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IDENTIFICATION OF RESIDUES INVOLVED IN NUCLEOTIDYLTRANSFERASE ACTIVITY OF JHP933 FROM *HELICOBACTER PYLORI* BY SITE-DIRECTED MUTAGENESIS

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Abstract: *Helicobacter pylori* is a well-known bacterial pathogen involved in the development of peptic ulcer, gastric adenocarcinoma and other forms of gastric cancer. Evidence has suggested that certain strain-specific genes in the plasticity region may play key roles in the pathogenesis of *H. pylori*-associated gastroduodenal diseases. Therefore there is considerable interest in the strain-specific genes located in the plasticity regions of *H. pylori*. JHP933 is encoded by the gene in the plasticity region of *H. pylori* strain J99. Recently, the crystal structure of JHP933 has confirmed it as a nucleotidyltransferase (NTase) superfamily protein and a putative active site has been proposed. However, no evidence from direct functional assay has been presented to confirm the active site and little is known about the functional mechanism of JHP933. Here, through superimposition with Cid1/NTP complex structures, we modelled the complex structures of JHP933 with different NTPs. Based on the models and using rational site-directed mutagenesis combined with enzymatic activity assays, we confirm the active site and identify several residues important for the nucleotidyl transferring function of JHP933. Furthermore, mutations of these active site residues result in the abolishment of the nucleotidyltransferase activity of JHP933. This work provides preliminary insight into the molecular mechanism underlying the pathophysiological role in *H. pylori* infection of JHP933 as a novel NTase superfamily protein.

Key words: JHP933; nucleotidyltransferase; site-directed mutagenesis; enzymatic assay; *Helicobacter pylori*; plasticity regions

INTRODUCTION

Helicobacter pylori is a widespread bacterial pathogen, which infects approximately 50% of the world's population. *H. pylori* infection plays an important role in chronic gastric inflammation, developing into a variety of diseases ranging from mild gastritis to peptic ulcers and some forms of gastric cancer [1, 2]. The complex pathology for the various clinical outcomes remains elusive. However, it has been proposed that genetic variability underlying the host adaptation differences of various *H. pylori* strains may manifest in various degrees of disease severities [3, 4].

It is demonstrated that nearly half of the strain-specific genes of *H. pylori* are preferentially located in the plasticity zones by genome sequence comparisons in the first fully sequenced *H. pylori* strains J99 and 26695 [5, 6]. Recently, there has been considerable interest in the strain-specific genes of plasticity regions and studies have suggested that some of these genes are associated with the pathogenesis of *H. pylori* related diseases [7-9]. As studied in *H. pylori* strain J99 (*jhp914-jhp961*), *jhp947* is thought to be significantly associated with duodenal ulcer and gastric cancer, and could become a good candidate marker for gastroduodenal diseases [7, 10]. *DupA (jhp917-jhp918)* is another pathogenicity-associated gene in the plasticity regions that encodes homologs of the VirB4 ATPase and could affect the development of duodenal ulcer and gastric cancer [11]. *Tfs3* and *tfs4*,

located in two different plasticity zones of *H. pylori*, have been characterized as a Type IV secretion system (T4SS) apparatus, playing important roles in DNA transfer and contributing to bacterial genetic variability [6,9,12,13].

Jhp933 is a conservative gene of the plasticity region of *H. pylori* and is associated with gastritis and peptic ulcer by analyses of clinical prevalence rate [12, 14,15]. Performing conserved domain search has suggested that JHP933 might be classified into the nucleotidyltransferase (NTase) superfamily. The NTase superfamily constitutes widely diverse proteins with distinct biological functions, including chromatin remodeling, RNA polyadenylation, RNA editing, DNA repairing, protein activity regulation, and antibiotic resistance [16-18]. Though NTase family proteins have different biological roles, it is recognized that almost all of them have the common function of transferring NMP from NTP to their distinct second substrate acceptor [16]. Recently, two similar crystal structures of JHP933 from the *H. pylori* strain J99 have been determined and the structural information indicated that JHP933 is an NTase superfamily protein with a characteristic αβαβαβα topology. Several conservative active site motifs, which are critical for maintaining the NTP hydrolysis function, have been suggested [19, 20]. However, the putative active site of JHP933 awaits confirmation by direct functional evidence and the NTP-binding property needs more studies for clarification. Moreover, the determination of the exact role and molecular mechanism of JHP933 remains to be done.

