bp downstream of the flgE start codon, creating a functional flgDE2::astA transcriptional fusion. The plasmid pDRH532 was then electroporated into DRH461 and DRH453, and kanamycin-resistant transformants were selected that resulted from the replacement of flgDE2 with flgDE2::nemo in the chromosome, creating DRH533 (81-176 ΔastA flgDE2::nemo) and DRH536 (81-176 ΔrpoN ΔastA flgDE2::nemo). C. jejuni 81-176 ΔastA containing the reporter gene fusion appeared as light blue colonies on MH agar containing the chromogenic substrate XS, whereas the ΔrpoN ΔastA derivative appeared as white colonies (data not shown). Arylsulphatase assays revealed that astA transcription from flgDE2::nemo was almost 100-fold higher in the 81-176 ΔastA background than in the ΔrpoN ΔastA mutant (Fig. 2). Complementation of the ΔrpoN ΔastA mutant with rpoN expressed from a chloramphenicol acetyltransferase (cat) promoter on

Fig. 1. Analysis of Cj0043 and Cj1729c in motility and flagellar biosynthesis. A. Motility phenotypes of DRH212 (wild-type 81-176 SmR), DRH963 (81-176 Cj1729c::cat-rpsL) and DRH619 (81-176 ΔCj0043) in MH motility media.

Fig. 2. Arylsulphatase assay of reporter strains containing flgDE2::nemo and establishment of astA as a reporter gene for C. jejuni. The first four bars represent arylsulphatase activity originating from the astA locus of each strain, and the last four bars represent arylsulphatase activity originating from flgDE2::nemo in each strain. Each strain was tested in triplicate, and the values reported represent the average arylsulphatase activity ± standard deviation from a representative assay. One arylsulphatase unit equals the amount of arylsulphatase necessary to generate 1 nmol of nitrophenol h⁻¹ per OD₆₀₀.

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Multiple insertions were identified in and insertions in other chromosomal loci in investigated no further. Fifty mutants contained transcription of the transcriptional reporter; these mutants were used as a transcriptional reporter gene in C. jejuni.

Identification of genes required for transcription of flgDE2::astA by transposon mutagenesis

To identify genes required for transcription of flgDE2::astA, we purified chromosomal DNA from strain DRH533 (81-176 ΔastA flgDE2::nemo) and subjected it to in vitro transposition with the picard transposon from pEnterpise2. Thirty-nine individual transposition reactions were performed, and the transposed chromosomal DNA was then transformed into DRH533 to obtain approximately 14 000 individual transposon mutants. The transposon mutants were recovered on MH agar containing chloramphenicol and the chromogenic substrate for AstA, XS. Eighty-three mutants were identified that appeared as white colonies indicating an AstA– phenotype.

DNA from each mutant was purified and sequenced to identify the location of picard in each derivative. Ten mutants contained transposon insertions between the flgDE2 promoter and the end of astA, resulting in destruction of the transcriptional reporter; these mutants were investigated no further. Fifty mutants contained picard insertions in other chromosomal loci in C. jejuni (Table 1).

<table>
<thead>
<tr>
<th>Gene designation and proposed function*</th>
<th>Identification/proposed function*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosomal loci</td>
<td>RNA polymerase σ54 subunit</td>
</tr>
<tr>
<td>rpoN (5)</td>
<td>Probable signal transduction regulatory protein</td>
</tr>
<tr>
<td>flgR (3)</td>
<td>Probable signal transduction histidine kinase</td>
</tr>
<tr>
<td>Cj0793 (3)</td>
<td>Probable flagellar biosynthesis protein</td>
</tr>
<tr>
<td>flhA (17)</td>
<td>Probable flagellar biosynthesis protein</td>
</tr>
<tr>
<td>flhB (2)</td>
<td>Probable flagellar biosynthesis protein</td>
</tr>
<tr>
<td>flgR (7)</td>
<td>Flagellar biosynthesis protein</td>
</tr>
<tr>
<td>fliP (5)</td>
<td>Probable flagellar biosynthesis protein</td>
</tr>
<tr>
<td>fliR (5)</td>
<td>Probable flagellar biosynthesis protein</td>
</tr>
<tr>
<td>Cj0883c (2)</td>
<td>Unknown/no identity</td>
</tr>
<tr>
<td>Cj1341</td>
<td>mal7; member of paralogous gene family implicated in phase variation of flagella</td>
</tr>
<tr>
<td>pVir loci</td>
<td></td>
</tr>
<tr>
<td>Cj01 (4)</td>
<td>virB8; type IV secretion/competence protein</td>
</tr>
<tr>
<td>Cj03</td>
<td>virB10; type IV secretion/competence protein</td>
</tr>
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<td>Cj06 (2)</td>
<td>H. pylori virD4 homologue</td>
</tr>
<tr>
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<tr>
<td>Cj10</td>
<td>H. pylori HP0937 homologue</td>
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<td>Cj11 (2)</td>
<td>locA; H. pylori topoisomerase 1 homologue</td>
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<tr>
<td>Cj13</td>
<td>ssb; single-stranded DNA binding protein</td>
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</tr>
<tr>
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<td>Unknown</td>
</tr>
<tr>
<td>Cj47</td>
<td>Unknown</td>
</tr>
<tr>
<td>Cj49</td>
<td>Homologue of H. pylori HP0996 and HP0942</td>
</tr>
<tr>
<td>Cj51 (2)</td>
<td>Unknown</td>
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</tbody>
</table>

a. Gene designation and proposed function are based on the annotated genome sequence from C. jejuni NCTC 11168 (Parkhill et al., 2000) and the annotated pVir sequence from C. jejuni 81-176 (Bacon et al., 2002).

b. Number indicates number of mutants identified with different picard insertions in the respective gene.

c. Each transposon mutant displayed at least a 10-fold reduction in flgDE2::astA transcription compared with the wild-type strain as determined by arylsulphatase assays (data not shown).

d. One picard insertion is in the coding sequence of Cj0883c and another is located 34 bp upstream of the start codon of this gene. Cj0883c is immediately upstream of fliA (Parkhill et al., 2000); the flgDE2::astA transcriptional defect by the picard insertions in these mutants may result from polar effects on the transcription of fliA.

tained a transposon insertion in mal7, which is a member of a family of genes in C. jejuni with products that have been implicated in phase variation of flagella (Karlyshev et al., 2002).

