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SHORT COMMUNICATION

Helicobacter pylori protein oxidation influences the colonization process

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Abstract

Dsb proteins control the formation and rearrangement of disulfide bonds during the folding of membrane and exported proteins. Here we examined the role of DsbI protein in *Helicobacter pylori* pathogenesis and demonstrated that a *dsbI* mutant impaired in disulfide bond formation revealed a greatly reduced ability to colonize mice gastric mucosa.

Keywords: *Helicobacter pylori*; Colonization; Protein oxidation

Introduction

Helicobacter pylori is a Gram-negative spiral-shaped ε-proteobacterium, which colonizes the gastric mucosa of humans. Infections caused by *H. pylori* are associated with different symptoms ranging from mild gastritis to ulcers. In addition, some chronic infections, acquired early in life, confer an increased risk of gastric cancer (Nardone and Morgner, 2003; Isakov and Malfertheiner, 2003). Although more than 50% of the human population is infected with *H. pylori*, only a subset develops a disease. Moreover, it has been documented that disease symptoms are correlated with the genotype of the two interacting organisms, the pathogen and the host. To date, numerous virulence factors responsible for the diversity of aspects of pathogen–host cells interaction have been described and characterized. The majority are extracytoplasmic proteins, mainly soluble periplasmic, membrane or exported proteins (Lamarque and Peek, 2003). Some essential virulence factors such as VacA or CagA are translocated into target cells via different secretion mechanisms (Hatakeyama, 2004; Montecucco and de Bernard, 2003). It was documented that the proper folding of a three-dimensional active structure of many extracytoplasmic virulence factors of Gram-negative pathogens require the complex action of Dsb proteins involved in disulfide bond formation and isomerization (Kadokura, 2003).

Materials and methods

Bacterial strains and culture conditions

H. pylori strain SS1 was grown under microaerobic conditions at 37 °C on blood agar base No. 2 containing 10% horse blood. The medium for the *H. pylori* mutant

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contained kanamycin (25 μg/ml). A preculture was used to inoculate liquid tryptone soy broth supplemented with 5% fetal bovine serum. The flasks were kept under microaerobic conditions at 37 °C with constant shaking (150 rpm), and bacterial cells were used for mice inoculation.

Recombinant DNA techniques

Gene replacement was used to construct the *H. pylori dsbI* isogenic mutant. The construction of the plasmid containing the *dsbI* gene disrupted by a kanamycin-resistant cassette (*Campylobacter jejuni aph(3′)-III* gene) was described by Raczko et al. (2005). This mutation does not have any polar effect on downstream genes. The Km cassette DNA fragment lacking a transcriptional termination signal was inserted in the orientation corresponding to HP595 transcription. Its orientation was checked by PCR. Additionally, we also investigated HP596 transcription and found that HP596 (located downstream) is transcribed from its own promoter. The *H. pylori* SS1 strain, naturally competent for genetic transformation, was used for generation of isogenic *H. pylori dsbI* mutants. The obtained kanamycin-resistant transformants were screened by hybridization using cloning vector DNA as a probe to confirm that no vector DNA was integrated into the chromosome through single cross-over and analyzed by PCR to prove that the predicted double crossover of the mutated allele had occurred. Further experiments showed that the lack of *dsbI* gene product is not lethal for bacterial cells. The mutant showed typical *Helicobacter* morphology and no alterations in growth rate.

Infection of mice and DNA preparation

C57BL/6, outbred specific pathogen-free mice (6–8-week-old) were experimentally infected with either *H. pylori* SS1 or isogenic *dsbI*-mutated *H. pylori* SS1. DNA from gastric mucosa was obtained as described previously (Dzwonek et al., 2004; Mikula et al., 2003). Non-infected 6–8-week-old C57BL/6 mice were used as a control.

