Colonization of chicks by motility mutants of *Campylobacter jejuni* demonstrates the importance of flagellin A expression

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***Campylobacter jejuni* strain 81116 contains two flagellin genes, *flaA* and *flaB*. Wild-type (WT) bacteria express *flaA* only, but *flaB* can be expressed under certain conditions. We have determined the importance of flagella for colonization of the avian caecum, which appears to be the natural environment for these bacteria. Mutants in which *flaA* or *flaB*, or both had been inactivated, and motility variants, were investigated. Flagella are not a requisite for colonization, but mutants lacking both flagellin genes colonized less efficiently than WT. Inactivation of the *flaB* gene, which had no effect on bacterial motility, enhanced chicken caecal colonization 1000-fold compared to WT. A variant (SF-1) with flagella composed of flagellin A, but with poor motility, also colonized better than WT. Conversely, mutants with an inactivated *flaA* gene colonized 100- to 1000-fold less efficiently than WT, regardless of their motility conferred by truncated or full-length flagellin B flagella. These results suggest that the presence of flagellin A, rather than motility, is essential for optimal bacterial colonization of chicken caeca.**

Introduction

Campylobacter jejuni infection is a major cause of diarrhoea in humans (Blaser & Reller, 1981). In contrast, the organism is a commensal of poultry, from which it enters the human food chain (Harris *et al.*, 1986; Stern *et al.*, 1988; Shane, 1992). In order to reduce the prevalence of *C. jejuni* in chickens, and thus decrease the risk of human infection, a better understanding of the factors involved in avian colonization is needed. Successful colonization involves a complex interaction between the host and bacterium. For *Campylobacter* colonization in the chicken, the factors involved in such interactions can only be speculative. However, a chicken colonization model has recently been developed (Stern *et al.*, 1988), which may allow bacterial colonization factors to be identified (Meinersmann, 1990).

The pathogenesis of campylobacteriosis is also largely unknown, but several virulence factors have been identified including cytotoxins (Guerrant *et al.*, 1987; Mahajan & Rodgers, 1990), adhesins (De Melo & Pechère, 1990) and flagella (Newell *et al.*, 1985; Morooka *et al.*, 1985). The flagella, which confer motility upon the bacteria, are, to date, the most investigated virulence factor. *In vitro* studies have established a role for flagella in invasion (Wassenaar *et al.*, 1991), possibly via adherence (Newell *et al.*, 1985). Furthermore, the importance of motility in colonization has been demonstrated in several animal models, including mice (Morooka *et al.*, 1985; Newell *et al.*, 1985), rabbits (Caldwell *et al.*, 1985; Pavlovskis *et al.*, 1991) and hamsters (Aguero-Rosenfeld *et al.*, 1990). Volunteer experiments also suggest a role for flagella in human infection (Black *et al.*, 1988), but as yet the role in chicken colonization has not been investigated.

Both *C. jejuni* and *C. coli*, possess two flagellin genes, *flaA* and *flaB* (Nuijten *et al.*, 1990; Guerry *et al.*, 1990). In *C. coli* strain VC167, both flagellin genes are expressed and their products are incorporated together into flagella, with flagellin A as the major component (Guerry *et al.*, 1990, 1991). In contrast, *C. jejuni* strain 81116 is able to produce two types of flagella, depending on which flagellin gene is expressed. In the wild-type (WT) bacteria of this strain, only *flaA* expression is detectable (Nuijten *et al.*, 1990). The *flaB* gene of this strain can be inactivated without loss of motility or invasiveness, as determined *in vitro* (Wassenaar *et al.*, 1991). In contrast, the inactivation of *flaA* leads to a 100-fold decrease in these properties. This suggests that the two types of

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Abbreviations: WT, wild-type; CC, caecal contents.