We were surprised to identify 23 mutants with picard insertions in pVir, a plasmid maintained in C. jejuni 81-176 that appears to encode components of a type IV secretion system (Bacon et al., 2000; 2002). These 23 insertions were found scattered around pVir including in genes encoding some of the type IV secretion proteins (such as virB8 and virB10) and numerous unknown genes (Table 1). However, upon purification of the mutant plasmids and transformation into 81-176 ΔastA flgDE2::nemo (which replaced the wild-type pVir with the mutant plasmids), we found that colonies derived from...
each mutant pVir transformation expressed flgDE2::astA to either wild-type level or not at all. Hence, the pVir mutants (but not the above chromosomal mutants) could not consistently reproduce defective transcription of flgDE2::astA. We note these pVir genes identified here simply in the interest of describing the picard mutagenesis findings completely.

Analysis of defined deletion mutants for motility, flagellar biosynthesis and transcription of flgDE2::astA

To characterize the role of specific chromosomal genes identified from the screen, we created in frame deletion mutations of flgR, flgS, fliA, fliP and fliR in 81-176 Sm® (DRH212) and in 81-176 ∆astA (DRH461; the deletion end-points are detailed in Table S1 in Supplementary material). In addition, we replaced codons 2–364 of fliB with a cat–rpsL cassette in 81-176 Sm®, thereby deleting a large portion of the gene and insertionally inactivating the deletion construct. In 81-176 ∆astA, we were able to create an in frame deletion mutant of fliB. We could make an in frame deletion of fliF in 81-176 Sm® but not in 81-176 ∆astA.

Mutations in flgR, flgS, fliA, fliB, fliF, fliP and fliR in 81-176 Sm® resulted in non-motile phenotypes (Fig. 3), which appeared to result from the inability of these mutants to synthesize flagella as determined by transmission electron microscopy (compare Fig. 4A–E to 81-176 Sm® in Fig. 1B). Mutants lacking flgR and fliB also did not produce flagella when analysed by electron microscopy as has been reported previously (data not shown; Jaganathan et al., 2001; Matz et al., 2002). When the ∆flgR mutant was complemented with pECO102 containing the flgR coding sequence, motility and production of flagella were restored (Fig. 3A; data not shown). We attempted to complement the flgS, fliA, fliB, fliF and fliP mutants with pECO102 derivatives containing the respective coding sequences of each gene. However, after multiple conjugation attempts, we were unable to recover transconjugants containing the complementing plasmids, suggesting that overexpression of these genes from pECO102 may be toxic to C. jejuni. We also attempted to make a pECO102 derivative containing the flIR coding sequence, but were unable to make this plasmid in Escherichia coli.

To examine the ability of the mutants to transcribe astA from the flgDE2::nemo reporter, each deletion mutant in the ∆astA background was electroporated with pDRH532 to replace flgDE2 with flgDE2::nemo and assayed for arylsulphatase activity. Deletion of rpoN, flgR and flgS resulted in approximately 100- to 250-fold reductions in transcription of flgDE2::astA (Table 2). Complementation of the ∆rpoN and ∆flgR mutants with plasmid-encoded rpoN and flgR, respectively, restored transcription of the reporter fusion, verifying the requirement of these genes in the transcription of flgDE2. Transcription of flgDE2::astA was dependent of the only other alternative σ factor, σ28 (encoded by fliA). Deletion of fliA, fliB, fliP and fliR also reduced transcription of flgDE2::astA approximately 10- to 60-fold depending on the mutation. These data verify the transposon screen for mutants defective in transcription of flgDE2::astA. Although not identified in our transposon mutagenesis screen, we also constructed a deletion of flgE (encoding the putative flagellar hook) in 81-176 ∆astA flgDE2::astA and found that transcription of the reporter was also defective in this mutant, implicating flgE in flgDE2 transcription along with the other flagellar assembly gene products. To analyse flgE transcription in 81-176 ∆fliF (DRH1056), we performed reverse transcription polymerase chain reaction (RT-PCR) with primers specific for flgE2 and detected decreased product, suggesting a role for FliF in transcription of this gene as well (data not shown).

Fig. 3. Motility phenotypes of C. jejuni flagellar mutants in MH motility media. A. DRH212 (wild-type 81-176 Sm®), DRH460 (81-176 ∆flgS), DRH737 (81-176 ∆flgR), and DRH737/pDRH818. B. DRH212 (wild-type 81-176 Sm®), DRH946 (81-176 ∆fliA), DRH822 (81-176 ∆fliB::cat–rpsL), and DRH1056 (81-176 ∆fliF). C. DRH212 (wild-type 81-176 Sm®), DRH1065 (81-176 ∆fliP) and DRH755 (81-176 ∆fliR).

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Analysis of transcription of flaB::astA and flaA::astA in defined mutants

Another σ^54-dependent flagellar gene in C. jejuni is flaB, which, in the scheme described for flagellar cascades in other bacteria, is predicted to be a class 3 gene, transcription of which is downstream of genes encoding flagellar hook proteins. To investigate whether the above deletion mutants were defective for transcription of flaB, as they were for transcription of the flgDE2 promoter, a flaB::astA reporter gene fusion was made and electroporated into each mutant, replacing flaB with the reporter construct. Similar to what we observed for flgDE2, transcription of flaB was reduced in all the mutants ranging from an approximately 50-fold reduction to over a 1000-fold reduction, depending on the mutation (Table 2). Transcription of flaB::astA was restored in the ΔrpoN and ΔflgR mutants by plasmid complementation. As predicted, σ^28 was not required for transcription of flaB::astA; in fact, we observed a 75% increase in flaB::astA transcription in the ΔfliA mutant. These data suggest that mutations in the flagellar secretion apparatus and in the putative regulatory genes flgR and flgS have a general negative effect on σ^54-dependent transcription of flagellar genes in C. jejuni.

In Salmonella typhimurium, mutations in the genes encoding proteins that constitute the flagellar secretory apparatus or the flagellar basal body and hook structures, such as flhA, flhB, fliP, fliR and flgE among others, lead to repression of σ^28-dependent transcription of flagellar genes such as the major flagellin (Kutsukake et al., 1990; Hughes et al., 1993). Repression is mediated by the FlgM anti-σ factor, which binds to σ^28 and prevents the formation of productive σ^28–RNA holoenzyme complexes necessary for transcription of σ^28-dependent flagellar genes (Ohnishi et al., 1992; Hughes et al., 1993; Chadsey et al., 1998). Upon completion of the hook and basal body element, FlgM is secreted from the bacterium, thus allowing σ^28 to express σ^28-dependent genes.