Real-time PCR

The infection with *H. pylori* was quantified 3 weeks post-challenge by real-time PCR of the 26-kDa species-specific antigen gene, as described previously (Mikula et al., 2003). The calculated amounts of total *H. pylori* DNA and the number of bacterial cells present in each sample were normalized to murine *GAPDH* DNA. Student's *t*-test was used to calculate the significance of the effects of the *dsbI* mutant on the colonization process. $p < 0.05$ was considered to be significant.

Bioinformatics analysis

The homology search and multiple alignment were performed using Psi-blast program (Altschul et al., 1997) and ClustalW (Thompson et al., 1997). The predictions of extracytoplasmic proteins of *H. pylori* were performed using the ConPred II server (Masafumi et al., 2004) and the LipoP server (Juncker et al., 2003).

Results and discussion

The most extensively studied disulfide oxidoreductases are those of *Escherichia coli* which generate two distinct pathways functioning in the periplasmic space: the oxidative pathway (DsbA, DsbB) and the isomerization pathway (DsbC, DsbD, DsbE) (Kadokura, 2003). We have previously identified and characterized a new *H. pylori* DsbB-like thiol-oxidoreductase encoded by the JHP0542 gene of J99 and HP0595 of 26695 (Raczko et al., 2005). HP0595 belongs to the DsbI protein family, which is paralogous to the DsbB family. DsbB accepts electrons from the periplasmic protein DsbA, while the substrates of DsbI still remain unknown. We used PSI-BLAST to search the genomes of ε-Proteobacteria for the orthologs of *dsbA*, *dsbB*, and *dsbI genes*, using their protein family members from *H. pylori* and *C. jejuni* (Thompson et al., 1997; Altschul et al., 1997). As expected, the results of our analysis (Table 1) show that the distribution of DsbA and DsbB is strongly correlated. The only notable exception is the lack of a DsbB ortholog in *C. lari*. Interestingly, the genome of this bacterium contains an additional copy of a DsbI ortholog. We hypothesize that one of the copies of DsbI in *C. lari* has taken over the function of the lost DsbB protein and acquired the ability to interact with DsbA. We have previously shown that in *C. jejuni*, DsbB can complement

Table 1. Distribution of homologs of *dsbI, dsbB* and *dsbA* in ε-proteobacteria

Organism	DsbI	DsbB	DsbA
Campylobacter coli RM2228[a]	+	+	+
Campylobacter jejuni RM1221	+	+	+
Campylobacter jejuni subsp. jejuni NCTC 11168	+	+	+
Campylobacter lari RM2100[a]	+ +	–	+
Campylobacter upsaliensis RM3195[a]	+	+	+
Helicobacter hepaticus ATCC 51449	–	–	–
Helicobacter pylori 26695	+	–	–
Helicobacter pylori J99	+	–	–
Wolinella succinogenes DSM 1740	–	–	–

+ or – indicate the presence or absence of a member of the respective family. + + indicates the presence of two members.

[a]Unfinished genome, whole genome shotgun sequence.

for the lack of DsbI, but not vice versa. Thus, it will be interesting to carry out complementation tests with both DsbI proteins from *C. lari*.

The analysis of both *H. pylori* strains (26695, J99) revealed the presence of DsbI proteins in the absence of DsbA, which suggests that "orthodox" DsbI proteins most likely act on other substrates. In this case, the oxygenation of a pair of cysteines in target proteins must be carried out by some other, yet unknown mechanism. This observation points to the differences in the interactions between Dsb proteins acting in two closely related microorganisms – *Campylobacter* and *Helicobacter* – and strongly implies a leading role for DsbI in oxidative folding of *Helicobacter* proteins.

In this report, considering the potentially essential physiological role of DsbI, we decided to analyze the influence of a *dsbI* gene knockout on the colonization process. To achieve this, an isogenic *dsbI*-mutated *H. pylori* SS1 strain was used for experiments on mice and the level of colonization was monitored by RT-PCR. *H. pylori* SS1 is a mouse-adapted strain, isolated by Lee et al. (1997). Although some pathological symptoms differ from those provoked by human infection, the murine model has been widely used to evaluate different aspects of *Helicobacter* pathogenesis. Recently, it has been shown that the immunoproteome of infected mice resembles that of infected humans, which further validated this experimental model (Bumann et al., 2002).

