Motility as a factor in the colonisation of gnotobiotic piglets by *Helicobacter pylori*

K. A. EATON, D. R. MORGAN* and S. KRAKOWKA

Department of Veterinary Pathobiology, College of Veterinary Medicine, The Ohio State University, Columbus, OH 43210 and *The Procter and Gamble Co., Miami Valley Laboratories, Cincinnati, OH 45239–8707, USA

Summary. Non-motile variants of *Helicobacter pylori* (strain 26695) occurred with a frequency of $1.6 \text{ (SD } 0.4) \times 10^{-4}$ variants/cell/division cycle, and reversion to the motile form occurred with a frequency of $< 10^{-7}$ variants/cell/division cycle. The two forms remained $> 90\%$ pure for up to 50 cell divisions and differed only in the presence or absence of motility and flagella. Bacteria were recovered from nine of 10 gnotobiotic piglets inoculated orally with motile *H. pylori*, but from only two of eight inoculated with the non-motile variant. The motile form survived for 21 days in infected piglets, but the non-motile variant survived for only 6 days. Bacteria recovered from piglets inoculated with the non-motile variant were non-motile. These data support the hypothesis that motility is a colonisation factor for *H. pylori*.

Introduction

Helicobacter pylori causes chronic active and lymphofollicular gastritis in human beings.¹ Experimentally, it causes lymphocytic gastritis in gnotobiotic piglets which closely resembles the human disease.^{2,3} This model has been used in studies of virulence and of colonisation factors,⁴ including urease production.⁵ The present report compares the colonising ability of motile and non-motile forms of a virulent strain of *H. pylori*.

Materials and methods

Bacteria

All bacteria used were derived from a human gastritis strain (26695) of *H. pylori*, and produced urease, catalase and oxidase. The strain had been maintained *in vitro* for approximately 4 years.

Cloning of motile and non-motile forms

Bacteria were inoculated into molten Brucella Broth (BBL, Cockeysville, MD, USA) with agar (Difco Laboratories, Detroit, MI, USA) 0.5% at 42°C. The medium was poured into petri dishes, allowed to solidify at room temperature, and incubated in CO₂

10% in air. This technique yielded clearly identifiable spreading and non-spreading colonies after incubation for 3 days at 37°C. Spreading colonies surrounded by a 1–5-mm halo contained motile populations assumed to have been derived from a single motile bacterium. Non-spreading colonies were *c.* 0.25 mm in diameter, lacked a halo, and contained non-motile populations, also assumed to have been derived from a single cell. Single spreading or non-spreading colonies were transferred by means of a sterile cotton-tipped swab to Brucella broth which, after incubation, was again inoculated into soft agar. From these cultures, single spreading or non-spreading colonies, as appropriate, were selected. This process was repeated five times to produce cloned populations containing either motile or non-motile organisms.

Inocula

The inocula for experimental animals were derived from motile or non-motile cloned cultures. Cloned populations were maintained in broth culture and used within 5–10 subcultures of cloning. Because strain 26695 had been maintained in culture for approximately 4 years before cloning, any slight difference between the number of subcultures undergone by the cloned motile and non-motile forms was unlikely to have been significant. Motility of the infecting strain and of the organisms recovered was quantitated by counting spreading and non-spreading colonies; bacteria were plated in soft agar and 100–500 colonies were counted. Populations with $\geq 30\%$ spreading colonies were considered motile, and those with $\leq 4\%$ non-motile.

Rate of variation

Spreading or non-spreading colonies from cloned populations were inoculated into Brucella broth. When each resulting culture contained *c.* 10^9 cfu/ml (as determined by direct counting in a haemocytometer chamber), a viable count was made by a standard plate technique and the number of division cycles (generations), *D*, was calculated according to the following formula: $D = [\log_{10}(x_2) - \log_{10}(x_1)] / \log_{10} 2$, where x_2 = the total number of bacteria in the final broth (cfu/ml $\times$ ml of culture). Since each colony in soft agar was presumed to be the progeny of a single bacterium, the starting number of bacteria, x_1 , was assumed to have been 1, and $\log_{10}(x_1) = 0$. Thus each broth culture was the result of approximately 30 division cycles. Portions of the broth culture were inoculated into soft agar in petri dishes to give a total of approximately 10000 colonies (200–500 colonies/plate) after incubation for 4–5 days in CO₂ 10%. Colonies were counted with an inverted phase contrast microscope at $\times 40$ magnification. Rate of variation, *R*, was calculated as follows: $R = V/T \times D$ variants/cell/division cycle, where *V* = the number of variant colonies (spreading colonies in a non-spreading population and non-spreading colonies in a spreading population), *T* = the total number of colonies counted and *D* = the number of division cycles. This gave an approximate value for *R*, based on the assumption that the populations counted were in exponential growth for most of their cycle and that the relative rate of proliferation of individual variants in the culture was uniform.