Our results suggest that a checkpoint control mechanism may exist for σ^54-dependent flagellar gene expression in C. jejuni, but we also wanted to determine whether, as in Salmonella species, blocking flagellar secretion represses σ^28-dependent flagellar gene expression in C.
jejuni. We examined transcription of flaA, which encodes the major flagellin under σ28 control (Hendrixson et al., 2001), by constructing a flaA::astA reporter. This reporter was electroporated into the mutant backgrounds to replace wild-type flaA with the reporter fusion. Using the flaA::astA reporter, we confirmed our earlier observation (made using RT-PCR) that flaA transcription results at least in part from σ28; there remains significant flaA::astA transcription even in the absence of σ28 (ΔfliaA; Table 2). Further evidence that σ28 is involved in flaA transcription includes the finding that overexpression of flaA from a plasmid led to elevated expression of flaA::astA (Table 2).

In contrast to σ24-dependent expression of flaDE2 and flaB, expression of flaA::astA was unaffected or reduced only slightly compared with wild type in strains with a disrupted flagellar secretion apparatus (ΔflhA, ΔflhB, ΔflIP and ΔfliaR mutants), with mutations in the regulatory genes for σ24-dependent flagellar gene expression (ΔrpoN, ΔflgR and ΔflgS mutants) or with a ΔflgE mutation, although these mutants do not produce flagella and are non-motile (Figs 1, 3 and 4; Hendrixson et al., 2001; Jagannathan et al., 2001). Similarly, transcription of flaA in 81-176 ΔflhF as determined by RT-PCR analysis was comparable to the level of flaA transcription in the wild-type strain (data not shown). Clearly, further study is warranted regarding the activities of the σ28-dependent promoter and the σ28-independent promoter on transcription of flaA. However, if we assume that transcription from the σ28-independent promoter is constant, these results suggest that, unlike in other bacteria, σ28 activity on transcription of flaA may generally be unaffected in mutants lacking a flagellar secretory apparatus or hook structure.

**Characterization of Cj1464 encoding an FlgM homologue in flaA transcription and motility**

Considering that the level of flaA::astA transcription was close to or at the wild-type level in all the flagellar mutants and higher than the level of flaA::astA transcription in the ΔfliaA mutant, we speculated that C. jejuni may lack an anti-σ28 factor like FlgM of other bacteria or may express a FlgM homologue that has only limited σ28-repressive activity. Colland et al. (2001) identified a FlgM homologue in Helicobacter pylori and suggested that Cj1464 encodes the corresponding gene product in C. jejuni. To test whether Cj1464 encodes an anti-σ28 factor, we deleted the entire coding sequence of Cj1464 (flgM) from 81-176 SmR and analysed flaA transcription by primer extension analysis. We detected two primer extension products that were one nucleotide different in size and dependent on σ28, as determined by their absence in the ΔfliaA mutant and appearance when this mutation was complemented by cloned flaA (Fig. 5, compare lane 1 with lanes 5 and 6). Relative amounts of σ28-dependent flaA transcription were unaffected by the absence or overexpression of flgM (Fig. 5, lanes 1–4). One prominent σ28-independent primer extension product was also identified. Except for the ΔfliaA mutant, the strains analysed by primer extension were fully motile and produced normal flagella; the ΔfliaA mutant showed reduced motility and produced shorter flagella as reported previously (Fig. 5B; data not shown; Hendrixson et al., 2001; Jagannathan et al., 2001).

As mutation of flgM did not affect expression of flaA, we explored whether FlgM could inhibit σ28-dependent transcription of flaA::astA in C. jejuni if overexpressed. We reasoned that, by overexpressing flgM from a plasmid in 81-176 ΔflhA flaA::astA (DRH1048), which presumably does not produce a functional flagellar secretory apparatus, FlgM will not only be present at higher levels than normal but should be retained in the cytoplasm. Arylsulphatase activity from the flaA::astA reporter in wild-type bacteria overexpressing flgM compared with that from bacteria with vector alone was reduced from 91.6 arylsulphatase units (Fig. 6), a level of inhibition that we were apparently unable to detect using primer extension (Fig. 5B, compare lanes 1 and 2).

**Table 2. Arylsulphatase activity of flaDE2–, flaB– and flaA–astA transcriptional fusions in C. jejuni strain 81-176.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>flaDE2::nemo&lt;sup&gt;a&lt;/sup&gt;</th>
<th>flaB::astA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>flaA::astA&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>17.29 ± 1.81</td>
<td>81.89 ± 5.85</td>
<td>87.05 ± 1.50</td>
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<tr>
<td>ΔflaA</td>
<td>21.87 ± 0.09</td>
<td>143.62 ± 4.07</td>
<td>42.38 ± 2.11</td>
</tr>
<tr>
<td>ΔflaA + vector</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
<td>37.67 ± 0.73</td>
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<tr>
<td>ΔflaA + flaA</td>
<td>ND</td>
<td>ND</td>
<td>338.18 ± 23.47</td>
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<tr>
<td>ΔrpoN</td>
<td>0.18 ± 0.08</td>
<td>0.08 ± 0.03</td>
<td>91.59 ± 1.11</td>
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<td>0.16 ± 0.10</td>
<td>0.09 ± 0.04</td>
<td>ND</td>
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<tr>
<td>ΔrpoN + rpoN</td>
<td>14.61 ± 1.34</td>
<td>70.21 ± 3.19</td>
<td>ND</td>
</tr>
<tr>
<td>ΔflgR</td>
<td>0.07 ± 0.01</td>
<td>0.10 ± 0.04</td>
<td>91.54 ± 3.78</td>
</tr>
<tr>
<td>ΔflgR + vector</td>
<td>0.07 ± 0.01</td>
<td>0.20 ± 0.11</td>
<td>ND</td>
</tr>
<tr>
<td>ΔflgR + flgR</td>
<td>15.92 ± 1.97</td>
<td>83.38 ± 6.37</td>
<td>ND</td>
</tr>
<tr>
<td>ΔflgS</td>
<td>0.09 ± 0.04</td>
<td>0.15 ± 0.10</td>
<td>64.13 ± 1.37</td>
</tr>
<tr>
<td>Δfla</td>
<td>0.30 ± 0.03</td>
<td>1.55 ± 0.28</td>
<td>71.34 ± 3.65</td>
</tr>
<tr>
<td>ΔflhB</td>
<td>0.13 ± 0.10</td>
<td>0.26 ± 0.01</td>
<td>72.82 ± 3.79</td>
</tr>
<tr>
<td>ΔflIP</td>
<td>1.46 ± 0.22</td>
<td>1.41 ± 0.42</td>
<td>75.12 ± 2.29</td>
</tr>
<tr>
<td>ΔflIR</td>
<td>0.07 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>63.03 ± 2.64</td>
</tr>
<tr>
<td>ΔflgE</td>
<td>0.07 ± 0.01</td>
<td>0.19 ± 0.01</td>
<td>101.66 ± 3.76</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results are from a typical assay with each sample performed in triplicate. Values are reported as arylsulphatase units. One unit equals the amount of arylsulphatase required to generate 1 nmol of nitrophenol-1–l OD600 of 1.
about 20% (Fig. 6), suggesting that FlgM activity is not a major checkpoint of transcription when cells are unable to assemble the flagellum properly. The response in flaA expression seen in cells that lack flhA is similar to the small magnitude of the FlgM effect in wild-type cells overexpressing flgM (Fig. 6). Only by overexpressing flgM in the ΔfliA mutant was expression of the flaA::astA reporter reduced to a level (59.8 arylsulphatase units) that approached that seen in the ΔfliA (s28) mutant (52 arylsulphatase units; Fig. 6). We draw two conclusions from these results: first, that transcription of flaA is not entirely dependent upon s28 and, secondly, that FlgM does not appear to play a major role in the net levels of flaA expression in C. jejuni, which is unlike what is seen with s28-
dependent transcription of the major flagellin in other bacteria.