Table 1. *A comparison of motility, invasive properties in vitro and colonization in vivo of the C. jejuni 81116 strains used in this study.*

Motility was determined on motility media. The genotype was determined by Southern blotting (Wassenaar *et al.*, 1991). The nature of the flagellin was determined on Western blots using monoclonal antibody specific for flagellin A (Nuijten *et al.*, 1991) and polyclonal antibody specific for flagellins A and B (Nuijten *et al.*, 1989; Wassenaar *et al.*, 1991). The average level of colonization of chicks dosed with approximately 5×10^3 or 10^5 c.f.u. is given. ND, Not determined.

Strain	Motility	Genotype	Composition of flagella	*In vivo* colonization: dosed at 5×10^3	*In vivo* colonization: dosed at 10^5
WT	+++	A^+B^+	FlaA*	10^6	10^9
R1	+	A^-B^+	FlaB†	ND	10^6
R1-V2	++	A^-B^+	FlaB	ND	10^4
R2	−	A^-B^-	None	ND	10^7
R3	+++	A^+B^-	FlaA	10^9	ND
SF-1	++	A^+B^+	FlaA*	10^9	ND

*No flagellin B was detectable, but the presence of minor amounts cannot be excluded.
†R1 produces truncated flagella, consisting of flagellin B.

flagella produced by *C. jejuni* strain 81116 have markedly different biological properties. The importance of these two types of flagella for survival in a natural environment is still unknown. To establish their role in colonization of the avian caecum, isogenic mutants expressing *flaA*, *flaB* or neither gene were investigated in the chick model.

Methods

Bacterial strains. *C. jejuni* 81116 has been extensively passaged *in vitro* since its isolation from a patient suffering from diarrhoea (Palmer *et al.*, 1983). Fully motile colonies from thioglycollate plates containing 0·4% agar (motility media) (Caldwell *et al.*, 1985), were cloned to purity and designated as wild-type (WT). Flagellin mutants R1, R2 and R3 were produced by homologous recombination with a vector containing a kanamycin-resistance gene flanked by flagellin sequences (Wassenaar *et al.*, 1991). Mutant R1 has an inactivated *flaA* gene and flagellin B is assembled into truncated flagella. Mutant R2 is a deletion mutant lacking part of *flaA* and *flaB*, and, therefore, has no remaining functional flagellin gene. Mutant R3 lacks a functional *flaB* gene and is completely motile by means of flagella consisting of flagellin A. Mutant R1-V2 is a spontaneous variant of R1 that has partly regained motility, due to a transcriptional upshift of *flaB* (T. M. Wassenaar and others, unpublished). SF-1 is a spontaneous mutant of WT that was originally described as a non-motile mutant, since it forms pin-point colonies in motility plates containing 0·7% agar (Newell *et al.*, 1984). However, in thioglycollate plates containing 0·4% agar this variant has a motility comparable to that of R1-V2. The properties of the WT strain and all mutants of *C. jejuni* 81116 used in this study are summarized in Table 1.

Culture conditions. All strains were cultured on Skirrow's selective media (Skirrow, 1977) with 10% (v/v) sheep blood at 42 °C in an atmosphere of 7% CO_2/8% O_2/85% N_2. For kanamycin selection, plates were supplemented with 30 μg kanamycin ml^{-1}. For *Campylobacter* selection from chicken caecal contents 30 μg cephaperazone ml^{-1} was added to suppress normal caecal flora. In each experiment, bacterial motility was monitored prior to inoculation and after colonization by colony size in motility media.

Infection of chickens. Chicks were colonized as previously described by Stern *et al.* (1988). Eggs from a Torbay 9 flock (Wickham Laboratories, UK) were hatched in isolators. Groups of six to ten birds were maintained in isolators and provided with unlimited food and water. *Campylobacter* strains were cultured overnight, harvested in phosphate-buffered saline (PBS; 6·5 mM-Na_2HPO_4, 1·5 mM-KH_2PO_4, 137 mM-NaCl, 1·5 mM-KCl, pH 7·3), and washed and diluted in PBS. Doses were estimated by measuring the A_{280} of an aliquot of lysed cells and determined against a standard curve. The actual doses were measured by performing viable counts on blood agar plates. Approximately 24 h after hatching, chicks were dosed by gavage with 0·1 ml bacterial suspensions or 0·1 ml PBS; 5 d after this inoculation, the birds were sacrificed by cervical dislocation. The chicks were weighed, the abdomen skinned and the abdominal contents gently removed to observe gross pathology. The contents (CC) of one caecum were gently extruded, weighed and diluted to give 0·1 g CC ml^{-1} in PBS. The weight of the caecal contents varied from 0·1 to 0·7 g. Sequential dilutions were plated out on selective blood agar plates, with and without kanamycin, and cultured for 48 h to give viable colony counts. In addition, dilutions were cultured in motility medium. The native micro-aerophilic faecal flora were quantified by performing viability counts on blood agar plates without antibiotics.

