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Helicobacter pylori cell translocating kinase (CtkA/JHP0940) is pro-apoptotic in mouse macrophages and acts as auto-phosphorylating tyrosine kinase

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ABSTRACT

The *Helicobacter pylori* gene JHP0940 has been shown to encode a serine/threonine kinase which can induce cytokines in gastric epithelial cells relevant to chronic gastric inflammation. Here we demonstrate that JHP0940 can be secreted by the bacteria, triggers apoptosis in cultured mouse macrophages and acts as an auto-phosphorylating tyrosine kinase.

Recombinant JHP0940 protein was found to decrease the viability of RAW264.7 cells (a mouse macrophage cell line) up to 55% within 24 h of co-incubation. The decreased cellular viability was due to apoptosis, which was confirmed by TUNEL assay and Fas expression analysis by flow-cytometry. Further, we found that caspase-1 and IL-1beta were activated upon treatment with JHP0940. These results point towards possible action through the host inflammasome. Our *in vitro* studies using tyrosine kinase assays further demonstrated that JHP0940 acts as auto-phosphorylating tyrosine kinase and induces pro-inflammatory cytokines in RAW264.7 cells. Upon exposure with JHP0940, these cells secreted IL-1beta, TNF-alpha and IL-6, in a dose- and time-dependent manner, as detected by ELISA and transcript profiling by q-RT-PCR.

The pro-inflammatory, pro-apoptotic and other regulatory responses triggered by JHP0940 lead to the assumption of its possible role in inducing chronic inflammation for enhanced bacterial persistence and escape from host innate immune responses by apoptosis of macrophages.

Introduction

Even 30 years after the discovery of *Helicobacter pylori* (Marshall and Warren, 1984) and establishment of its role in gastric diseases, more than 80% of the population in developing countries (Hunt et al., 2011) as compared to 30–50% in developed countries (Covacci et al., 1999) is infected with the bacterium. However, the prevalence of *H. pylori* infection varies from geographic region to region throughout the world (Ferlay et al., 2010) which is more likely correlated with socioeconomic status of the population (Malaty and Graham, 1994) and sex; because in many populations males have 20–30% higher rates of infection than females (Replogle et al., 1995). Since *H. pylori* is the main cause of gastric cancer, WHO has classified the bacterium as a class I carcinogen in 1994 and severity of the disease in the present scenario is so high that gastric cancer is the second leading cause of cancer related deaths worldwide and constitutes the fourth most common cancer type (Ferlay et al., 2010).

H. pylori causes infection of the gastric mucosa (Marshall and Warren, 1984) and is found to be the most successful colonizer of the human stomach causing chronic gastritis, duodenal ulcer, intestinal metaplasia and gastric cancer as a consequence of long term colonization (Graham, 1997; Uemura et al., 2001). Although the severity of chronic inflammation depends on host genetic factors, environmental parameters and bacterial factors play an important role in the onset of gastric diseases. Among the bacterial factors encoded by various clinical *H. pylori* strains, one of the two most studied proteins is CagA which is encoded within the cytotoxin-associated genes (*cag*) pathogenicity island (PAI) and is about 40 kb in size (Covacci et al., 1993). It has been established that most of the genes from the *cag*PAI act as components of a type IV

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secretion system (T4SS) forming a syringe like structure that facilitates translocation of CagA and probably other macromolecular bacterial factors into the host cells (Backert et al., 2010). Moreover, the strains that harbour the *cag*PAI are associated with more severe gastric inflammation and overt outcomes as compared to *cag*PAI-negative strains. The other most studied *H. pylori* virulence factor is VacA (vacuolating cytotoxin A) which induces cytoplasmic vacuolation by forming membrane channels in epithelial membranes *in vitro* (Cover and Blaser, 1992). Besides this activity, it has a multifunctional role in colonization, virulence and immunomodulation (Cover and Blanke, 2005; Pachathundikandi et al., 2013). Apart from these two cardinal effectors, virulence genes such as BabA, SabA, SabB, OipA etc. (Basso et al., 2010; Odenbreit et al., 2009) code for the other important factors which facilitate adhesion of the bacterium to the gastric epithelium. In addition, IceA that carries a restriction endonuclease activity was also found to be associated with gastritis and duodenal ulcer (Xu et al., 2002). There is an on-going interest in another putative virulence factor, DupA, on account of its association and envisaged role in gastric diseases (Hussein et al., 2010; Shiota et al., 2010; Schmidt et al., 2009). Furthermore, findings on some other *H. pylori* gene products such as protease HtrA (Hoy et al., 2012), AnsB (asparginase), GGT (γ-glutamyl transpeptidase) (McGovern et al., 2001), HP0986 (Alvi et al., 2011), HP-NAP (Satin et al., 2000) and HorB (Snelling et al., 2007) revealed their association with inflammation and pointed to their role in gastric diseases. Given this, the data available about these new generation bacterial factors do not seem to be adequate to fully understand pathogenesis of gastric inflammation and cancer. Therefore, it seems prudent to focus on the discovery of novel virulence factors and their biological roles relevant to the development and progression of gastric diseases. JHP0940 or CtkA is one such potential virulence factor that has been discovered and partly characterized (Rizwan et al., 2008) and its crystal structure has been solved (Kim do et al., 2010).

