Role of HP1076 in type IV secretion pathway in *Helicobacter pylori*: abridged secondary publication

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KEY MESSAGES

1. *Helicobacter pylori* HP1076 is essential for translocation and phosphorylation of CagA.
2. Deletion of HP1076 inhibits transcription of CagD and also affects the assembly of Cag T4SS components CagT and CagM.
3. It is likely that the effects of HP1076 on cag T4SS assembly and activity are mediated by down-regulation of CagD.

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Introduction

Helicobacter pylori infection is recognised as a primary risk factor for gastric adenocarcinoma and gastric lymphoma.[1] Strains of *H pylori* harbouring cag pathogenicity island (PAI) are typically associated with a higher rate of disease.[2] Cag PAI encodes about 27 proteins, some of which comprise a type IV secretion system (T4SS) for the translocation of CagA effector into gastric epithelial cells. Upon translocation, CagA interacts with various host cell proteins, resulting in abnormal proliferation, deregulation of cell-cell contacts and induction of proinflammatory cytokines. Little is known about the structural organisation of T4SS and the mechanistic details of CagA translocation. Notably, nine cag PAI genes essential for CagA delivery are unique in *H pylori*, suggesting that cag T4SS in *H pylori* differs considerably from T4SSs in other bacterial species.

This study focuses on the functional role of an uncharacterised protein HP1076 in cag T4SS. HP1076 is reported to be a co-chaperone of flagellar export chaperone FliS.[3] Interestingly, HP1076-null mutant has no significant effect on flagella synthesis and bacterial motility (unpublished data). Rather, our data show that translocation and phosphorylation of CagA is reduced in gastric epithelial cells infected with *HP1076*-null mutant. We therefore hypothesise that HP1076 may involve in the other secretory pathways, in particular cag T4SS.

Methods

We obtained a collection of 25 Cag antibodies using purified recombinant proteins and commercially synthesised peptides for immunisation. We characterised the expression of Cag proteins, CagA translocation and phosphorylation by immunoblotting upon different infection dosages. In addition, assembly of T4SS was studied by limited proteolysis. Attempts to isolate potential relay proteins of HP1076 were carried out by pull-down assays and 2-D gel electrophoresis.

Results

Cloning, expression and antibody production of Cag T4SS proteins

In this study, all 27 genes for T4SS from *H pylori* were successfully cloned into *E coli* expression vector. Expression and purification of individual Cag proteins were also tested and optimised. Currently, a total of 11 recombinant Cag proteins including Cag3, Cag5, VirB11-like cag protein, CagZ, Cag10, CagX, CagT, CagS, CagM, CagF, CagD were purified for immunisation for polyclonal antibody production. Some of the cag proteins including Cag1, Cag2, Cag4, CagW, CagV, CagU, CagQ, CagN, CagL, CagI, CagH, CagG, CagE, CagC, CagB could not be purified in sufficient quantity for immunisation. Antibodies against these Cag proteins were raised using synthetic peptides.

Characterisation of Cag T4SS protein expression level in wild-type strain and *HP1076*-null mutant

To determine whether HP1076 is linked to the biosynthesis of cag T4SS, expression levels of Cag proteins were analysed by immunoblotting. Our results show that expression levels of cag proteins between G27 wild-type strain and *HP1076*-null mutant were comparable, with an exception of CagD, which was completely undetectable in *HP1076*-null mutant.

Since HP1076 possess co-chaperone activity

towards FliS,[3] we questioned whether HP1076 had a stabilising effect on CagD, preventing it from degradation. Our result showed that there was no direct biophysical contact between HP1076 and CagD, suggesting that the down-regulation of CagD in *HP1076*-null mutant was likely at transcription level. Transcription inactivation of CagD was further confirmed by real-time PCR, that the mRNA level of CagD was reduced by about 70%.

Characterisation of Cag T4SS assembling by limited proteolysis

We further characterised the effect of HP1076 on T4SS assembly by trypsin-limited proteolysis. For T4SS surface exposed proteins, they are accessible to proteolytic attack; therefore, comparison of the trypsin digestion pattern will allow us to probe for any changes in T4SS maturation and assembly. Our results show that the digestion profiles of CagI, CagT and CagX from the G27 strain and HP1076-null mutant were comparable; however, it appears that CagI in *HP1076*-null mutant were slightly resistant to proteolysis. Significant differences of digestion patterns were revealed when cells were first treated with osmotic shock that the bacterial outer membrane was permeabilised before proteolysis. Specifically, CagT in *HP1076*-null mutant was completely digested in small quantity of trypsin. CagM in *HP1076*-null mutant was also shown to be more susceptible to trypsin digestion, though the extent was less severe when compared to CagT. Taken together, these results suggested that deletion of HP1076 interrupted the assembly of T4SS.