To determine the stability of motile populations, bacteria were maintained for up to 200 division cycles. Single colonies were selected and grown in Brucella broth. When the culture reached the late logarithmic phase the bacteria were counted, the percentage of spreading colonies was determined, and a portion of the culture was diluted 1 in 100 in 25 ml of fresh Brucella broth. The number of division cycles in each subculture was calculated as above, where x_1 was the number of bacteria in the broth immediately after dilution, and x_2 was the number of bacteria in the final culture. Each final culture was the result of *c.* 9–12 division cycles.

Electronmicroscopy

Bacteria were grown in Brucella broth, washed, stained with phosphotungstic acid and examined with a Phillips 300 transmission electronmicroscope, as previously described.⁴

Analytical methods

Profiles of total bacterial proteins were prepared and analysed by methods described previously.⁶

Briefly, the proteins were separated by electrophoresis on polyacrylamide 10% gels.⁷ Gels were scanned with an Ultrosan XL laser densitometer (LKB Pharmacia, Piscataway, NJ, USA). Protein patterns were compared by cluster analysis with a computer. The relatedness of bacterial strains was expressed as percentage similarity. Based on previous studies,⁶ bacterial strains with > 90% similarity were considered identical.

Restriction endonuclease analysis was performed as described previously.⁴ Bacterial DNA was isolated by protease digestion and ethanol precipitation. Purified DNA was incubated with *Hind*III and fragments were separated in an agarose 0.7% gel.

Animals

Eighteen outbred, Yorkshire cross piglets from five litters were obtained by caesarian section, maintained in sterile isolation units for 3 days, and inoculated orally with 10^9 cfu as described previously.² They were randomly divided into five experimental groups. Groups of six and two piglets received "non-motile clones" which contained, respectively, 0% and 4% of organisms giving rise to spreading colonies. Groups of three, four and three piglets received "motile clones" that contained, respectively, 30%, 63% and 74% of motile organisms. Five piglets given motile clones and three given non-motile clones were killed 6 days after inoculation and the remainder were killed 21 days after inoculation. At necropsy, fragments of gastric mucosa 1–2 mm in diameter from each of the four regions of the stomach (cardia, fundus, antrum and pylorus) were inoculated on to blood agar plates for the recovery of *H. pylori*. In addition, each stomach was divided longitudinally along the greater and lesser curvatures. One half of each stomach was weighed and the mucosa was removed and homogenised in sterile Brucella broth. Serial 10-fold dilutions of the homogenate were plated in duplicate for counting *H. pylori*. The extent of colonisation was expressed as cfu/g of gastric mucosa. The remaining half of each stomach was immersed in neutral buffered formalin 10% for histopathological examination.

Results

Bacterial motility and rate of variation

In three trials, the rate of variation from motile to non-motile for populations originating from a single spreading colony was 1.6 (SD 0.4) $\times 10^{-4}$ variants/cell/division cycle. In five trials, the rate of variation from non-motile to motile was $< 10^{-7}$ variants/cell/division cycle (only 0–3 motile revertant colonies out of approximately 10000 colonies counted in each trial). Because the initial reversion rate was low, motile

Fig. 1. Long-term reversion of motile *H. pylori*; points represent means of three separate determinations.

Fig. 2. Densitometer scan of SDS-PAGE of total bacterial protein of *H. pylori* variants: 26C, motile form; 26B, non-motile form; 26695, parental strain. All peaks are present in all strains.

populations contained > 90% motile organisms for 50–60 division cycles. However, after passage for > 100 division cycles motile variants reverted to a population giving rise to more non-spreading than spreading colonies (fig. 1).

Characteristics of motile and non-motile variants

Ultrastructural studies of non-motile *H. pylori* demonstrated the absence of the polar tuft of sheathed flagella seen in the motile form. Otherwise, the two variants were morphologically indistinguishable. SDS-PAGE of total bacterial protein from motile and non-motile variants revealed similar patterns, and densitometry scans of the polyacrylamide gels

demonstrated that the variants were > 90% similar (fig. 2). The restriction endonuclease patterns of the variants were indistinguishable (fig. 3).

