

Role of the *Helicobacter pylori* outer-membrane proteins AlpA and AlpB in colonization of the guinea pig stomach

Ramon de Jonge,^{1,2} Zarmina Durrani,³ Sjoerd G. Rijpkema,³
Ernst J. Kuipers,¹ Arnoud H. M. van Vliet¹
and Johannes G. Kusters¹

Correspondence

Johannes G. Kusters

j.g.kusters@erasmusmc.nl

¹Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center Rotterdam, Rotterdam, the Netherlands

²Department of Gastroenterology, VU University Medical Center, Amsterdam, the Netherlands

³Division of Bacteriology, National Institute for Biological Standards and Control, Potters Bar, UK

The human gastric pathogen *Helicobacter pylori* expresses several putative outer-membrane proteins (OMPs), but the role of individual OMPs in colonization of the stomach by *H. pylori* is still poorly understood. The role of four such OMPs (AlpA, AlpB, OipA and HopZ) in a guinea pig model of *H. pylori* infection has been investigated. Single *alpA*, *alpB*, *hopZ* and *oipA* isogenic mutants were constructed in the guinea pig-adapted, wild-type *H. pylori* strain GP15. Guinea pigs were inoculated intragastrically with the wild-type strain, single mutants or a mixture of the wild-type and a single mutant in a 1 : 1 ratio. Three weeks after infection, *H. pylori* could be isolated from stomach sections of all animals that were infected with the wild-type, the *hopZ* mutant or the *oipA* mutant, but from only five of nine ($P = 0.18$) and one of seven ($P = 0.02$) animals that were infected with the *alpA* or *alpB* mutants, respectively. The *hopZ* and *oipA* mutants colonized the majority of animals that were inoculated with the strain mixture, whereas *alpA* and *alpB* mutants could not be isolated from animals that were infected with the strain mixture ($P < 0.01$). Specific IgG antibody responses were observed in all animals that were infected with either the wild-type or a mutant, but IgG levels were lower in animals that were infected with either the *alpA* or the *alpB* mutants, compared to the wild-type strain ($P < 0.05$). In conclusion, absence of AlpA or AlpB is a serious disadvantage for colonization of the stomach by *H. pylori*.

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INTRODUCTION

The human pathogen *Helicobacter pylori* persistently colonizes the gastric mucosa of more than half of the world's population. Colonization by *H. pylori* always results in active gastritis and is associated with the development of peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (Kuipers, 1997). Several *H. pylori* factors are commonly believed to be required for efficient, lifelong colonization of the gastric mucosa, including urease activity (Eaton & Krakowka, 1994), motility by polar sheathed flagella (Eaton *et al.*, 1996) and adherence to gastric epithelial cells (Hessey *et al.*, 1990; Guruge *et al.*, 1998; Yamaoka *et al.*, 2002). Adherence to gastric

epithelial cells protects *H. pylori* against peristalsis and mucosal shedding and warrants access to nutrients that are released from damaged epithelium.

It appears that mucosal adhesion of *H. pylori* is associated directly with mucosal damage (Hessey *et al.*, 1990; Guruge *et al.*, 1998). Adhesion of *H. pylori* is mediated in part via specific adhesins, such as the sialyl Lewis^x-binding protein SabA (Mahdavi *et al.*, 2002) and the Lewis^b blood group antigen-binding protein BabA (Boren *et al.*, 1993; Ilver *et al.*, 1998). However, the *H. pylori* genome also encodes several other outer-membrane proteins (OMPs) that have been associated with adhesion, including OipA, HopZ, AlpA and AlpB (Alm *et al.*, 1999; Evans & Evans, 2000; Tomb *et al.*, 1997). The role of these individual OMPs in adhesion of *H. pylori* is still relatively poorly understood. *H. pylori* strains with reduced expression of OipA, SabA, SabB and HopZ displayed limited colonization of the murine stomach when two or more of these OMPs were not expressed, due to phase

Abbreviations: Kan^R, kanamycin-resistance; OMP, outer-membrane protein.

variation (Yamaoka *et al.*, 2002). AlpA and AlpB may function as adhesins, as isogenic *alpA* and *alpB* mutants displayed reduced adherence to human gastric tissue and gastric epithelial cells when tested *in vitro* (Odenbreit *et al.*, 1999, 2002a).