In the present study, we attempted to investigate the functional roles of JHP0940 which is located in a plasticity region gene cluster of the chromosome. The plasticity region is a highly variable zone that was identified by comparison of complete genome sequences of the two *H. pylori* strains (26695 and J99) that contain approximately 50% strain specific genes (Alm et al., 1999). Some of the genes in this region are thought to be acquired by *H. pylori* through horizontal gene transfer from some environmental bacteria (Datta et al., 2003). Thus, the plasticity zone genes provide an assumption that the proteins encoded by them may contribute to virulence or modulation of host immune responses. The association of jhp940 gene with gastric cancer (Occhialini et al., 2000; Yakoob et al., 2010), expression of JHP940 protein in response to interaction of *H. pylori* with the gastric mucosa in Mongolian gerbils *in vivo* (Graham et al., 2002) and its antigenicity profile prompted us to look at its virulence properties in more detail. Previous observations indicated that JHP940 can induce pro-inflammatory cytokines IL-8 and TNFα and increases translocation of transcription factor NF-κB in the human macrophage cell line Thp1 (Rizwan et al., 2008). Consequently, it was reported that JHP0940 also acts as a cell-translocating Ser/Thr Kinase and could indirectly upregulate the phosphorylation of NF-κB at serine residue 276 in human gastric epithelial cells (Kim do et al., 2010).

In the present study, we further extend the functional acumen of the JHP0940 protein and report its interaction with the host immune apparatus as evident from serum antibody titres present in patients infected with JHP0940-positive *H. pylori*. We also found that JHP940 acts as an auto-phosphorylating tyrosine kinase. This is perhaps the first described tyrosine kinase in *H. pylori* and we could demonstrate that it induces strong pro-inflammatory responses as well as Fas-mediated apoptosis in murine macrophage cells.

Materials and methods

Bacterial strains

The *H. pylori* wild-type strains J99 (Alm et al., 1999), SJM180 (Kersulyte et al., 2003) and P1 (Mueller et al., 2012) were used in the study. *H. pylori* was grown in thin layers on horse serum GC agar plates supplemented with vancomycin (10 μg/ml), nystatin (1 μg/ml), and trimethoprim (5 μg/ml) as described previously (Tegtmeyer et al., 2013). All antibiotics were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bacteria were grown at 37 °C for 2 days in an anaerobic jar containing a Campygen gas mix of 5% O_2, 10% CO_2, and 85% N_2 (Oxoid, Wesel, Germany) (Tegtmeyer et al., 2011).

JHP0940 (Ctk) secretion assays

Bacterial wild-type strains were grown in BHI broth medium supplemented with 10% FCS for 12 h starting with an $OD_{600nm} \sim 0.2$. The supernatant and the cell pellets were separated by centrifugation at 4000 rpm, and the supernatant was further purified from remaining bacterial cells by passage through a 0.21 μm sterile filter (Sigma Aldrich). The resulting bacterial pellets and supernatants were analysed by immunoblotting (Boehm et al., 2012). Absence of live bacteria in the supernatant was also confirmed by incubation on agar plates showing no growth.

Cell culture

Mouse macrophage RAW264.7 cell line was obtained from National Centre for Cell Science (Pune, India) and maintained in RPMI 1640 medium (Hyclone, USA) supplemented with 10% heat inactivated fetal bovine serum (Gibco, USA), 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were trypsinized and seeded for the experiments.

Expression and purification of recombinant JHP0940 protein

The *jhp0940* gene was cloned in pRSET-A expression vector and the protein was expressed in *Escherichia coli* BL21 (DE3) cells, induced with 0.1 mM IPTG. His-tagged JHP0940 was purified as described earlier (Rizwan et al., 2008) with minor modifications, using Cobalt based Talon® resin (Clontech, USA). The homogeneity of the protein was confirmed by SDS-PAGE (12%) and the amount of protein estimated by bicinchoninic acid assay (BCA). The purified protein JHP0940 was treated with polymyxin B beads for 4 h and separated by centrifugation at 10,000 rpm for 10 min. LPS contamination in polymyxin B treated protein was quantitated by *Limulus* amebocyte lysate assay using Chromogenic Endotoxin Quantitation Kit (Pierce Thermo Scientific).

Treatment of cells with JHP0940 and preparation of cell lysate

RAW264.7 cells were cultured in 60 mm culture dish at a density of 4×10^6 per plate and treated with JHP0940 protein. Before harvesting, the cells were washed in cold PBS, scraped by cold plastic cell scraper, transferred to cold microfuge tube and lysed with ice cold lysis buffer (20 mM Tris HCl pH 7.4, 137 mM NaCl, 10% Glycerol, 1% NP-40, 2 mM EDTA, PI cocktail 1 μl/1 × 10^6 cells, 1 mM PMSF, 1 mM DTT, 1 mM Na_3VO_4) by intermittent vortexing for 40–60 s and repeated 5 times after an interval of 10 min and followed by centrifugation at 14,000 rpm for 10 min at 4 °C. Supernatant was collected and quantified by BCA method for western blotting.