Here we have modelled the complex structures of JHP933 with different nucleotide substrates by superimposition of JHP933 structure with Cid1/NTP complex structures. Based on the structural information, we have performed the rational site-directed mutagenesis and enzymatic activity assays on pinpointed residues; the results confirm the active site of JHP933 and identify several conservative residues key to the nucleotidyltransferase activity of JHP933. Our work paves a preliminary way towards exploiting the functional mechanisms underlying the role of JHP933 in *H. pylori* pathogenesis.

MATERIALS AND METHODS

Homology modelling of JHP933/NTP complex structures

By superimposition of the JHP933 structure (protein data bank (PDB) code: 4O8S) with the known complex structures of Cid1/NTP (PDB codes: 4FH5, 4FHV, 4FHW, 4FHY) as a reference template, 4 different NTPs (ATP, GTP, UTP, CTP) were superimposed onto the structure of JHP933, respectively. The superimposition was performed by using PyMOL (www.pymol.org) and CCP4 LSQ [21].

Recombinant DNA constitution

The gene encoding the full-length JHP933 was amplified from DNA of the *H. pylori* strain J99 by PCR. The amplified gene was fitted with *Bam*HI and *Xho*I sites at the 5' and 3' ends, respectively. The cassette was cloned into modified pET32a with inserted N-terminal Trx and Histidine-tag. Based on the constituted pET32a-JHP933, different point mutations were introduced using the QuikChange® II mutagenesis kit.

Expression and purification of proteins

Proteins were produced into *E. coli* BL21(DE3) cells. Cells were induced with 0.3 mM IPTG (isopropyl β-D-thiogalactoside) and harvested after expression for 12-16 h at 16°C. For purification, cells were lysed by ultrasonication on ice in a buffer containing 50 mM Tris (Ph 8.0), 300 mM NaCl, 0.1% Triton-X 100 and 5% glycerol. After centrifugation at 39000*x*g for 20 min, the soluble N-terminally Trx and Histidine-tagged proteins were bound to nickel-sepharose affinity resin. The tags of the eluted proteins were removed by cleavage with TEV protease, then further purified with size exclusion chromatography at 50 mM Tris (pH 8.0), 100 mM NaCl and 1 mM $MgCl_2$. The purity of the proteins was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Enzymatic activity assays

The EnzCheck® pyrophosphate assay kit from Invitrogen was used to analyze the activity of the wild-type and mutant JHP933 proteins based on a previously described method [22]. The standard 1 mL reaction mixture contains:720 μL-x μL dH_2O, 50 μL 20X reaction buffer containing $MgCl_2$, 200 μL MESG substrate solution, 10 μL NTP, x uL protein, 10 μL purine nucleoside phosphorylase and 10 μL inorganic pyrophosphatase. The components are added to the reaction mixture in the order listed above, then incubated for 30-60 min at 22°C. The absorbance at 360 nm was measured and corrected for a control reaction lacking JHP933.

Site-directed mutagenesis

QuikChange ® II Site-Directed mutagenesis (Stratagene) was used to generate mutants G39A, D55Q, D57Q and E113N of JHP933 in a pET32a-JHP933 vector as per the manufacturer's instructions. The mutagenesis results were confirmed via DNA sequencing. The recombinant DNAs were transformed into *E. coli* Top10 and proteins were expressed in *E. coli* BL-21(DE3).

RESULTS AND DISCUSSION

Homology modelling of JHP933 for NTP binding

The crystal structure of JHP933 points to a putative active site for substrate binding [23]. However, the special second substrate is unknown, except that NTP is recognized to be a common first substrate for most of the NTase superfamily proteins. By superimposition with the known complex structures of Cid1/NTP, the structures of JHP933 complexed with magnesium ions and 4 different NTPs (ATP, GTP, UTP, CTP) were modelled (Fig. 1). A groove conserved in both structures of JHP933 and Cid1 was identified, which is formed mainly by β-strands β1, β2, β5 and α-helices α2, α4, α5 in JHP933 (the numbering of the secondary structure follows the definition in the structure of JHP933 [19]), and is large enough to accommodate