Discussion
In this report, we elucidated key portions of the flagellar regulatory cascade in C. jejuni by exploiting a new reporter gene system based on the astA gene encoding arylsulphatase in combination with transposon mutagenesis. This reporter gene may be a valuable tool in future gene regulation studies in C. jejuni. The use of lacZ as a reporter for C. jejuni has been demonstrated previously (Wösten et al., 1998; Bailon et al., 1999; van Vliet et al., 2001; Matz et al., 2002), but these studies have been
conducted with reporter gene fusions present on plasmids not the chromosome, which is not always ideal. We were unable successfully to create lacZ reporter fusion constructs on the chromosome of C. jejuni, eliminating the possibility of studying regulation of genes in single copy. An additional limitation of lacZ fusions in C. jejuni is the reported inability of C. jejuni derivatives harbouring lacZ reporter genes to grow on media containing the chromogenic substrate 5-bromo-4-chloro-3-indoly-β-galactose (Xgal; Wösten et al., 1998). By adapting astA as a reporter in C. jejuni strains lacking the native astA locus, we have developed a reporter gene system that allows for easy monitoring of gene expression on agar plates containing the chromogenic substrate XS and spectrophotometrically in whole-cell lysates.

By combining the use of flagellar gene–astA fusions, transposon mutagenesis and defined deletion mutagenesis, we were able to reveal critical elements of the flagellar transcriptional cascade in C. jejuni. Our model for the flagellar transcriptional cascade is depicted in Fig. 7. In S. typhimurium, C. crescentus and V. cholerae, master regulators (such as FlhDC, CtrA and FlrA) are at the top of the flagellar transcriptional cascade and control the transcription of many flagellar genes including genes for the flagellar secretory apparatus (Kutsukake and Iino, 1994; Quon et al., 1996; Prouty et al., 2001). Annotation of the C. jejuni genome sequence suggests that these master regulators are absent from the bacterium, which may imply that fliA and genes encoding the putative flagellar secretory apparatus in C. jejuni such as fliA, fliB, fliP and fliR are transcribed constitutively or regulated differently from other bacteria. We do not believe that σ54 is responsible for transcription of these genes as σ54 binding sites appear to be absent from the promoter regions of these genes; future experiments will address this point directly.

The extent of RpoN (σ54) control over flagellar motility in C. jejuni is not fully determined as yet. The ΔrpoN mutant is non-motile and lacks flagella (Hendrixson et al., 2001; Jagannathan et al., 2001). The genes that we have shown to require σ54 for transcription include flgDE2 and flaB (Hendrixson et al., 2001). However, a mutant with a transposon insertion in flgD has reduced motility, and disruption of flgE2 and flaB do not affect motility (Wassenar et al., 1991; Hendrixson et al., 2001). As the ΔrpoN mutant has a more severe motility defect than the flgD mutant, it seems likely that other flagellar genes required for motility are dependent on σ54 for transcription. By scanning the genome sequence of C. jejuni (Parkhill et al., 2000), we identified potential σ54 binding sites in the promoter regions of flgE and many other flagellar genes encoding putative flagellar basal body, ring and hook-associated proteins, such as flgK (Cj1466), fliE (Cj0526c), flgC (Cj0527c), flgB (Cj0528c), flgH (Cj0687c), flgG2 (Cj0697), flgG (Cj0698) and flgI (Cj1462); a previous study determined that flgK and flgB mutants of C. jejuni are non-motile (Golden and Acheson, 2002). These genes are indicated in the box located in Fig. 7. Analysis of transcription of these genes may further elucidate the flagellar regulatory cascade and the role of σ54 in flagellar gene expression and motility.

In our model, we propose three classes of σ54-dependent genes represented by flgE, flgDE2 and flaB (Fig. 7). We present a proposed order of transcription of these three classes; however, our data alone do not suggest that this temporal regulation is absolutely correct. We did observe that transcription of flgDE2 and flaB was dependent on intact flgE, suggesting that this gene is upstream of the transcriptional cascade. The possibility of studying regulation of genes in single copy allows for easy monitoring of gene expression on agar plates containing the chromogenic substrate XS and spectrophotometrically in whole-cell lysates.

Fig. 7. Model of flagellar transcriptional cascade in C. jejuni. Genes with a promoter containing putative σ54 binding sites that have not been shown to date to be dependent on σ54 for transcription are indicated in a box. The proteins indicated to the left of the figure within a bracket are postulated to play a role in transcriptional control of the boxed σ54-dependent genes and in post-translational control over FlgS function. The weak FlgM repressive activity for σ28 is indicated by a dashed line. See Discussion for details.

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of these other two flagellar genes in the transcriptional cascade. Additionally, genes for flagellins such as flaB are usually transcribed downstream of flagellar hook-associated genes in the flagellar transcriptional cascade of other bacteria, leading us to suggest that transcription of flaB in C. jejuni may be dependent upon flgDE2 as well. If the proposed temporal regulation of transcription is indeed correct, this finding would suggest that there are unknown factors present in C. jejuni controlling the ordered regulation of these three σ54-dependent flagellar genes.