Several animal models have been employed for study of *H. pylori* infection, using non-human primates, gerbils, mice and guinea pigs (Marchetti *et al.*, 1995; Dubois *et al.*, 1996; Hirayama *et al.*, 1996; Guruge *et al.*, 1998; Shomer *et al.*, 1998). The guinea pig model for *H. pylori* infection has some attractive features, such as ease of husbandry and animal size. The guinea pig stomach has several features in common with the human stomach that are lacking in the mouse model, such as sterility and the production of IL8 (Shomer *et al.*, 1998; Sturegard *et al.*, 1998).

In this study, we have employed the guinea pig model of *H. pylori* infection to characterize the role of the OMPs AlpA, AlpB, OipA and HopZ in colonization of the stomach by *H. pylori*. Isogenic mutants in the *alpA*, *alpB*, *oipA* and *hopZ* genes were created in the guinea pig-adapted *H. pylori* strain GP15 and used to infect guinea pigs with either single mutants or 1 : 1 mixtures of the wild-type strain and a single mutant. Three weeks after inoculation, colonization efficiency and local and systemic antibody responses were measured. We thus observed that both AlpA and AlpB make important contributions to gastric colonization by *H. pylori*.

METHODS

Bacterial strain and culture conditions. The guinea pig-adapted *H. pylori* strain GP15 (Rijpkema *et al.*, 2001) and isogenic mutants in the four OMP-encoding genes were cultured routinely on Columbia agar medium plates that were supplemented with 5 % defibrinated horse blood or 7 % saponin-lysed horse blood and Dent selective supplement (Oxoid), further referred to as Dent plates. *H. pylori* was grown routinely for 72–96 h at 37 °C in an atmosphere of 5 % O₂, 10 % CO₂ and 85 % N₂. Broth cultures were grown in Brucella broth that was supplemented with 3 % newborn calf serum (Gibco-BRL) and Dent supplement (Oxoid). When appropriate, *H. pylori* growth media were supplemented with kanamycin to a final concentration of 20 µg ml⁻¹.

Construction of site-directed mutants and recombinant DNA techniques. Standard recombinant DNA techniques were performed as described by Sambrook *et al.* (1989). Plasmid pJMK30 (van Vliet *et al.*, 1998) was used as the source of the kanamycin-resistance (Kan^R) cassette. PCR fragments that contained an internal part of the *alpA*, *alpB*, *oipA* or *hopZ* genes were amplified by PCR using the primers listed in Table 1 and were subsequently cloned into pGEM-T Easy vector (Promega). The Kan^R cassette was inserted into the unique *Eco*47III, *Sma*I, *Hind*III and *Sma*I sites in the *alpA*, *alpB*, *oipA* and *hopZ* genes, respectively. The resulting plasmids were used to transform *H. pylori* strain GP15 by natural transformation, as described previously (Bijlsma *et al.*, 1999). Correct integration of the Kan^R cassette was verified by restriction analysis and PCR amplification, using primers located outside the amplified region that was used for creation of the respective mutants (Table 1).

Animal experiments and serology. Infection experiments were performed essentially as described previously (Rijpkema *et al.*, 2001).

Table 1. Oligonucleotide primers used in this study

Gene	Gene number in strain*		Primer†	Sequence (5'→3')
	26695	J99		
<i>alpA</i>	HP0912	JHP0848	AlpA-F1	ACAACACCGGAGGCAATGTC
			AlpA-R1	GATAGGAGCGGCTAAAGATG
			AlpA-F2	AGGAGCATCCCATTTGATAAG
			AlpA-R2	TGCCTTGCACCAAACCTGTA
<i>alpB</i>	HP0913	JHP0849	AlpB-F1	CTTATGACCTTTGGGTATG
			AlpB-R1	GAGCGGCTAAACACATTGTA
			AlpB-F2	AGCCTAGCGTTAAGCTTGAG
			AlpB-R2	TTCATGCGTAACCCACATG
<i>oipA</i>	HP0638	JHP0581	OipA-F1	CACGCTGAAAGGAACGGATT
			OipA-R1	AAGATAAACGCTCACCCTC
			OipA-F2	GATTGATTAAGGCGTGGTG
			OipA-R2	CTATCAAGGAGAGCGAAAGA
<i>hopZ</i>	HP0009	JHP0007	HopZ-F1	CCTTACACCTCTGCTGATAG
			HopZ-R1	ATCTTCACGCCTAGTTCCAC
			HopZ-F2	AGTTTAGCGCCGTTACTAGC
			HopZ-R2	GAGTTGAGTGCTAGCAAAG