The mutation in *dsbI* almost eliminated the ability of *H. pylori* to colonize the stomach ($p<0.01$). These results support the importance of the disulfide bond formation process in the infection. In one out of seven mice, real-time PCR confirmed a low level of residual infection, but the remaining animals remained uncolonized. The extrapolated amount of bacterial DNA quantified by real-time PCR corresponded to 2230–18975 bacterial cells/100 ng of mouse DNA in gastric samples from seven control mice (infected by the wild-type strain SS1) and 0–183 bacterial cells/100 ng of mouse DNA in samples from seven mice infected with the isogenic mutant in *dsbI* (Fig. 1). Mucosa specimens were also checked for *H. pylori* colonization using the qualitative rapid urease test (CLO test) which was read routinely 1 h after gastric mucosa specimen collection and, if negative, 3 and 24 h later. Development of a pink color was indicative of a positive test. In each mouse infected by SS1 wild-type strain the CLO test was positive, whereas it was negative in the case of all mice infected by the mutated strain. The complementation experiment (*dsbI* mutant transformed with *dsbI* gene on a plasmid) was not performed due to the fact that *H. pylori* strain SS1 is poorly tolerant to non-self DNA (Gorrell et al., 2005). A complementation test was conducted using *H. pylori* J99 as described in the previously published paper (Raczko et al., 2005) but this strain does not colonize mice.

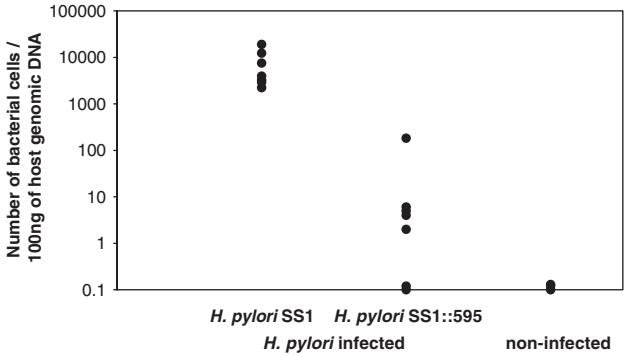

Fig. 1. Gastric colonization level of *H. pylori* in mice infected by the SS1 strain and by SS1/HP0595::Km.

Proteins, which are affected by *dsbI* mutations are still unknown, but as in many pathogenic bacteria, the *dsbI* defect may have an adverse effect on the conformation of many virulence determinants resulting in their misfolding and instability. We carried out a prediction of extracytoplasmic proteins in *H. pylori*. The criteria used were: identification of putative transmembrane helices (Masafumi et al., 2004) or identification of an SpI- or SpII-type signal sequence (Juncker et al., 2003). We found 366 extracytoplasmic proteins in *H. pylori*, 211 of which contain at least two cysteines. This number represents the upper estimation of a set of possible targets of DsbI.

Analysis of experimental data from two-hybrid assays of *H. pylori* available in the http://dpi.nhri.org.tw/hp/ database (Lin et al., 2005) reveals that HP0595 interacts with: HP0025 (Omp2), HP0060, HP0870 (FlgE), HP1052 (EnvA), HP1173, and HP1430. Among these proteins, perhaps the most interesting in terms of pathogenesis is HP1173. It bears a resemblance to a periplasmic protein LipoP, predicted to be anchored with its N-terminus in the inner membrane. HP1173 has two Cys residues, which are most probably located on the surface of the protein (according to our preliminary structure prediction), suggesting that they could be oxygenated by HP0595. It is noteworthy that HP1173 has an ortholog in the J99 strain, but not in any other bacterial genomes analyzed. HP1173 itself interacts with four proteins, which may be involved in pathogenicity of *Helicobacter*.