In our model, FlgR, presumably upon activation as a result of phosphorylation by FlgS under appropriate signals, functions as a transcriptional activator required by σ54 to initiate transcription. Mutants lacking σ54, FlgR and FlgS failed to transcribe the σ54-dependent flagellar genes flgDE2 and flaB. Because of the homology that FlgS and FlgR exhibit with other sensor kinases and NtrC-like response regulators, respectively, we propose that the two proteins constitute a two-component regulatory system governing σ54-dependent transcription of flagellar genes in C. jejuni.

A major observation from our studies is that σ54-dependent transcription of flagellar genes in C. jejuni is apparently linked to the formation of the flagellar secretory apparatus (encoded in part by flhA, flhB, fliP and fliR). This aspect of flagellar gene regulation in C. jejuni is similar to that seen in C. crescentus. In this bacterium, transcription of hook, basal body and flagellin genes is dependent not only on σ54 and the σ54-dependent transcriptional activator FlbD, but also on components believed to function as the flagellar secretory apparatus (such as FlhA, FliP and FliR) and the MS ring and flagellar switches (such as FliF, FliM, FliG and FliN; Benson et al., 1994; Ramakrishnan et al., 1994; Wu et al., 1995; Wu and Newton, 1997). However, activation of transcription of the flagellar genes by FlbD is not well understood, and no sensor kinase has yet been identified controlling the activity of FlbD.

Given that FlgS is a putative sensor kinase that may control the activation of the σ54-dependent pathway potentially through the activation of FlgR by phosphorylation in C. jejuni, we speculate that FlgS may be the direct sensor determining whether conditions are appropriate for transcription of σ54-dependent flagellar genes to ensue. In this context, FlgS may be able directly to detect proper formation of the flagellar secretory apparatus, initiating σ54-dependent transcription of flagellar genes. Biochemical analysis regarding the activity of FlgS and the phosphorylation state of FlgR in wild-type bacteria and the various flagellar mutants may help to elucidate the connection between the formation of the flagellar secretory apparatus and the activation of transcription of σ54-dependent flagellar genes in C. jejuni. Of relevance to this model is the subcellular localization of FlgS; the hydropathy plot of the protein sequence is not conclusive, although it appears that the protein lacks extensive transmembrane domains by the Kyte–Doolittle criteria. Thus, it would seem that whatever sensing role FlgS may play could take place in the cytoplasm. Further work on this protein is needed before a mechanism for its role in flagellar regulation can be ascertained.

Another feature of the flagellar transcriptional cascade of C. jejuni demonstrated in our study is the lack of significant repression of flaA transcription in our flagellar secretory apparatus mutants. One likely reason why we did not see complete repression of σ28-mediated transcription of flaA in these mutants is that FlgM in C. jejuni 81-176 appears to be a weak repressor of σ28 transcriptional activity. In many of our flagellar mutants, we observed either no or only a slight reduction in transcription of the flaA::astA reporter, which was still above the level of transcriptions seen in a ΔfliA mutant. Even under the non-physiological conditions in which FlgM was overproduced in a ΔflhA mutant, the level of flaA::astA transcription was still slightly above that found in a ΔfliA mutant. Based on our experimental results, it is questionable whether FlgM acts in a significant way to regulate σ28 in C. jejuni, as originally suggested by Colland et al. (2001), who identified a putative anti-σ28 factor signature in FlgM of C. jejuni. In addition to the limited effect of FlgM, there is also considerable σ28-independent expression of flaA in C. jejuni 81-176 that would probably be outside the influence of anti-σ factor function.

We can only speculate why FlgM does not significantly reduce σ28 activity in C. jejuni. One possibility is that the protein has diverged sufficiently so that it no longer interacts with σ28. Alignment of the C. jejuni FlgM and σ28 proteins with homologous proteins from other bacteria reveals a significant amount of amino acid sequence divergence (data not shown; Colland et al., 2001). This sequence divergence in the two proteins may suggest that FlgM and σ28 do not have a high affinity for each other in C. jejuni, preventing a productive interaction required to repress σ28 activity. However, FlgM and σ28 from C. jejuni are most closely related to their homologues in H. pylori, and FlgM of H. pylori can both prevent σ28 and RNA polymerase from interacting and repress σ28 transcriptional activity in this bacterium (Colland et al., 2001). Plus, despite the apparent divergence in amino acid sequences between the FlgM and σ28 proteins of H. pylori and S. typhimurium, the H. pylori FlgM could complement an S. typhimurium flaA mutant to repress σ28 transcriptional activation of the major flagellin by one-third (Josenshans et al., 2002), suggesting that sequence divergences of one or both the proteins through evolution has not prevented the formation of repressive FlgM–σ28 complexes.
Another reason for transcription of flaA in our flagellar regulatory and secretory apparatus mutants is that approximately half the transcription of flaA results from a σ^28-independent promoter, based on the activity of the flaA::astA reporter in the wild-type strain versus the ΔflaA mutant. Currently, we do not know the location of the promoter or the start of transcription for this σ^28-independent activity of flaA transcription. In our primer extension analysis, we did detect a fainter product, the 5' end of which is located close to the flaA start codon, but we do not currently know whether this is the true start point of transcription from a σ^28-independent promoter. Additional analysis of flaA transcription and FlaA protein levels in a ΔflaA mutant may reveal the identity of this promoter and its contribution to FlaA levels.

A feature of flagellar biosynthesis in the Salmonella model, in which σ^28-dependent transcription of the major flagellin gene is inhibited in flagellar secretory apparatus mutants, may generally not hold for Campylobacter and Helicobacter species. In Campylobacter coli, which is closely related to C. jejuni, primer extension analyses suggested that, in a flgE mutant, transcription of both flaA and flaB (which as in C. jejuni are presumed to involve σ^28 and σ^54 for transcription respectively) was unaffected (Guerry et al., 1990; 1991; Kinsella et al., 1997). Furthermore, in H. pylori, mutation of flgR, a homologue of the C. jejuni flgR, which is required for transcription of certain flagellar basal body and hook proteins, did not lead to reduced σ^28-mediated transcription of flaA and actually resulted in a slight increase in transcription of this gene (Sphohn and Scarlato, 1999). In addition, disruption of flgE in H. pylori did not appear to decrease FlaA protein levels significantly (O'Toole et al., 1994). Evidence contrary to these findings includes the observation that, in an H. pylori flaA mutant, no flaA transcripts were detected (Schmitz et al., 1997). Allan et al. (2000) also showed that, in other H. pylori flagellar mutants such as an flhB mutant, flaA transcripts were reduced two- to fourfold, but were still detectable. Each of these H. pylori studies used different strains, perhaps suggesting lack of conservation of repressing flaA transcription in this bacterium.