*Primer sequences were based on the complete genome sequences of *H. pylori* strains 26695 and J99 (Tomb *et al.*, 1997; Alm *et al.*, 1999).

†F1/R1 primer combinations were used to amplify gene fragments for insertion of the Kan^R cassette; F2/R2 primer combinations are located outside the F1/R1 fragment and were used to verify correct integration of the Kan^R cassette into the *H. pylori* genome.

Female Dunkin–Hartley guinea pigs (200–300 g) were housed on grids and fed with water and food *ad libitum*. All animal experiments were performed according to the guidelines of the Home Office (1986) and were approved by the local ethics committee. Guinea pigs were fasted overnight before being given an intragastric dose of either wild-type *H. pylori* strain GP15, its isogenic mutants or a mixture of both at a ratio of 1 : 1. Either two or three doses of approximately 10^9 c.f.u. *H. pylori* per dose were given to animals intragastrically on alternate days (Rijpkema *et al.*, 2001). Animals were dissected 21 days after inoculation and serum, bile and gastric mucosa of the antral region were collected. The stomach was rinsed briefly with PBS and a small section of the gastric antrum was streaked out on Dent plates to detect wild-type *H. pylori*, and on kanamycin-supplemented Dent plates to detect *H. pylori* mutants. Colonies were identified as *H. pylori* by morphology and urease activity. Antibodies were extracted from antral tissue as described previously (Bergquist *et al.*, 2000). Specific IgG and IgA were detected by ELISA, using plates that were coated with *H. pylori* GP15 outer-membrane fraction, as described previously (Rijpkema *et al.*, 2001; Durrani & Rijpkema, 2003).

Statistical analysis. The Mann–Whitney U test for non-parametric data was used to compare differences between experimental groups. All data are presented as mean \pm SD. *P* values of < 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Characterization of *H. pylori* OMP mutants

To evaluate the contribution of the AlpA, AlpB, OipA and HopZ proteins in gastric colonization by *H. pylori*, isogenic mutants in the respective genes were created in *H. pylori* strain GP15. Parts of the *alpA*, *alpB*, *oipA* and *hopZ* genes were amplified by PCR, using primer F1/R1 combinations (Table 1), and cloned in an *E. coli* vector; the coding region of the genes was interrupted by the insertion of a Kan^R cassette. Subsequently, the mutated genes were used for allelic exchange after natural transformation of *H. pylori* and kanamycin-resistant colonies were selected. Correct replacement of the wild-type gene by the mutated copy was confirmed by PCR using F2/R2 primer combinations (Table 1). Absence of one of these OMPs did not affect growth under standard growth conditions, as the growth rate of the mutants was similar to that of the wild-type strain. There was also no significant difference between the mutants and the wild-type strain in the ability to induce IL8 production in gastric epithelial cells (data not shown), confirming the previously reported phenotype of the mutants (Ando *et al.*, 2002; Odenbreit *et al.*, 2002b).

Effect of OMP gene mutation on gastric colonization by *H. pylori* in guinea pigs

The role of the AlpA, AlpB, OipA and HopZ proteins in gastric colonization *in vivo* was evaluated by using the guinea pig model for *H. pylori* infection (Rijpkema *et al.*, 2001; Durrani & Rijpkema, 2003). Ability to colonize was first assessed by comparing single infections with the wild-type strain GP15 and single isogenic mutants in two independent infection experiments. The results of these experiments are shown in Fig. 1a. Wild-type *H. pylori* GP15 and the *oipA* and

hopZ mutants colonized all inoculated guinea pigs, confirming previous observations made by using a mouse model of *H. pylori* infection (Yamaoka *et al.*, 2002). The *alpA* mutant could only be isolated from five of nine inoculated guinea pigs, but this difference was not significant ($P = 0.18$). In contrast, the *alpB* mutant could only be isolated from one of seven inoculated animals ($P = 0.02$).

