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Reversible expression of flagella in *Campylobacter* spp.

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1. SUMMARY

The in vitro phase variation of flagella and the transition rates between flagellate and aflagellate phenotypes in *Campylobacter* species including *C. jejuni, C. coli, C. lari* (thermophilic campylobacters), *C. fetus* subsp. *fetus, C. fetus* subsp. *venerealis* and *C. hyointestinalis* were investigated. The change from the flagellate to aflagellate phenotype was detected in all of the 12 *Campylobacter* strains studied. When measured in a motility medium, flagellate to aflagellate transition in thermophilic campylobacters, *C. fetus* and *C. hyointestinalis* strains occurred at a rate of 1.8×10^{-3} to 7.5×10^{-3}, 3.0×10^{-4} to 7.8×10^{-4} and 1.8×10^{-5} to 7.7×10^{-6} per cell per generation, respectively. Transition from aflagellate to flagellate phenotype occurred at a rate of 5.8×10^{-6} to 9.3×10^{-6} per cell per generation in thermophilic campylobacters and 1.0×10^{-6} to 1.5×10^{-6} in *C. fetus* strains. No reversion from aflagellate to flagellate phenotype could be detected in *C. hyointestinalis* strains. It was concluded that the ability to reversibly express flagella was inherent in the wild-type strains and the transition rates for both directions were consistent for each strain.

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2. INTRODUCTION

Campylobacter species are important pathogens in man and animals [1,2]. These organisms have polar flagella which confer a characteristic darting motility. Experimental evidence suggests that flagella have an important role in the pathogenesis of *Campylobacter* infections [3,4] and contribute significantly to serotyping schemes based on heat-labile antigens [5]. Some strains of campylobacter have been shown to undergo both phase and antigenic variations of flagella [6–8]. Phase variation refers to a bidirectional transition between flagellate (Fla^+) and aflagellate (Fla^-) phenotypes. Reversible expression of flagella in *C. jejuni* is regulated at transcriptional level and influenced by the environment of bacteria [9]. When both motility phenotypes (Fla^+ and Fla^-) of *C. jejuni* are used to infect either rabbits [6], hamsters [10] or humans [11], only flagellate or-

ganisms can be recovered from faecal samples. When measured in culture medium, however, transition from flagellate to aflagellate form is more frequent [6,10].

Flagellar phase variation has been described only for *C. jejuni*. However, our preliminary observations indicated that flagellar phase variations occurred among other *Campylobacter* species. We therefore investigated the in vitro phase variations of flagella in *C. jejuni* and other pathogenic *Campylobacter* species and the transition rates between flagellate and aflagellate phenotypes.

3. MATERIALS AND METHODS

3.1. Bacterial strains and growth conditions

The *Campylobacter* species used in this study were originally isolated from humans and animals. *C. jejuni* BE119, NA167 and *C. coli* HT42 were isolated from faeces of diarrhoeic children. *C. jejuni* CF48, *C. coli* CF49 and *C. lari* CF80 were isolated from faeces of chickens. *C. fetus* subsp. *fetus* OA5 and OA15 were isolated from aborted ovine fetuses and *C. fetus* subsp. *venerealis* BA1 and BA2 from aborted bovine fetuses. *C. hyointestinalis* CE1 and CE9 were isolated from calves with enteritis. Wild-type strains were flagellate and motile. Strains were kept frozen in Brucella broth containing 15% (v/v) glycerol at −70°C. Cultures were grown on blood agar plates at 37°C in an atmosphere containing 5% oxygen and 10% carbon dioxide.