Another interesting protein interacting with HP0595 is Omp2 (HP0025). Forty paralogs of *hp0025* were found in the *H. pylori* genome, 35 of these paralogs do not have a homolog in other hitherto sequenced genomes. Proteins from the HP0025 family possess characteristic Cys residues, which again make them possible substrates of HP0595. Moreover, 27 of these proteins are, like HP0595, extracytoplasmic proteins (according to LipoP analysis). However, no experimental data are available, to suggest that other members of the HP0025 family interact with HP0595.

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References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Bumann, D., Holland, P., Siejak, F., Koesling, J., Sabarth, N., Lamer, S., Zimny-Arndt, U., Jungblut, P.R., Meyer, T.F., 2002. A comparison of murine and human immunoproteomes of *Helicobacter pylori* validates the preclinical murine infection model for antigen screening. Infect. Immun. 70, 6494–6498.

Dzwonek, A., Mikula, M., Woszczynski, M., Hennig, E., Ostrowski, J., 2004. Protective effect of vaccination with DNA of the *H. pylori* genomic library in experimentally infected mice. Cell Mol. Biol. Lett. 9, 483–495.

Gorrell, R.J., Yang, J., Kusters, J.G., van Vliet, A.H., Robins-Browne, R.M., 2005. Restriction of DNA encoding selectable markers decreases the transformation efficiency of *Helicobacter pylori*. FEMS Immunol. Med. Microbiol. 44, 213–219.

Hatakeyama, M., 2004. Oncogenic mechanisms of the *Helicobacter pylori* CagA protein. Nat. Rev. Cancer 4, 688–694.

Isakov, V., Malfertheiner, P., 2003. *Helicobacter pylori* and nonmalignant diseases. Helicobacter 8, 36–43.

Juncker, A.S., Willenbrock, H., Von Heijne, G., Brunak, S., Nielsen, H., Krogh, A., 2003. Prediction of lipoprotein signal peptides in Gram-negative bacteria. Protein Sci. 12, 1652–1662.

Kadokura, H., 2003. Protein disulfide bond formation in prokaryotes. Annu. Rev. Biochem. 72, 111–135.

Lamarque, D., Peek, R.M.J., 2003. Pathogenesis of *Helicobacter pylori* infection. Helicobacter 8, 21–30.

Lee, A., O'Rourke, J., De, U.M.C., Robertson, B., Daskalopoulos, G., Dixon, M.F., 1997. A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. Gastroenterology 112, 1386–1397.

Lin, C.Y., Chen, C.L., Cho, C.S., Wang, L.M., Chang, C.M., Chen, P.Y., Lo, C.Z., Hsiung, C.A., 2005. hp-DPI: *Helicobacter pylori* database of protein interactomes – embracing experimental and inferred interactions. Bioinformatics 21, 1288–1290.

Masafumi, A., Hironori, M., Masami, I., Jun-Xiong, X., Takashi, K., Masanobu, S., Toshio, S., 2004. ConPred II: a consensus prediction method for obtaining transmembrane topology models with high reliability. Nucleic Acids Res. 32, W390–W393.

Mikula, M., Dzwonek, A., Jagusztyn-Krynicka, E.K., Ostrowski, J., 2003. Quantitative detection for low levels of *Helicobacter pylori* infection in experimentally infected mice by real-time PCR. J. Microbiol. Methods 55, 351–359.

Montecucco, C., de Bernard, M., 2003. Molecular and cellular mechanisms of action of the vacuolating cytotoxin (VacA) and neutrophil-activating protein (HP-NAP) virulence factors of *Helicobacter pylori*. Microbes Infect. 8, 715–721.

Nardone, G., Morgner, A., 2003. *Helicobacter pylori* and gastric malignancies. Helicobacter 8, 44–52.

Raczko, A.M., Bujnicki, J.M., Pawlowski, M., Godlewska, R., Lewandowska, M., Jagusztyn-Krynicka, E.K., 2005. Characterization of new DsbB-like thiol-oxidoreductases of *Campylobacter jejuni* and *Helicobacter pylori* and classification of the DsbB family based on phylogenomic, structural and functional criteria. Microbiology 151, 219–231.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.