The attenuating effect of the OMP mutations was further defined by using competitive infection experiments with wild-type GP15 and each of the single OMP mutants in a 1 : 1 ratio. Wild-type and OMP mutants could be distinguished by the kanamycin resistance of mutant cells. The wild-type strain was isolated from all inoculated animals, irrespective of the OMP mutant included in the 1 : 1 mixture (Fig. 1b). The *hopZ* and *oipA* mutants could also be isolated from more than half of the inoculated animals, indicating that neither mutant had a major selective disadvantage when compared to the wild-type strain. In contrast, the *alpA* and *alpB*

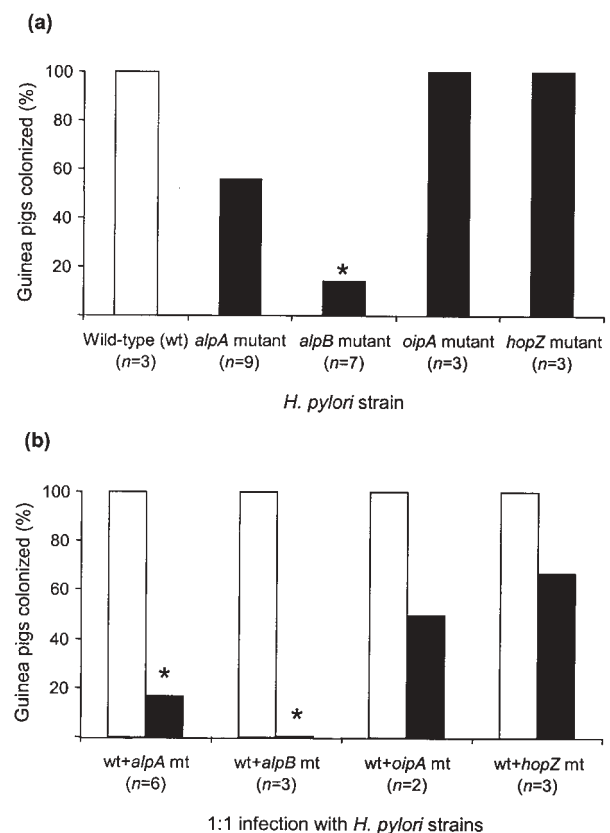


Fig. 1. Colonization of guinea pigs by wild-type *H. pylori* GP15 and isogenic OMP mutants. (a) Single inoculation of either wild-type *H. pylori* strain GP15 (empty bar) or *alpA*, *alpB*, *oipA* or *hopZ* mutants (filled bars). (b) Results of competitive infection experiments, using 1 : 1 mixtures of wild-type GP15 (empty bars) and OMP mutants (filled bars). Asterisks indicate a significant difference between the number of animals colonized after inoculation with *H. pylori* OMP mutants, compared to infection by the wild-type strain GP15 ($P < 0.05$).

mutants could only be isolated from one of six ($P = 0.01$) and none of three ($P = 0.03$) infected guinea pigs, respectively.