Contrary to our findings showing that flaA::astA transcription is not affected in a flhB, flgR or rpoN mutant, others have reported that, in C. jejuni strains other than 81-176, flaA transcription was reduced or FlaA protein levels were abolished in strains lacking one of these genes (Jagannathan et al., 2001; Matz et al., 2002). Jagannathan et al. (2001) reported loss of detectable flagellin (both FlaA and FlaB) by mutation in either rpoN or flgR and suggested that neither flagellin gene was transcribed in either mutant. On the contrary, our results show that flaA transcription is not affected in these mutants, whereas flaB transcription is abolished. Using the flaA::astA or flaB::astA reporter fusions, we were able specifically to analyse transcription from either the flaA or the flaB promoter and reliably make conclusions regarding the levels of transcription of flaA and flaB in various flagellar mutants. Use of these reporters in other strains of C. jejuni may provide insight whether this apparent FlgM independence of σ^28 activity is a common feature among C. jejuni strains.

In this work, we have provided insight into the flagellar transcriptional cascade in C. jejuni. We have identified flagellar genes and regulatory factors that are required for activation of the σ^54 arm of the flagellar transcriptional pathway. A major factor that allowed us to accomplish these goals was the creation of astA as a reporter gene in C. jejuni combined with recent developments in random transposon mutagenesis and defined deletion mutagenesis in the organism (Hendrixson et al., 2001). Whereas new insights in the C. jejuni flagellar transcriptional cascade were revealed, many questions remained unanswered regarding how many other flagellar genes are regulated in the organism. Biochemical analysis of the putative FlgS–FlgR two-component regulatory system will increase our understanding regarding how σ^28-dependent transcription of flagellar genes is accomplished. Considering our findings, it is apparent that the flagellar transcriptional cascade in C. jejuni has aspects similar to that of C. crescentus with regard to requirements for activation of transcription of σ^54-dependent flagellar genes, but also includes σ^28 in transcription of the major flagellin, similar to what is seen in other bacterial organisms such as S. typhimurium. Despite similarities to these two systems, our current understanding of the flagellar transcriptional cascade in C. jejuni suggests that the bacterium does not entirely follow the flagellar regulatory hierarchy of either organism.

**Experimental procedures**

**Bacterial strains and plasmids**

All bacterial strains and plasmids used in this study are described in Table S1 in Supplementary material. C. jejuni was grown in microaerophilic conditions at 37°C on Mueller–Hinton (MH) agar. Microaerophilic conditions were generated by inflating plastic sealed bags with a gas mixture containing 85% nitrogen, 10% carbon dioxide and 5% oxygen. For C. jejuni, antibiotics were used at the following concentrations: trimethoprim, 10 μg ml^-1; cefoperazone 30 μg ml^-1; kanamycin, 50 μg ml^-1; chloramphenicol, 15 μg ml^-1; and streptomycin, 0.5, 1 or 2 mg ml^-1. For detection of arysulphate, XS was added to a final concentration of 100 μg ml^-1 in MH agar. All C. jejuni strains were stored at −80°C in MH broth containing 15% glycerol. E. coli DH5α and DH5αpir were grown in Luria–Bertani (LB) agar or broth. For E. coli, antibiotics were used at the following concentrations: ampicillin, 100 μg ml^-1; kanamycin, 50 μg ml^-1; and chloramphenicol, 15 μg ml^-1. All E. coli strains were stored at −80°C in LB containing 20% glycerol.
Construction of defined deletion mutants

In addition to using a cat–rpsL cassette to facilitate the creation of defined deletion mutants, a kan–rpsL cassette was also created. A Smal-digested apbA-3 cassette from plpL600 (Labigne-Roussel et al., 1988) was cloned into the HincII site of pDRH172 (Hendrixson et al., 2001) to insert apbA-3 next to the cloned rpsL gene. This plasmid was designated pDRH436. The kan–rpsL cassette was then amplified by polymerase chain reaction (PCR) with primers containing 5′ Smal restriction sites, purified and cloned into Smal-digested pUC19 to create pDRH437. This plasmid was the source of the kan–rpsL cassette.

The method for construction of defined deletion mutants in C. jejuni has been described previously (Hendrixson et al., 2001). To construct a defined deletion mutation of astA in C. jejuni strain 81-176, pDRH138 harbouring the astA locus (Wiesner et al., 2003) was digested with EcoRV, and the Smal-digested cat–rpsL cassette was ligated into this site to interrupt the gene. This plasmid, pDRH424, was electroporated into 81-176 SmR (DRH212) to replace astA with astA::cat–rpsL to create DRH435. A SOEing reaction (Higuchi, 1990) was performed to fuse the upstream and downstream DNA fragments surrounding astA, creating an in frame fusion linking the start codon of astA to codon 611 of the gene. This fragment was cloned as a Psfl fragment into pUC19 to create pDRH449. This plasmid was electroporated into DRH435 by the method of van Vliet et al. (1997) to replace astA::cat–rpsL with the astA deletion construct in the chromosome, creating DRH461 (81-176 SmRΔastA).

Chromosomal DNA from C. jejuni strain 81-176 was used in PCR with primers containing specific 5′ restriction sites to amplify various fragments containing flgR, flhA, flhF, flhP, flhR, flgE and flgM. The fragments were cloned into either pUC19 or pBR322 (for a description of the plasmids, see Table S1 in Supplementary material). Each gene was then interrupted by ligation of a Smal-digested cat–rpsL cassette from pDRH265 (Hendrixson et al., 2001) or a Smal-digested kan–rpsL cassette from pDRH437 into an appropriate restriction site to create plasmids used to electroporate 81-176 SmR (DRH212) or 81-176 ΔastA (see Table S1 in Supplementary material). Because of the lack of suitable restriction sites in flhF, pUC19 harbouring flhF (pDRH416) was subjected to site-specific PCR mutagenesis (Makarova et al., 2000) to create an EcoRV site 703 bp into the flhF coding sequence. This plasmid, pDRH434, was then digested with EcoRV to insert the cat–rpsL cassette to interrupt flhF and create pDRH468. The electroporated plasmids replaced the chromosomal copies of the wild-type genes with the respective cat–rpsL- or kan–rpsL-interrupted genes. These strains were the intermediate strains that were used in the final step to create the defined deletion chromosomal mutations. Sequencing of the cloned flhP and flgE loci of C. jejuni 81-176 revealed significant differences compared with the respective genes in C. jejuni NCTC11168. The 81-176 flhP and flgE sequences have been deposited in GenBank with the accession numbers Y1277719 and Y1277720 respectively.