3.2. Selection of aflagellated variants

The non-flagellated variants were selected according to the method of Newell et al. [12]. Briefly, aflagellate organisms were enriched in the wild-type population by sequential subculture from the centre of semi-solid nutrient gelatin agar (1% (w/v) peptone, 0.33% (w/v) yeast extract, 0.5% (w/v) sodium chloride, 0.8% (w/v) gelatin and 0.75% (w/v) agar) stab cultures. Aflagellate colonies were identified by the type of growth on motility medium (thioglycolate medium supplemented with 0.33% (w/v) agar), confirmed by dark-field microscopy and by light microscopy with flagella staining [13]. The spreading colonies composed of flagellate organisms were readily distinguished from the pin-point, compact colonies formed by aflagellate organisms. Aflagellate

Table 1

Variation of motility phenotypes in *Campylobacter* spp.

Strain			Fla$^+$ to Fla$^-$ transition			Fla$^-$ to Fla$^+$ transition		
			No. of variants	No. of cells examined [a]	Transition rate [b]	No. of variants	No. of cells examined [a]	Transition rate [b]
C. jejuni	BE119		3780	8841	7.5×10^{-3}	11	26687	7.2×10^{-6}
	NA167		2340	10260	4.0×10^{-3}	14	27914	8.7×10^{-6}
	CF48		2743	8445	5.7×10^{-3}	15	28021	9.3×10^{-6}
E. coli	HT42		975	9120	1.8×10^{-3}	10	28347	6.1×10^{-6}
	CF49		2721	9258	5.1×10^{-3}	14	27572	8.9×10^{-6}
C. lari	CF80		1742	10116	3.0×10^{-3}	9	26940	5.8×10^{-6}
C. fetus (subsp. *fetus*)		OA5	272	9636	4.9×10^{-4}	4	52862	1.3×10^{-6}
		OA15	440	9810	7.8×10^{-4}	5	56663	1.5×10^{-6}
(subsp. *venerealis*)		BA1	158	8475	3.2×10^{-4}	4	57115	1.2×10^{-6}
		BA2	195	11200	3.0×10^{-4}	3	52433	1.0×10^{-6}
C. hyointestinalis		CE1	13	12140	1.8×10^{-5}	0	102265	0 [c]
		CE9	6	13522	7.7×10^{-6}	0	101678	0 [c]

[a] The numbers for the transitions are the totals from three separate experiments.

[b] The ratio of the number of variants to the total number of cells plated gave the fraction of cells which had undergone transition. This ratio was divided by the approximate number of generations the population had undergone to yield the transition rate.

[c] In the limit of this experiment transition could not be detected; this does not mean that transition does not exist. The frequency of transition might be too low to be detected in the limit of colony number counted.

variants were subcloned at least six times through motility agar to confirm their phenotypic stability.

3.3. Rate of flagella phase variation

A pour plate technique was used to determine the flagella phenotype of large numbers of organisms [6]. Thioglycolate medium supplemented with 0.33% agar was autoclaved and cooled to 45°C. A 24-h culture of given flagellar phenotype was suspended in sterile saline, diluted and added to the motility medium to achieve a final density of approximately 200–400 colonies per plate. The plates were allowed to solidify at room temperature for 2 h and were incubated at 37°C for 48 h. Both colony types were counted and variants were examined by flagellar stain [13] for confirmation. The transition experiments were performed in triplicate. Three clones of a single variant were used to measure transition in triplicate experiments. As the final passage, sufficient bacteria were plated to examine at least 8000 colonies per strain per Fla^+ to Fla^- experiment and at least 26 000 colonies per strain per Fla^- to Fla^+ experiment. The ratio of the number of variants to the total number of cells counted in triplicate experiments gave the fraction of cells which had undergone transition. This ratio was divided by the approximate number of generations that the population had undergone to yield the transition rate.

4. RESULTS AND DISCUSSION

Phenotypic analysis of the randomly selected colonies of the wild-type and the variants of each strain showed that all phenotypic characteristics (more than 30 including antibiotic susceptibility) of wild-type and variant strains were identical except for flagellar expression. The fact that generation time was the same for both Fla^+ and Fla^- organisms, indicated that the apparent rate of phase variation was not influenced by differences in growth rates of the two phases.