Results

The colonization dose response (of between 54 and 5×10^5 bacteria) in 1-d-old chicks was determined for *C. jejuni* 81116 WT (Fig. 1). Six groups of chicks were infected with various doses of bacteria and the level of colonization was determined after 5 d. No *Campylobacter* colonization was detectable in any of the control birds dosed with PBS. An infective dose of 54 colony-forming units (c.f.u.) *Campylobacter* per chick colonized three out of eight animals. The detection level of colonization was 10^2 c.f.u. (g CC)$^{-1}$. As expected, there was a direct relationship between infective dose and colonization levels. All chicks dosed with 5×10^3 c.f.u. or more were infected. Neither weight loss nor gross

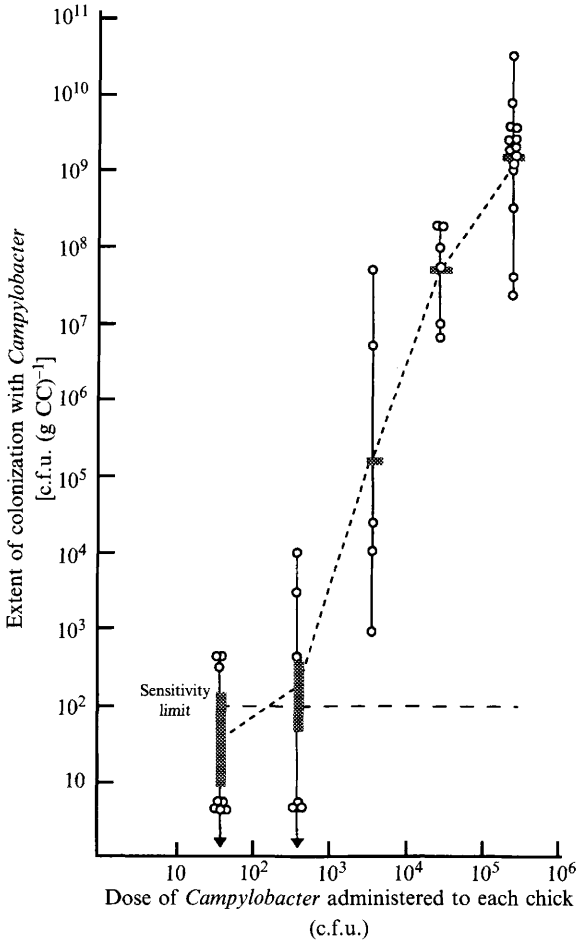

Fig. 1. Caecal colonization of chicks orally infected with various numbers of *C. jejuni* WT. Colonization of each chick with *Campylobacter* (○) is given per g CC 6 d post-infection. The geometric mean of the colonization levels of each group (▩) and mean infective dose response curve (------) are given. In the first two groups a further five and three chicks, respectively, had fewer than 10^2 c.f.u. (g CC)$^{-1}$, which is the sensitivity limit. The means of these groups were therefore calculated with the maximum variation of 0 to 99 c.f.u. (g CC)$^{-1}$ for these chicks.