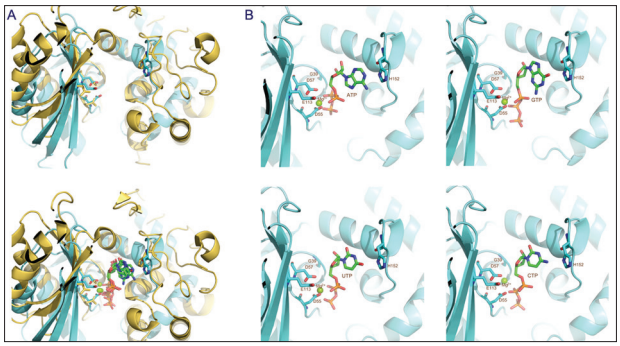

Fig. 1. Modelled JHP933/NTP complex structures. A. superimposition of structures of JHP933 (PDB code: 4O8S) in cyan and Cid1/NTP (PDB codes: 4FH5, 4FHV, 4FHW, 4FHY) in gold, with (top) and without (bottom) NTP substrates. B. modelled complex structures of JHP933 with 4 different NTPs (ATP, GTP, UTP, CTP); NTPs, magnesium ion and conservative active site residues are labelled and shown in a ball-and-stick presentation.

NTP, divalent ions and the unidentified second substrate. Zooming into the active site for NTP-binding, the strictly conservative residues among the orthologs in *H. pylori*, including G39, D55, D57 and E113, were pinpointed according to the modeled complex structures. These residues mainly contribute to chelating magnesium ions and coupling the interaction with triphosphate tail of NTP (Fig. 1) [23]. In all 4 modeled complex structures, the base of NTP is located adjacent to the loop connecting α-helices α4 and α5 containing residue H152, implying this loop region may play an important role in NTP substrate recognition, and the flexibility property of this loop may endow the capability of JHP933 for accommodating different NTP substrates.

NTP transferring property of JHP933

To test the nucleotidyltransferase activity of JHP933, pyrophosphate assays of wild-type JHP933 for 5 different NTPs were performed. The results indicated that JHP933 could catalyze all five nucleotides and liberate the inorganic pyrophosphate at a high level with no obvious preference (Fig. 2). This provides the first direct evidence that JHP933 has the capacity to hydrolyze NTP to NMP, and the *in vitro* nucleotidyl transferring function of JHP933 is likely independent of its second substrate.

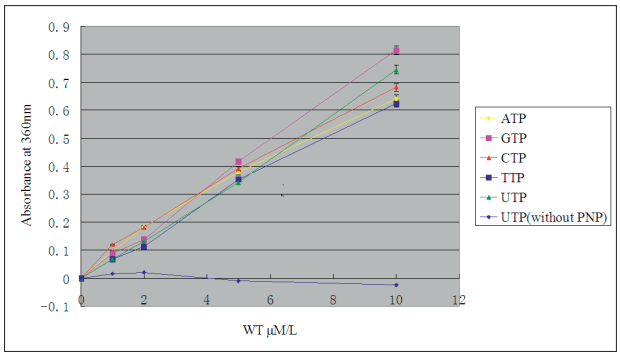

Fig. 2. Nucleotidyltransferase activity of JHP933 for different NTPs. The absorbance of OD_{360} measurement of JHP933 catalytic activity for 5 different NTPs, including ATP, GTP, CTP, TTP, UTP, with UTP (without purine nucleoside phosphorylase) as a control. The following reagents were combined in 1 mL reaction volumes: 1 mM NTP, 0.2 mM MESG, 1 U purine nucleoside phosphorylase, 0.03 U pyrophosphatase, 1× reaction buffer and various dilutions of JHP933. UTP (without PNP) was used to confirm that the protein had no absorbance at 360 nm. After incubating at 22°C for 45 min, the absorbance at 360 nm was measured and corrected for absorbance at 360 nm of a control reaction lacking JHP933. Values represent the mean of three independent experiments; bars represent standard error of mean (SEM); $p<0.05$.

