BACTERIAL PATHOGENICITY

***flaA* mRNA transcription level correlates with *Helicobacter pylori* colonisation efficiency in gnotobiotic piglets**

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In-vivo passage of the gastric pathogen *Helicobacter pylori* in gnotobiotic piglets results in a greater colonisation efficiency in subsequent infections. The rate of colonisation steadily increases with the number of passages. To determine if this increased efficiency is related to the level of expression of *flaA*, a gene which encodes the major subunit of flagella, this study evaluated the level of *flaA* expression at five points during serial in-vivo passage of strain 26695. Semi-quantitative reverse transcriptase polymerase chain reaction and Northern dot-blot analysis of *flaA* mRNA levels (expressed as a ratio to the level of *ureA* mRNA) revealed a positive correlation between *flaA* expression and colonisation efficiency; *flaA* mRNA levels increased incrementally with in-vivo passage as did colonisation rates. Immunoblots of outer-membrane vesicles from pig-passaged and laboratory-passaged strains also demonstrated marked differences in the amount of flagellin present in poor and efficient colonisers.

Introduction

Helicobacter pylori, a common human pathogen, is a gram-negative, spiral shaped, micro-aerophilic organism that inhabits gastric mucosa. It is the causative agent of chronic superficial gastritis and is implicated in gastric and duodenal ulceration [1]. *H. pylori* is uniquely adapted to life in the gastric mucus. Its ability to colonise mucus is most likely due, at least in part, to strong flagellar motility. It has been shown that gene products specifically associated with motility are essential for colonisation. An insertional mutation in the *flaA* gene, which encodes the major subunit of flagella, severely hinders colonisation of gnotobiotic piglets or ferrets by *Helicobacter* spp. and a mutation in *flaB* decreases colonisation of piglets [2, 3].

It has been observed that in the gnotobiotic piglet model, serial in-vivo passage results in a dramatic increase in colonisation efficiency of the parent strain, human isolate 26695 [4]. Because we and others have demonstrated the importance of FlaA in colonisation [2, 3], this study was undertaken to determine if a correlation exists between the colonisation rate of

H. pylori as it is passed *in vivo* and the transcriptional level of *flaA*. The publication of the genomic sequence for strain 26695 [5] and our experience with this strain in numerous gnotobiotic piglet studies prompted the choice of strain 26695 as the experimental organism.

Materials and methods

Animals and organisms

Gnotobiotic piglets were infected orally with 2 ml of human isolate 26695 at a titre of 10^9 cfu/ml. For quantification of colonisation efficiency, data from several different isolates were used for each pig-passage number and piglets were killed 28 days after inoculation [4]. One half of each stomach was placed in a sterile petri dish and gastric mucosa was removed with a sterile scalpel blade, weighed and homogenised in 5 ml of Brucella broth. Ten-fold serial dilutions from 10^1 – 10^4 were plated in duplicate on Trypticase Soy Agar plates supplemented with sheep blood 5% (Becton Dickinson, Cockeysville, MD, USA) and incubated at 37°C under micro-aerobic conditions. Colonies were counted after 4–6 days and colonisation efficiency was determined by dividing the number of colonies obtained by the weight of the mucosa (cfu/g of mucosa). Recovered bacteria were stored in Brucella broth with glycerol 15% at –70°C until used for ensuing infections or RNA isolations. All experiments

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involving piglets were approved by the OSU Laboratory Animal Care and Use Committee.

Outer-membrane proteins

Outer-membrane proteins (OMPs) of the laboratory-passaged strain 26695 and one of its pig-passaged derivatives (passaged >11 times) were isolated by the method of Blaser *et al.* [6]. Two-dimensional gel electrophoresis with equal amounts of OMPs was performed in the Mini-Protean system according to the manufacturer's instructions (BioRad, Hercules, CA, USA). Resultant gels were then either silver-stained or proteins were immobilised on uncharged nylon membranes. Flagellins were identified by immunoblotting with a monoclonal antibody which cross-reacts with FlaA and FlaB (72c, kindly donated by Dr Trevor Trust) [7].

Estimation of *flaA* mRNA

To assess the amount of *flaA* mRNA present in isolates of strain 26695 frozen stocks of bacterial strains were cultured on blood-agar plates and total RNA was isolated with TRIzol reagent (Gibco-BRL, Gaithersburg, MD, USA). The isolates used in this study included the laboratory-passaged human isolate (p0), an isolate which had been passaged once in germ-free piglets (p1), one which had been passaged 11 times (p11), one which had been passaged between 11 and 20 times (11 < p < 20), and one which had been passaged >20 times (p > 20).

Reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA (1 µg) was treated with amplification grade DNAase and reverse transcribed with Superscript RNase H⁻ Reverse Transcriptase (Gibco-BRL) and random hexamers to prime cDNA synthesis. Reverse transcription reactions without Superscripts were also included to ensure that subsequent amplification products were not the result of undigested DNA. Two µl (10%) of the cDNA preparations were amplified in a multiplex PCR with 100-pmol of the following primers: *flaA*, 5'-ATGGCTTTTCAGGTC-AATAC-3' and 5'-GCCTTAAGATATTTGTTGAA-CG-3' [8]; and *ureA*, 5'-GCCAATGGTAAATTAGTT-3' and 5'-CTCCTTAATTGTTTTAC-3' [9]. Amplifications were performed in a 100-µl volume with a model 480 thermocycler (Perkin-Elmer, Norwalk, CT, USA) under the following conditions: 1× PCR buffer, 0.8 mM dNTPs, 1.5 mM MgCl₂, 2.5 U of Taq polymerase; 3 min incubation at 94°C followed by 28 cycles of 94°C for 1 min, 50°C annealing for 1 min and 72°C for 2 min. The reaction was then extended by a further 10 min at 72°C (all reagents from Gibco-BRL). Fifteen µl of the reaction products were analysed on agarose 1% gels stained with ethidium bromide. Gels were digitised on a Gelprint 2000i (BioPhotronics, Ann Arbor, MI, USA) and band intensities were quantified with ImageQuant software (Molecular Dynamics,

Sunnyvale, CA, USA). Corrections were made to compensate for the difference in size between the *flaA* and *ureA* amplicons by dividing their intensities by their respective number of base pairs. Two separate batches of RNA were derived for analysis by PCR. From these samples, a total of three reverse transcription reactions and four amplification reactions were performed.

Northern blot analysis. Total RNA was isolated and treated with DNAase as above, extracted with phenol/chloroform and precipitated with 3 M sodium acetate (pH 5) and ethanol; 100 ng of each RNA preparation were amplified with the *flaA* primers as above for 35 cycles to ensure that no endogenous DNA was present in the samples. Total RNA from each of the five isolates used in this study was diluted to 280 ng/µl and each sample was five-fold serially diluted to a final concentration of *c.* 2 ng/µl. Two µl of each dilution were spotted on two different uncharged nylon membranes and RNA was immobilised with a UV Stratalinker (Stratagene, La Jolla, CA, USA). Each membrane was pre-hybridised for 1 h at 41°C in Standard buffer with formamide 50% (5 × SSC; 1/5 vol blocking solution, 10 × concentrate, Boehringer Mannheim, Indianapolis, IN, USA; SDS 0.02% w/v; N-lauroylsarcosine 0.1% w/v; deionised formamide 50%). Probes for *flaA* and *ureA* were synthesised from PCR products which had been gel purified by the GeneClean II Kit (Bio101, Inc., Vista, CA, USA), ethanol precipitated and verified by restriction analysis. The PCR products for *flaA* and *ureA* were digoxigenin (DIG)-labelled with the DIG High Prime Labeling and Detection Starter Kit I (Boehringer Mannheim) according to the manufacturer's recommendations. Each duplicate membrane prepared above was then hybridised overnight at 41°C in pre-hybridisation solution containing either the *flaA* probe (80 ng/ml final concentration) or the *ureA* probe (26.4 ng/ml final concentration). Membranes were then washed, blocked, incubated with an anti-DIG alkaline phosphatase-conjugated antibody for 1 h and detected colorimetrically according to the manufacturer's recommendations. The membranes were digitised on a Gelprint 2000i (Biophotonics) and spot intensities were quantified with ImageQuant software (Molecular Dynamics).

Results and discussion

Table 1 illustrates the increases in colonisation rates from the initial passage in piglets of human isolate 26695 (p0), through intermediate passages, until the final in-vivo passage used in this study (p > 20). These data are extensions of an earlier, more limited study that demonstrated increased colonisation with pig-passage [4]. The first increase, from p0 (10³) to p1 (10⁵) is almost certainly a reflection of the adaptation of this organism from one host to another, albeit in a similar gastric environment. What is interesting to note

Table 1. Comparisons of strain 26695 in-vivo passage numbers, colonisation rates and ratios of *flaA/ureA* mRNA expression

In-vivo passages	Number of piglets infected/total*	cfu/g of mucosa	<i>flaA/ureA</i> ratios	
			PCR†	dot-blot
p0	8/11	$1 \times 10^{3\ddagger}$	0.44(0.11)	0.73
p1	8/8	1×10^5	0.77(0.04)	1.58
p11	18/18	1.7×10^6	0.88(0.07)	1.72
11 < p < 20	13/13	5.6×10^6	1.42(0.62)	1.42
p > 20	8/8	2×10^7	2.40(0.60)	2.59

*Data include some piglets previously reported [4].

†Mean (SD) for four trials.

‡Colonisation in many piglets was too low to be exactly quantified, see reference [4].

is that even after this initial adaption *H. pylori* continued to improve its colonisation efficiency by c. 20–40% each passage. Fig. 1 illustrates the second piece of evidence which prompted this study. Although equal amounts of OMPs from laboratory-passaged and serially in-vivo passaged isolates of the same parent strain were loaded on 2-D gels, immunoblots of the OMPs demonstrated flagellar proteins as clearly prominent in the pig-passaged isolate but barely detectable in the laboratory-passaged isolate.