Because of difficulty in cloning flgS and because of the lack of suitable restriction sites in flhB, SOEing reactions (Higuchi, 1990) were performed to generate fusions of the upstream and downstream DNA regions of flgS and flhB. In creating the fusions, an in frame Stul restriction site was created to link the two DNA segments. For flgS, ~750 bp of upstream and downstream DNA sequence was fused with a Stul site at the junction of the fusion. This fragment replaced the entire coding sequence of flgS with the Stul site. The fragment was cloned as a BamHI fragment into pBR322 to create pDRH425. The Smal-digested cat–rpsL cassette was cloned into Stul-digested pDRH425 to create pDRH426. For flhB, a 1.5 kb SOEing product was generated that fused an in frame Stul site linking the start codon to codon 365, deleting the intervening coding sequence of flhB. This fragment was cloned as a BamHI fragment into pUC19 to create pDRH742. The Smal-digested cat–rpsL cassette was cloned into Stul-digested pDRH742 to create pDRH781. Plasmid pDRH781 was electroporated into C. jejuni 81-176 SmR (DRH212) or 81-176 ΔastA (DRH461) to replace flhB with flhB::cat–rpsL. Plasmid pDRH426 was electroporated into 81-176 SmR (DRH212) to replace flgS with cat–rpsL. These strains were the intermediate strains that were used in the final step to create the defined deletion chromosomal mutations.

SOEing reactions (Higuchi, 1990) or deletion mutagenesis PCR (Makarova et al., 2000) were performed to generate fusions of the upstream and downstream DNA segments surrounding flgR, flhA, flhF, flhP, flhR, flgE and flgM. Each deletion fragment was cloned into pUC19 (the constructed plasmids are described in Table S1 in Supplementary material). These plasmids were electroporated into the respective 81-176 SmR (DRH212) and 81-176 ΔastA (DRH461) intermediate strains to replace the cat–rpsL mutations with the deletion constructs. Transformants were recovered on MH agar containing 0.5, 1 or 2 mg ml−1 streptomycin and then selected for chloramphenicol sensitivity on MH agar containing 15 μg ml−1 chloramphenicol. All mutants were verified for deletion of appropriate genes by colony PCR.

To delete astA in DRH311 (81-176 ΔflIA), DRH321 (81-176 ΔapoN) and DRH460 (81-176 ΔflgS), DRH311 and DRH321 were first electroporated with pDRH424 to replace astA with astA::cat–rpsL, and DRH460 was electroporated with pDRH444 to replace astA with astA::kan–rpsL. The obtained mutants were then electroporated with pDRH449 to replace the insertionally inactivated astA with the astA deletion construct as described above.

To generate complementing plasmids for C. jejuni to express genes from the C. jejuni chloramphenicol acetyltransferase (cat) promoter, the coding sequences from the second codon to the stop codon of rpoN, flIA, flgR and flgM were amplified by PCR from the chromosomal DNA of C. jejuni strain 81-176. Primers used for the amplification contained 5′ BamHI restriction sites in frame to codon 2 and the stop codon of each gene. The amplified fragments were purified and cloned into the BamHI site of pCECO102 (Wiesner et al., 2003). Plasmids were screened to ensure that each fragment was inserted into the correct orientation in the plasmid allowing for expression of the genes from the cat promoter. In each plasmid, expression of the gene results in a protein encoding a methionine, followed by a glycine and a serine (resulting from the codons provided by the in frame BamHI site), and then followed by the amino acids encoded by codons 2 to the stop codon of each gene. Each plasmid
Construction of the astA–kan reporter gene cassette and the nemo transposon

To construct an astA–kan cassette, aphA-3 (encoding kanamycin resistance) was purified as a Smal fragment from plL600 and ligated into HincII-digested pUC19 to create pDRH371. Primers containing 5’ PstI restriction sites were constructed to amplify a promoterless astA fragment from the chromosome of C. jejuni 81-176. This fragment extended from 14 bp upstream of the start codon to the stop codon of astA and retained the ribosomal binding site of the gene. This fragment was digested with PstI and ligated into PstI-digested pDRH371 to create pDRH466. The astA–kan fragment was then amplified by PCR with primers containing 5’ MluI sites and ligated into MluI-digested pFalcon (Hendrixson et al., 2001). Digestion of pFalcon with MluI followed by ligation of the MluI-digested astA–kan cassette replaced the kan cassette in the solo transposon backbone with the astA–kan cassette, creating pNautilus harbouring the transposon nemo.

To insert nemo into flgE2, a 4.2 kb fragment harbouring flgDE2 was amplified by PCR from the chromosome of strain C. jejuni 81-176 with primers containing 5’ KpnI sites. This fragment was digested with KpnI and ligated into KpnI-digested pUC19 to create pDRH351. Himar1 C9 transposase was purified from DH5α/pMALC9 (Akerley and Lampe, 2002). Transposition reactions contained 1 µg of purified pNautilus, 2 µg of pDRH351 and 500 ng of purified Himar1 C9 transposase in a total volume of 80 µl as described previously (Hendrixson et al., 2001). Plasmid DNA was purified by phenol–chloroform extraction, ethanol precipitated and transformed into DH5α. One plasmid, pDRH532, was recovered that contained nemo inserted 515 bp downstream of the flgE2 start codon in the correct orientation to generate a flgDE2::astA transcriptional fusion. This plasmid was then electroporated into 81-176 ΔastA (DRH461), replacing flgDE2 with flgDE2::nemo to generate the reporter strain 81-176 ΔastA flgDE2::nemo (DRH533).