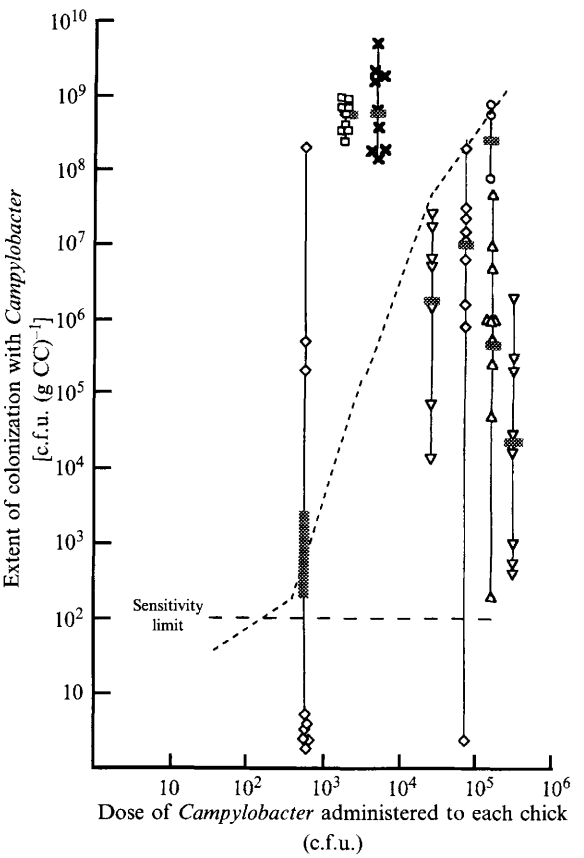

Fig. 2. Caecal colonization of chicks orally infected with WT (○), SF-1 (□), R3 (×), R2 (◇), R1 (△) and R1-V2 ▽). Chicks were infected with R2 and R1-V2 at two different doses. Colonization of each chick with *Campylobacter* is given per g CC 6 d post-infection. The geometric means are given (▩). For comparison, the mean values (------) obtained with various doses of WT, as determined in Fig. 1, is given.

pathological changes were observed in the colonized chicks.

The presence of *Campylobacter* did not lead to a decrease in the native micro-aerophilic microflora of the caecum, as determined by colony counts on plates without selective antibiotics. In the highest dosed groups, *Campylobacter* outnumbered the native flora by a factor of 100.

Chicks were inoculated with the motility mutants. The target dose for R1 and R2 was 10^5 c.f.u. per chick, which was above the 100% colonization dose for WT because these mutants were expected to colonize poorly. Mutant R3, which was expected to colonize as efficiently as WT, and variant SF1, which had been shown to colonize as well as WT in the infant mouse model (Newell *et al.*, 1985), had target doses of 10^3 c.f.u. per chick. Mutant R1-V2 was tested at two doses (4×10^4 and 2×10^5 c.f.u. per chick) because there was no information available about its colonization potential. For each experiment, the WT strain was included as a positive control at a dose of approximately 10^5 c.f.u. per chick. In each experiment the colonization potential of WT was reproducible. The ability of the mutants to colonize the chicks varied significantly (Fig. 2). Mutant R2, lacking both flagellin genes (Table 1), colonized only slightly less efficiently than WT. To determine whether this relatively high colonization level was due to the high dose, R2 was also tested at the lower dose of 10^3 c.f.u. per chick. This resulted in a mean colonization level equal to that of WT, but only three out of nine birds were colonized. Mutant R3, lacking a *flaB* gene, colonized chicks 1000 times more efficiently than WT at a dose of 5×10^3 c.f.u. per chick. Conversely, mutant R1, which produces truncated flagellin B flagella (Table 1), colonized chicks 1000 times less efficiently than WT. Similarly, the motility variant of R1 (R1-V2) colonized to about the same extent as R1

(between 10- and 10^4-fold less than WT), depending on the dose. Unlike WT, colonization by mutant R1-V2 apparently reached a maximum at a dose of $3{\cdot}4 \times 10^4$ c.f.u. per chick, since a dose of 2×10^5 c.f.u. per chick did not further increase colonization levels. Interestingly SF-1, which has a motility comparable to R1-V2, was able to colonize the chicken caeca much better, to the same extent as R3. Mutants R1 and R2 had a wider range of colonization than the other mutants or WT. The reasons for this are unknown.