Fig. 2 is an example of a gel used to compare the levels of *flaA* mRNA present in isolates differing in in-vivo passage number. The level of *flaA* mRNA

Fig. 1. Detection of flagellins in pig-passaged (A) and laboratory-passaged (B) strains by immunoblot. Similar amounts of OMPs from parent strain 26695 and an isolate that had been multiply passaged *in vivo* were loaded on 2-D gels. Arrows indicate the location of flagellins.

Fig. 2. A typical agarose gel comparing *flaA* and *ureA* mRNA expression levels between strains after different numbers of in-vivo passages. As the passage number increases serially (represented from left to right on the gel) the ratio of *flaA* to *ureA* mRNA increases (see Table 1). Lane S, Hi-lo DNA ladder (Minnesota Molecular, Minneapolis, MN, USA); 1, *H. pylori* human isolate 26695, 0 pig passages; 2, 1 passage; 3, 11 passages; 4, between 11 and 20 passages; 5, >20 passages.

present in each total RNA sample was expressed as a ratio to *ureA* mRNA present in the same sample; *ureA* is believed to be expressed constitutively [10] except, perhaps, under variable nitrogen availability [11]. As culture conditions were not varied in the in-vivo or in-vitro experiments, *ureA* served as an endogenous internal control in both multiplex PCR and the dot-blot analysis to compensate for any variation between samples which may have been the result of handling. For multiplex amplification, 28 was the lower limit of cycles necessary to visualise both amplicons and was used, therefore, for all amplifications. Table 1 illustrates the increase in *flaA* mRNA content, as determined by PCR, which occurred after multiple in-vivo passages. There is an approximately two-fold initial increase in *flaA* mRNA after the first passage in

pigs which, like colonisation efficiency mentioned above, is related to the strain's adaptation to a new host. It is likely that a significant proportion of the laboratory-passaged human isolate, in the absence of selective pressure favouring motility, had lost its ability to synthesise *flaA* mRNA efficiently and was subsequently eliminated during the first passage in pigs. However, the level of *flaA* mRNA continued to increase incrementally well after the first passage and, at the final endpoint of >20 passages, the level of *flaA* mRNA was approximately three times greater than the level after one passage. A linear regression curve plotting the cfu/g of mucosa against the expression ratios as determined by PCR returned a *p* value of 0.01 (data not shown).

These data are supported by the RNA dot-blot analysis (Table 1), the RNA for which had been isolated independently from that used for the PCR analysis. There was an initial jump in the level of *flaA* mRNA (again expressed as a ratio to *ureA* mRNA) after the first passage. This upward trend continued as evidenced by the ratios present after 11 passages and after >20 passages.

This study showed that *flaA* mRNA levels monitored *in vitro* correlated with growth *in vivo* and that selection for increased proliferation in piglets was associated with increased FlaA protein and increased *flaA* transcript levels. Previous studies have shown that phenotypic expression of motility also increases with animal passage [12], but such expression appears to be an insensitive indicator of FlaA expression. Motility expression jumped from 30% in strain 26695 to 100% motile colonisers after a single animal passage [12], while FlaA expression continued to increase with continued animal passage.

Although the basis for the increased expression of *flaA* in in-vitro passaged strains has not yet been formally investigated, at least two possibilities seem plausible. It has been determined that the *flaA* gene has a sigma-28 like promoter [8] and it is possible that, with piglet passage, natural random genetic mutation alters this region such that the promoter operates with an increased efficiency. This increased efficiency may confer a selective advantage to bacteria which are able to produce more *flaA* mRNA and, presumably, more FlaA. Another cause may be found in the hierarchical nature of flagellar synthesis. Schmitz *et al.* have recently described a gene in *H. pylori* (*flbA*), which is involved in the regulation of flagellar biogenesis [13]. It has been hypothesised that a similar membrane-associated protein in *Caulobacter crescentus* (FlbF) acts as a signal transducer that coordinates flagellar expression either directly or by activation of intermediate transcriptional regulators [14]. If increased levels of *flaA* mRNA augment the ability of *H. pylori* to colonise its host, bacteria with adaptations in this system that confer an increased

ability of *H. pylori* to synthesise *flaA* mRNA would also have a selective advantage.

It is likely that other genes besides *flaA* will demonstrate a correlation between transcription levels and colonisation efficiency during in-vivo passage. The increased ability of *H. pylori* to colonise may be due to varying transcription levels of a number of colonising factors. Previous studies have shown that numerous bacterial virulence factors are often regulated together [15] and subtle changes in the efficiencies of these networks may contribute to the incremental increases in colonisation efficiency which have been observed in the gnotobiotic piglet model. The data from the present study lead to the inference that events are occurring at the transcriptional level that improve colonisation rate. Components of a system which impact transcriptional levels of important motility genes may prove to be useful targets for immunisation strategies against *H. pylori*. Further studies investigating the relationships between *flaA* transcript levels, the mRNA levels of other genes, and their correlation to colonisation efficiencies during in-vivo passages will be valuable to the understanding of this nearly ubiquitous pathogen.

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