Antibody responses to infection by OMP mutants

Differential effects of the OMP mutations were apparent in the antibody response in serum, as well as in locally produced antral mucosa IgG and bile IgA (Fig. 2). Serum IgG and antral IgG antibody responses were significantly lower in animals that were inoculated with the *alpB* mutant, compared to the wild-type strain ($P = 0.01$ in both cases). Serum IgG, but not antral mucosal IgG, was also decreased in animals that were inoculated with the *alpA* mutant ($P = 0.01$). This finding may be due to incomplete colonization (56 %) of animals that were inoculated with the *alpA* mutant (Fig. 1). There was no difference between the levels of specific IgG antibodies in animals that were inoculated with wild-type GP15 and the *oipA* and *hopZ* mutants. Interestingly, bile IgA levels were significantly lower in animals that were inoculated with the *alpB*, *oipA* and *hopZ* mutants (P values of 0.01, 0.03 and 0.03, respectively), but not with the *alpA* mutant ($P = 0.12$) (Fig. 2). We currently have no explanation for this unexpected difference between the IgG and IgA responses with the *oipA* and *hopZ* mutants. Antibody responses in animals that were infected with 1:1 ratios of wild-type and single OMP mutants were similar to those infected with the wild-type GP15 strain (data not shown), which is not surprising in view of the total colonization by the wild-type strain.

DISCUSSION

Our findings extend previous observations (made *in vitro*) that the *alp* operon (which consists of *alpA* and *alpB*) is involved in adherence to gastric epithelial cells and gastric tissue sections (Odenbreit *et al.*, 1999, 2002a) and demonstrate that the Alp OMPs contribute significantly to successful infection of guinea pigs by *H. pylori*. The marked decrease in colonization efficiency of *alpA* and *alpB* mutants can be attributed to a deficiency of the mutants to adhere to gastric epithelial cells *in vivo*, as was demonstrated previously *in vitro* by using gastric biopsies (Odenbreit *et al.*, 2002a). As a consequence of inactivation of these two OMP-encoding genes, *H. pylori* can probably be removed easily from the

stomach by gastric emptying and may therefore be unable to persist at the epithelial surface and cause inflammation of gastric mucosa.

The *alpA* gene is transcribed *in vivo* in human gastric tissues, as well as in murine gastric tissues, in the first 3 months of infection (Rokbi *et al.*, 2001), indicating that the Alp proteins play an active role in establishing and maintaining gastric colonization. Transcription of *alpA* was tenfold higher 1 h post-infection than 1 week post-infection; by comparison, transcription of *ureA* and 16S rRNA varied much less over time (Rokbi *et al.*, 2001). The role of AlpA and AlpB in the early stages of infection may make them important candidates for a prophylactic vaccine. Indeed, vaccination studies in mice have indicated that an AlpA vaccine reduced bacterial load significantly when given as a prophylaxis, but was ineffective when given as a therapeutic treatment (Sanchez *et al.*, 2001).

Conclusion

In this study, it is demonstrated that the *H. pylori* OMPs AlpB and, to a lesser extent, AlpA, are required for gastric colonization of the guinea pig stomach, most probably by mediating adherence to gastric epithelial cells. Further work is required to determine whether *alpA* or *alpB* mutants may serve as an attenuated live vaccine. Characterization of the receptors and functions of individual *H. pylori* OMP molecules may provide further insights into mechanisms that are essential for *H. pylori* colonization of human gastric mucosa.

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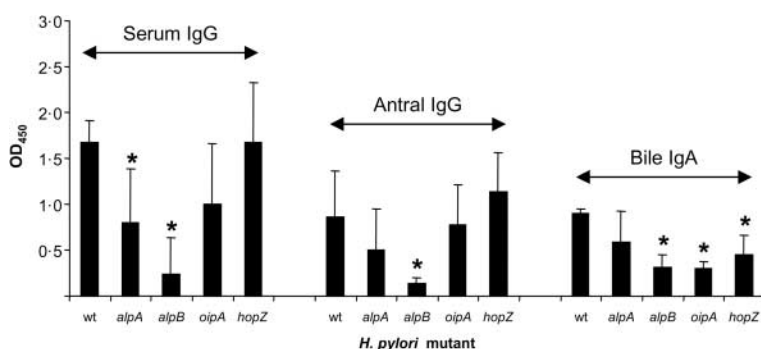


Fig. 2. Serum and local antibody responses to colonization by wild-type strain *H. pylori* GP15 (wt) or single OMP mutants (*alpA*, *alpB*, *oipA* or *hopZ*). Data are means of the OD₄₅₀. Error bars denote SD; asterisks indicate a significant difference between the antibody response after infection by OMP mutants, compared to infection by the wild-type strain GP15 ($P < 0.05$).

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