An overview of results is shown in Table 1. The change from the flagellate to aflagellate phenotype could be detected in all *Campylobacter* strains studied. These data suggested that the ability to reversibly express flagella was inherent in the wild type strains. The transition rates obtained from triplicate experiments were similar. This suggested that flagellar phase variation was consistent for each strain. When measured in motility medium, the Fla^+ to Fla^- transition in thermophilic campylobacters (*C. jejuni, C. coli* and *C. lari*), *C. fetus* (subsp. *fetus* and subsp. *venerealis*) and *C. hyointestinalis* occurred at a rate of 1.8×10^{-3} to 7.5×10^{-3}, 3.0×10^{-4} to 7.8×10^{-4} and 1.8×10^{-5} to 7.7×10^{-6} per cell per generation, respectively. The transition from Fla^- to Fla^+ was less frequent. This type of transition occurred at a rate of 5.8×10^{-6} to 9.3×10^{-6} per cell per generation in thermophilic campylobacter strains and 1.0×10^{-6} to 1.5×10^{-6} per cell per generation in *C. fetus* strains. However, no in vitro reversion from Fla^- to Fla^+ phenotype could be detected in two *C. hyointestinalis* strains when examining up to 100 000 colonies for each strain. This finding, however, does not mean that this transition does not exist. Although, the mechanism for flagellar phase variation in campylobacters is not known, point mutation in the *fla* gene, or an accessory gene, instead of reversible gene regulation can explain the non-detectable reversion to the Fla^+ state in *C. hyointestinalis*. Alternatively, the frequency reversion may be too low to be detected by the method applied.

It was interesting to note the differences in transition rates among particular groups or species of *Campylobacter*. The transition rates of certain strains which were close systematically to each other, accumulated in particular ranges. Our suggestion is compatible with those of *Salmonella* flagellar phase variation reported previously, in which the phase changes are on the order of 10^{-3} to 10^{-5} per cell per generation for some strains of *S. typhimurium* but may be as low as 10^{-7} for strains of *S. abortus-equi* [14,15].

Although there have been several reports on the instability of certain traits in campylobacters, few in vitro studies have been conducted to determine the reversible expression of flagella in these organisms. Caldwell et al. [6] have reported that some strains of *C. jejuni* undergo a bidirectional transition between Fla^+ and Fla^- pheno-

types. Aguero-Rosenfeld et al. [10] have observed the variation only in the Fla^+ to Fla^- direction in a *C. jejuni* strain. However, Harris et al. [7] did not detect Fla^+ to Fla^- transition in a strain of *C. coli*. Our findings confirmed the results of Caldwell et al. [6]. Similar phase variations have been described for various characteristics in *Neisseria gonorrhoeae* [16], *Bordetella pertussis* [17] and *Escherichia coli* [18].

The frequency of the shift from Fla^+ to Fla^- was very high but the frequency of the shift from Fla^- to Fla^+ was very low during growth in laboratory media. Previous studies have also showed that the prevalence of *Campylobacter* flagellar phase is influenced by the environment of the bacteria [6]; when isolated from living organisms as the natural habitat, *Campylobacter* spp. were almost always capable of forming flagella and Fla^- to Fla^+ expression was predominant during in vivo growth. Presumably, the ability to undergo such surface changes confers advantages to the pathogen as it encounters multivarious environments. In fact, thermophilic campylobacters which had the highest transition rate in both directions have the broadest host range in nature among the *Campylobacter* species. In contrast, *C. fetus* and *C. hyointestinalis* which had lower transition rates have relatively narrow host range. Thus, it may be proposed that phase variations in flagella play a role in the adaptation of *Campylobacter* spp. to grow in vitro. Furthermore, the demonstration of reversible flagellar expression may have a significant impact on the interpretation of results obtained with serotyping schemes based on heat-labile antigens.

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