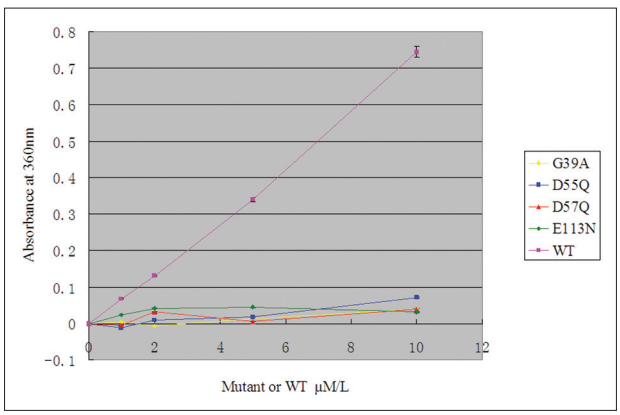

Fig. 3. Comparison of nucleotidyltransferase activity of wild-type and mutant JHP933. The absorbance of OD_{360} measurement of wild-type and mutant JHP933 catalytic activity for UTP. The following reagents were combined in 1 mL reaction volumes: 1 mM UTP, 0.2 mM MESG, 1 U purine nucleoside phosphorylase, 0.03 U pyrophosphatase, 1× reaction buffer and various dilutions of wild-type and mutant JHP933, respectively. After incubating at 22°C for 45 min, the absorbance at 360 nm was measured and corrected for absorbance at 360 nm of a control reaction lacking wild-type or mutant JHP933. Values represent the mean of 3 independent experiments; bars represent standard error of mean (SEM); $p<0.05$.

Active site residue mutations abolish JHP933 nucleotidyltransferase function

Based on the structural information of the different JHP933/NTP models and the sequence homology analysis, the strictly conservative residues critical for divalent ion chelation and NTP triphosphate tail interaction including G39, D55, D57 and E113 were mutated for further investigation of the NTP-binding and enzymatic activity property of JHP933. All these variants were shown to maintain correct monomer folding when purified with a size exclusion column (Fig. S1). Using the pyrophosphate assay of wild type for reference and UTP as a NTP substrate to test the enzymatic activity of JHP933 mutants, all mutations of these residues individually almost completely abolished the NTP nucleotidyl transferring activity of JHP933, demonstrating their important role in NTP hydrolysis (Fig. 3). This work also confirms that this putative active site is involved in the NTP-binding of JHP933. The signature motifs, including these residues, are highly evolutionary conservative NTase superfamily proteins, implying that the NMP transferring function might be prerequisite and essential for NTase family proteins to play their different biological roles.

For almost all known members of NTase superfamily proteins, with the common function of transferring NMP from NTP to their different acceptor, some NTase fold proteins may have preference for their NTP substrate selectivity, such as poly(A) polymerases (PAPs), poly(U) polymerases (PUPs), terminal uridil transferases (TUTases) and CCases. However, some may not have any selectivity. By structural comparison with another NTase protein, *S. pombe* Cid1, it has been suggested that JHP933 may have a substrate preference for molecules resembling UTP [20]. However, this hypothesis needs further investigation due to a lack of any direct evidence. Cid1 was first identified as a PAP and later reclassified as a PUP [24-26]. The structures of Cid1 have demonstrated that it can still accommodate 4 different NTPs at the active site and a residue mutation H336N can switch its selectivity from UTP to ATP [27]. Here, our en-

zymatic assay of JHP933 on different NTP substrates failed to show JHP933 has a preference for nucleotide recognition *in vitro*. The modelled JHP933/NTP complex structures indicated that the nucleotide recognition motif loop is located between the α-helices α4 and α5, which may give JHP933 certain flexibility in accommodating different NTP substrates, and thus might be a better strategy for *H. pylori* to survive during the evolution process. With the rational site-directed mutagenesis and enzymatic assay, we further pinpointed the residues key to JHP933's nucleotidyl transferring function and confirmed the active site for JHP933, thus providing a preliminary insight into the molecular mechanisms of the nucleotide-binding and transferring activity of JHP933. However, to elucidate the exact roles played by active site residues and to decipher the biological roles of JHP933, the three-dimensional structure determination of the JHP933/NTP/second substrate complex is needed.

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Conflict of interest disclosure: The authors declared that they have no conflicts of interest to this work.

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SUPPLEMENTARY MATERIAL (Available online)

Supplementary Fig. S1. Size exclusion chromatography of constructs of JHP933. The JHP933 variants were purified and assessed by size-exclusion chromatography (Superdex 75 pg, column volume 120 mL), the single sharp Gaussian peak migrated to 31 kDa as compared to a standard chromatogram test, implicating that the variants were well-folded and stable.
Available at: http://serbiosoc.org.rs/arch/files/SupplFig1S.jpg