Colonising ability of the variants

Of 10 piglets inoculated with motile *H. pylori* (group A), nine became infected as indicated by the recovery of *H. pylori* at necropsy (table). In contrast, only two out of eight piglets inoculated with non-motile *H. pylori* (group B) became infected. In group A, bacteria were recovered from piglets killed both 6 and 21 days after inoculation. However, in group B, bacteria were recovered only from piglets killed 6 days after in-

Fig. 3. *Hind*III restriction endonuclease analysis of motile and non-motile forms of *H. pylori*: A, non-motile form; B, motile form; C, parental strain 26695.

oculation. Furthermore, 6 days after inoculation, piglets infected with motile *H. pylori* differed from those given the non-motile variant in being colonised more heavily (table). In piglets given the motile form, there was no relation between the extent of colonisation and the proportion of spreading colonies that grew from the inoculum. Thus, the mean bacterial colonisation in piglets killed 21 days after receiving *H. pylori* with 30% motile cells was 1.5 (SD 1.8) $\times 10^5$ cfu/g of gastric mucosa; and of the two piglets given the 74% motile clone, one remained infected (8.4×10^2 cfu/g) 21 days after inoculation. Finally, motility of the recovered strains varied between the

groups (table). All of the isolates from group A piglets gave rise to a higher percentage of spreading colonies than did the inocula. In contrast, the bacteria recovered from the two group B piglets were non-motile (fig. 4).

Discussion

Like *Campylobacter jejuni*, *H. pylori* was able to change reversibly from a motile to a non-motile form. The rate of conversion from motile to non-motile was approximately 10-fold lower than both the rate reported for *C. jejuni*⁸ and the rate of flagellar phase variation reported for *Salmonella* spp.⁹ The rate of reversion from non-motile to motile was $< 10^{-7}$ variants/cell/division cycle and could not be measured precisely, but again was less than that reported for *C. jejuni*.⁸ Since the conversion rate from motile to non-motile was much greater than that from non-motile to motile, steady state populations would be expected to be largely non-motile. This is demonstrated by prolonged passage of populations derived from a single spreading colony.

Motile and non-motile variants did not differ ultrastructurally (except for the presence or absence of flagella), or in their DNA restriction endonuclease patterns, or in their SDS-PAGE patterns of total bacterial protein. The $> 90\%$ similarity observed in densitometry scans of protein profiles demonstrated strain identity,⁶ and suggests that although non-motile variants did not express intact flagella, nevertheless, they contained flagellin.

Flagella assist colonisation in a number of gastrointestinal pathogens including *Vibrio cholerae*,^{10,11} *C. jejuni*¹² and *Salmonella* spp.¹³ It has been suggested that flagellar adhesins play a role, or that motility facilitates penetration of intestinal mucus.^{10,13} In addition, motility may allow non-adherent bacterial species to maintain their location within the mucus layer of the stomach or intestine.¹⁴ In this study, motile *H. pylori* differed from non-motile variants in colonising the gastric mucosa more heavily and for longer periods. Bacteria recovered from piglets also gave rise to more spreading colonies than did the infecting

Table. Colonising ability of motile and non-motile *H. pylori* in gnotobiotic piglets

Piglet group	Interval (days) between inoculation and necropsy	Number of piglets from which <i>H. pylori</i> was recovered	Mean bacterial count ($\log_{10}$ cfu/g) in gastric mucosa	Percentage motility of organisms in inocula	Percentage motility in organisms recovered
A	6	5/5	5.2 (SD 5.2)	63–74	96–99
A	21	4/5	3.0 (SD 3.0)	30–74	89
B	6	2/3	1.9 (SD 1.9)	0	< 1
B	21	0/5	0	0–4	NR

NR, bacteria not recovered.

Fig. 4. Motility of *H. pylori* recovered from infected piglets. Bacteria were plated in soft agar and photographed after incubation for 5 days. **A**, *H. pylori* recovered from piglet inoculated with motile form; motility is indicated by spreading halo surrounding each colony. **B**, *H. pylori* recovered from piglet inoculated with non-motile form; colonies lack spreading halo (irregular smudging of some colonies is present, →).

cultures. These results demonstrate that motility promoted colonisation.

It is conceivable that the differences in colonisation seen in this study could be attributed to different degrees of subculture of the variants. However, all the variants were derived from the same parental strain, which had been subcultured repeatedly for 4 years. Therefore, it is unlikely that the small number of subcultures necessary to prepare the inoculum had an effect on colonising ability. Furthermore, in other (unpublished) studies, up to 10 subcultures had no effect on colonising ability.

Selection for motility *in vivo* was reported previously for *H. pylori* in gnotobiotic piglets⁴ and for *C. jejuni* in rabbits,⁸ but the mechanism was undetermined. Selection for motility may be due either to better survival of motile variants or to the reversion *in vivo* of the non-

motile to motile form. In the present work, the persistence of the non-motile form in piglets inoculated with non-motile *H. pylori* suggests that this form colonises, but does so poorly and is probably unable to compete with co-existing motile variants. This is consistent with the observation that non-motile *H. pylori* did not persist beyond 6 days, but that motile *H. pylori* persisted for 21 days in these experiments and up to 87 days in subsequent studies.³ Thus, it is likely that selection for motility is due to enhanced survival of motile variants *in vivo* rather than to reversion from non-motile to motile forms.

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