Isolation and identification of mutants defective for transcription of flgDE2::astA

The picard transposon was amplified from pEnterprise (Hendrixson et al., 2001) by PCR using primers that added 5’ Pmel restriction sites. The amplified fragment contained picard with 98 bp of 5’ sequence and 217 bp of 3’ sequence. After digestion of pUC19 with EcoRI and HindIII, blunt ends were generated by filling in the overhangs generated by restriction enzyme digestion with T4 DNA polymerase (Invitrogen), and the Pmel-digested PCR fragment containing picard was ligated into the plasmid to generate pEnterprise2. Chromosomal DNA from strain DRH533 was purified and used in 39 individual in vitro transposition reactions containing 2 µg of chromosomal DNA, 1 µg of pEnterprise2 (harbouring the picard transposon) and 500 ng of purified Himar1 C9 transposase. Transposition reactions and repair and transposition reactions were performed as above and as described previously (Hendrixson et al., 2001). Approximately 14,000 transformants were recovered on MH agar containing 15 µg ml⁻¹ chloramphenicol and 100 µg ml⁻¹ XS. C. jejuni mutants defective for transcription of flgDE2::astA were identified as white colonies after growth on this agar. The sequence surrounding the picard transposon in each mutant was determined by direct sequencing of DNA from chromosomal preparations or by inverse PCR (Ochman et al., 1988), followed by sequencing of the generated PCR products.

Construction of chromosomal transcriptional reporter gene fusions

To create a convenient astA–kan cassette for cloning into various genes to create functional astA transcriptional fusion constructs, astA–kan from pDRH466 was amplified by PCR with primers that contained 5’ Smal restriction sites. The amplified DNA was purified, digested with Smal and ligated into Smal-digested pUC19 to create pDRH580.

Chromosomal DNA from C. jejuni strain 81-176 was used to amplify by PCR a 2.1 kb fragment containing flaA and a 4.3 kb fragment containing flaAB. For each fragment, specific primers were used that contained 5’ BamHI sites. Each fragment was purified, digested with BamHI and ligated to BamHI-digested pUC19. The plasmid containing flaA was designated pDRH517, and the plasmid containing flaAB was designated pDRH519. The Smal-digested astA–kan cassette from pDRH580 was then ligated into EcoRV-digested pDRH517 and HpaI-digested pDRH519 to insert the cassette into the coding sequence of flaA and flaB respectively. The constructs were screened to ensure that the astA–kan cassette was inserted into the correct orientation to create functional transcriptional fusions of flaA and flaB to astA. The plasmid containing the flaA::astA fusion was designated pDRH608, and the plasmid containing the flaB::astA fusion was designated pDRH610. Each plasmid was purified and electroporated into appropriate C. jejuni ΔastA mutants to replace flaA or flaB with the respective transcriptional reporter gene constructs.

Motility assays

To examine the motility phenotype of various C. jejuni 81-176 derivatives, strains were streaked on MH agar (or MH agar containing 15 µg ml⁻¹ chloramphenicol when appropriate) and grown at 37°C under microaerophilic conditions for 48 h. Each strain was then restreaked heavily onto three appropriate MH agar plates and grown at 37°C under microaerophilic conditions for 16 h. Each strain was resuspended and diluted in MH broth to an OD₆₀₀ of 0.7 and then stabbed with a needle into MH motility media containing 0.4% agar. Motility phenotypes were examined after incubation of plasmids at 37°C under microaerophilic conditions for 48 h.

Transmission electron microscopy

To examine the presence of flagella on C. jejuni 81-176 derivatives, strains were grown on MH agar for 48 h at 37°C
under microaerophilic conditions. Nickel-coated copper grids were soaked in 0.1% poly L-lysine for 1 min, and then excess poly L-lysine was removed. The copper side of each grid was touched to a colony on an MH agar plate, and the grid was then soaked in 4% paraformaldehyde containing 0.2% gluteraldehyde solution for 1 min. Grids were soaked twice in water for 30 s and then stained in 1% uranyl acetate for 1 min. The grids were visualized with a Philips CM100 transmission electron microscope at 60 kV.

Arylsulphatase assays

Arylsulphatase in whole cells of *C. jejuni* was analysed by protocols based on the methods of Henderson and Milazzo (1979) and Yao and Guerry (1996). *C. jejuni* strains were streaked on MH agar and grown at 37°C under microaerophilic conditions for 48 h. Each strain was then streaked heavily onto three separate MH agar plates and grown for 16 h at 37°C under microaerophilic conditions. Each plate was considered as a different sample for each strain so that each strain was tested in triplicate in each assay. Growth from each plate was resuspended in PBS to an OD$_{600}$ between 0.6 and 1. Each sample was then divided into two 1 ml aliquots. One aliquot was washed once in arylsulphatase buffer 1 (AB1: 0.1 M Tris, pH 7.2) and then resuspended in 1 ml of AB1. The other aliquot was washed once in arylsulphatase buffer 2 (AB2: 2 mM tyramine, 0.1 M Tris, pH 7.2) and then resuspended in 1 ml of AB2. Two hundred microlitres of each sample was then added to 200 µl of freshly prepared arylsulphatase buffer 3 (AB3; 20 mM nitrophenylsulphate, 0.1 M Tris, pH 7.2). Reactions were incubated for 1 h at 37°C and then stopped by the addition of 800 µl of 0.2 N NaOH. To measure arylsulphatase activity, 1 ml of each sample was measured spectrophotometrically at OD$_{410}$ to obtain a reading for the amount of nitrophenol released. The samples resuspended in AB1 served as blanks for the respective samples resuspended in AB2 when arylsulphatase activity was measured spectrophotometrically. To determine the amount of nitrophenol released, values were compared to a standard curve of OD$_{410}$ readings from known concentrations of nitrophenol. One arylsulphatase unit is defined as the amount of enzyme catalysing the release of 1 nmol of nitrophenol h$^{-1}$ per OD$_{600}$ of 1.

RT-PCR and primer extension analysis

Purification of RNA using Trizol reagent (Invitrogen) and RT-PCR using random primers and Superscript II reverse transcriptase (Invitrogen) was performed according to the manufacturer’s instructions and as described previously (Hendrixson et al., 2001). Amplification of products in the subsequent PCRs involved the use of specific primers for flaA, flagE2 and rpoA. For primer extension reactions, a primer for flaA (5’-AGCATCTAAACTTCTGATTAGCAT-3’) binding 81 bp downstream of the start codon was end labelled with $[y^{-32}P]$-ATP by T4 DNA kinase (Invitrogen). Primer extension reactions were performed as described previously using 11 µg of RNA (Higgins and DiRita, 1994). Products from the primer extension reactions were run on a 6% polyacrylamide sequencing gel alongside a sequencing ladder (generated using the Thermo Sequenase radiolabelled terminator cycle sequencing kit; USB Corporation) of flaA from pDRH517 using the above flaA primer.

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Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/mmi3731/mmi3731sm.htm

Table S1. Bacterial strains and plasmids used in this study.

References


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