All bacterial strains recovered from the caeca displayed the original colony morphology on motility media, indicating that there was no *in-vivo*-induced or selected change in motility. Variant R1-V2 can revert to the R1 phenotype at a low frequency *in vitro* (T. M. Wassenaar and others, unpublished). Nevertheless, R1-V2 and R1 recovered from caecal contents of chicks retained their original phenotype, so a switch in flagellin phenotype from R1 to R1-V2 or vice versa does not appear to be inducible by colonization.

Discussion

C. jejuni appears to be non-pathogenic in chickens, even though chicken caeca can be highly colonized (Stern *et al.*, 1988; Shane, 1992). The bacterial factors involved in the colonization of the avian caeca are unknown. However, it seemed likely that effective motility is a prerequisite of survival in such a mucoid micro-environment (Lee *et al.*, 1986). Motility in *Campylobacter* is mediated by the products of two flagellin genes, *flaA* and *flaB*. In this study, we made use of isogenic mutants of *C. jejuni* 81116 to differentiate between the relative importance for caecal colonization of motility and the mere presence of the two types of flagellin. Our results clearly demonstrate that flagellin expression and motility are not essential for colonization, although the nature of the flagellin expressed has a significant effect on the ability of the bacterium to colonize chicken caeca.

The lack of correlation between bacterial motility and avian caecal colonization (Table 1) is exemplified by the difference in motility between R1 and R1-V2, which is not reflected in their colonization capacity. Moreover, SF-1, displaying a motility similar to R1-V2, colonizes 1000-fold better than WT, and the completely non-motile mutant R2 colonizes as efficiently as R1. The finding that motility is not required for colonization is in contrast to data from previous animal model studies (Newell *et al.*, 1985; Morooka *et al.*, 1985; Pavlovskis *et al.*, 1991). Possible explanations for this anomaly are the differences in the animal models and the motility mutants used. Morooka *et al.* (1985) and Newell *et al.* (1985) used non-defined motility mutants and variants to demonstrate the importance of motility for colonization in a suckling mouse model. Such mutants may have had concomitant changes in other colonization factors. An isogenic aflagellate mutant was unable to colonize rabbits in either an oral or a RITARD model (Pavlovskis *et al.*, 1991). However, the artificial nature of the RITARD model, competition with pre-existing gut flora, and host and site differences in mucous composition, are likely reasons for the discrepancy.

The chicken colonization model is a reflection of the micro-environment that *C. jejuni* naturally encounters. The results obtained in this study indicate the relative unimportance of bacterial motility for survival in such a unique niche.

Although motility is not a prerequisite for colonization, the nature of the flagellin still has an effect on colonization potential. Mutants expressing flagellin B (R1 and R1-V2) display colonization levels that are at least 100-fold lower than WT, SF1 and R3, all expressing flagellin A.

The expression of flagellin B by *C. jejuni* is apparently not advantageous to caecal colonization. Flagellin mutant R3, lacking the *flaB* gene, colonizes much more efficiently than WT. This was unexpected since *flaB* expression was undetectable in W1 (Nuijten *et al.*, 1990). Nevertheless, this anomaly could be explained by a low expression level of *flaB* in WT, which in some undefined way affects colonization potential. Another, more likely, explanation is that inactivation of *flaB* may influence the activity of other, as yet unidentified, colonization factors.

The mutants expressing flagellin B used in this study contain an inactivated *flaA* gene. However, we have observed that culturing wild-type *C. jejuni* 81116 *in vitro* can result in a switch to flagellin B expression (T. M. Wassenaar and others, unpublished). It can therefore be hypothesized that bacteria surviving an *in vitro* environment partly express flagellin B. The colonization potential of R1 and R1-V2 suggests that such bacteria are less able to colonize chickens than bacteria expressing flagellin A.

The importance of factors other than flagella in the colonization of chicken caeca remains to be investigated. Some *Campylobacter* strains undergo antigenic changes during chicken colonization (Meinersmann *et al.*, 1990), indicating that novel bacterial components are expressed during colonization. The occurrence of such antigenic changes, which could be due to the selection of pre-existing mutants or the induction of genes coding for these components, is currently under investigation.

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