Characterisation of CagA phosphorylation and IL-8 production in infected AGS cells

We next evaluated the functional importance of HP1076 and its association with T4SS in bacterial infection, translocation and phosphorylation of CagA in host cells were examined. At early phase of infection, gastric epithelial cells infected with HP1076-null mutant showed a lower level of translocated CagA. After 2 hours of infection, there was no significant difference in CagA level between cells infected with G27 strain and *HP1076*-null mutant. Noteworthy, phosphorylation of CagA was almost completely suppressed in cells were infected with *HP1076*-null mutant. These results suggested that effective translocation and phosphorylation of CagA was HP1076 dependent. Additionally, HP1076-null mutant led to a reduction of IL-8 secretion that may link to inhibition of CagA phosphorylation.

Isolation of HP1076 interacting partners in *H pylori* and AGS cells by pull-down assays

To understand the mechanistic details of HP1076 on T4SS, and CagA secretion and phosphorylation, we applied pull-down assays to isolate potential interacting partner of HP1076 in *H pylori* lysate and gastric epithelia cells. However, no extra band was detected.

Additionally, protein profiling of *H pylori* G27 strain and HP1076-null mutant were analysed by 2D-PAGE. Down-regulation of 9 proteins involved in amino acid metabolism and protein synthesis, and up-regulation of 6 proteins involved in antioxidant defence mechanism were found in HP1076-null mutant.

Discussion

Disease outcomes of *H pylori* infection are tightly associated with bacterial strains that possess cag pathogenicity island, which encodes protein components of a T4SS, an effector protein CagA and various proteins that are unique but with unknown function in *H pylori*. Our research team has previously found that HP1076 knockout strain had a reduced secretion of CagA to gastric epithelial cells (unpublished data). Since secretion of cagA is fully dependent on cag T4SS, we hypothesised that HP1076 may be involved in T4SS. In the present study, we successfully obtained a repertoire of Cag antibodies using purified recombinant proteins and commercially synthesised peptides. Deletion of HP1076 led to transcription inactivation of CagD. CagD has been reported to be essential for CagA translocation.[4] It is unclear whether the inhibitory effect of HP1076 on CagA secretion is a direct mechanism or indirectly mediated through down-regulation of CagD. Since the crystal structure of HP1076 does not display any positively charged groove for nucleotide binding,[3] we speculate that HP1076 may inactivate the transcription of CagD via unidentified role.

In general, cag PAI encodes numerous protein orthologues of VirB/VirD4 system that the two T4SS may share similar core structure. However, little is known about T4SS that secrete effector proteins, especially cag T4SS. Recently study has isolated the membrane spanning core complex of cag T4SS which is composed of CagM, CagT, Cag3, CagX and CagY,[5] among which CagM, CagT and Cag3 are unique to *H pylori*. Here, our results from limited proteolysis indicated that HP1076 may interfere the structural assembly of cag T4SS, especially CagT and CagM. Since both Cag proteins are key structural components required for T4SS activity,[5] it implies that the reduction of CagA translocation in HP1076-null mutant may also be due to defect in T4SS assembly, in addition to the down-regulation of CagD.

Our study further demonstrated that deletion of HP1076 not only suppressed CagA translocation. Interestingly, tyrosine-phosphorylation of CagA was severely inhibited. CagA phosphorylation is catalysed by Src family protein kinases and c-Abl

kinase. Taken together with the results from the pull-down assays, it is unlikely that the HP1076 directly binds on the two kinases and inhibits their CagA phosphorylation activity. A previous genetic study showed that CagD is necessary for both translocation and phosphorylation of CagA,[4] we speculate that the reduced CagA phosphorylation is mediated through down-regulation of CagD induced by HP1076 knockout. In contrast, though CagD is associated with the maximal IL-8 release,[4] our results did not reveal any difference in IL-8 secretion between wild type G27 strain and HP1076-null mutant.

Collectively, our results suggest that HP1076 is necessary for cag T4SS activity, especially CagD expression. Since CagD can be found as a secreted protein, cytosolic protein and inner membrane protein, it may play a multifunctional role in T4SS. Probably, the effects of HP1076 on T4SS assembly, and CagA translocation and phosphorylation are primary due to the inactivation of CagD gene expression, though direct function of HP1076 on T4SS cannot be eliminated and needs further investigation. We attempted to isolate interacting partner of HP1076 in both gastric epithelial cells and *H pylori*, however no stable complex can be obtained. Comparison of protein profiling between the G27 strain and HP1076 revealed various differentially expressed proteins. Yet, none of them have been linked to T4SS that further in-depth studies are required in the future. In conclusion, the present study provides an example to demonstrate the regulation of T4SS beyond the cag pathogenicity island.

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