Supplement information

Characterization and use of *Helicobacter pylori* SecAN68 ATPase for probing SecA inhibitors as antibacterial agents

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1 Material and Methods

1.1 Materials

1.1.1 Plasmids and Strains

E. coli DH5α and *E. coli* BL21 (DE3) are from our collection. Primers, the recombinant expression plasmid pET-28a(+)-*Hp*SecAN68 fragment (a fragment of 610 amino acid residues at the N-terminal end of *H. pylori* SecA protein, about 69 kDa in size) are synthesized by Tsingke Biotechnology Company (TBC).

1.1.2 Reagents

The plasmid extraction kit (DP103) was from TBC, the BCA protein concentration assay kit (BL521A) was purchased from Biosharp, Ni-NTA (C600033) was from BBI, protein pre-staining Maker was from TBC, Restriction endonuclease was from NEB (Beijing), Isopropyl-β-D-thiogalactoside (IPTG) and Kanamycin (Kana) were from BioFroxx.

1.2 Methods

1.2.1 Construction of recombinant strains

The *Hp*SecAN68 gene fragment with 6×His-tag coding sequence was chemically synthesized, and *BamH* I and *Xho* I digest sites were added at both ends of the sequence and cloned into the pET-28a(+) vector. *E. coli* BL21 (DE3) was transformed by the heat-activated method, and the strain was identified by double digestion using the pET-28a(+) vector and preserved.

1.2.2 Expression of recombinant plasmids

BL21(DE3)-pET-28a(+)-*Hp*SecAN68 strain was grown on LB solid medium (containing 50 μg/mL Kana), and positive colonies were picked to prepare saturated overnight cultures (SONC). SONC was transferred to fresh media and incubated until the OD_{600} was 0.6, then 1 mM IPTG was added and induced for another 4 h at 37 °C and 200 rpm/min. 1 mL of the culture was centrifuged and the cell pellet was resuspended in PBS the cells were broken by ultrasonication. The suspension was centrifuged at 4 °C, 11000 r/min for 20 min, to separate the supernatant and precipitate. Finally, uninduced whole bacteria protein, the supernatant and precipitation of induced whole bacterial protein, and the induced whole bacterial supernatants and pellets after ultrasound are collected for SDS-PAGE analysis.

1.2.3 Optimization of expression conditions

The IPTG concentration gradient was set as 0 mM, 0.1 mM, 0.25 mM, 0.5 mM, 0.75 mM, and 1 mM to induce protein expression. The starting OD_{600} was set as 0.4, 0.6, 0.8, and 1.0 to induce expression. The induction time was set as 4 h, 10 h, 16 h, and 24 h to induce expression. The remaining steps of protein expression are the same as in 1.2.2.

1.2.4 Protein purification

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The bacterial cells were collected by centrifugation at 5000 rpm, 4 °C, for 15 min, and resuspended with 10 mL binding solution (10 mM Tris-HCl, pH 7.9, 500 mM NaCl, 20 mM Imidazole), then in an ice bath broken for 40 min with sonication. The supernatant after centrifugation at 4 °C, 11000 rpm for 15 min, was then loaded onto a Ni-NTA affinity column. After washing, the His-tag *Hp*SecAN68 protein was eluted with 200 mM Imidazole. Protein concentration was measured by the BCA Protein Test Kit and proteins were analyzed by SDS PAGE.

1.2.5 Analysis of the enzymatic properties of recombinant *Hp*SecAN68

SecA catalyzes the production of ADP and phosphate ions from ATP. So the catalytic activity of *Hp*SecAN68 ATPase is measured by the change in light absorption value at 340 nm to reflect the rate of NADH consumption. The enzymatic reactions use pyruvate kinase (PK) and lactate dehydrogenase (LDH) as coenzymes[1].

$$\mathrm{ATP} \xrightarrow{Hp\mathrm{SecAN68}} \mathrm{ADP} + \mathrm{Pi}$$
$$\mathrm{ADP} + \mathrm{PEP} \xrightarrow{\mathrm{PK}} \mathrm{ATP} + \mathrm{Pyruvic\ acid}$$
$$\mathrm{Pyruvic\ acid} + \mathrm{NADH} \xrightarrow{\mathrm{LDH}} \mathrm{Lactic\ acid} + \mathrm{NAD^+}$$

The methods of Schulte[2] and Wang[3] et al were modified: a 200 μL enzyme activity assay system contained 100 mM Hepes (pH=7~8), 1 mM phosphoenolpyruvate (PEP), 5 U pyruvate kinase, 5 U lactate dehydrogenase, 3~8 μg *Hp*SecAN68, 0~2.5 mM NADH, 0~20 mM $MgCl_2$, 0~12.5 mM $MnCl_2$, 0~12.5 mM ATP, and the blank control was an equal volume of ddH_2O without *Hp*SecAN68. The reaction mixtures were incubated in a 96-well plate at 37 °C for 50 min, and the ΔmOD_{340}/min was calculated for 60 min.

1.2.6 Determination of kinetic parameters of recombinant *Hp*SecAN68

*Hp*SecAN68 ATPase activity was measured at different ATP substrate concentrations (0-12.5 mM), the Molar Absorbance Coefficient (ε) is 6.22 $L \cdot mmol^{\cdot -1} \cdot cm^{-1}$ in 340 nm. The double inverse curve was plotted using Lineweaver-Burk method. Origin 2019b software was fitted to the Mie equation V=Vmax* [S]/(Km+[S]), calculating the Mie affinity constant (Km) and the maximum reaction rate (Vmax) of the enzyme.

1.2.7 Recombinant *Hp*SecAN68 for the inhibitor screening assay

*Hp*SecAN68 catalyzes ATP, releasing energy for protein and polypeptide chain transport. The complex formed between malachite green, molybdate, and free orthophosphate has light absorption at 660 nm, and the catalytic reaction is shown below.

$$\mathrm{ATP} \xrightarrow{Hp\mathrm{SecAN68}} \mathrm{ADP} + \mathrm{Pi}$$
$$\mathrm{Pi} + \mathrm{Malachite\ green} + \mathrm{Ammonium\ molybdate\ tetrahydrate(H+)} \xrightarrow{\mathrm{LDH}} \mathrm{Malachite\ green} - \mathrm{Ammonium\ phosphomolybdate}$$

The enzyme system of Hsieh[4] et al, was modified: 1 μL 10× enzyme buffer, 0.8 μg/μL *Hp*SecAN68, 6 mM ATP, and finally ddH_2O to a total reaction mixture of 10 μL, and the blank control was an equal volume of ddH_2O instead of

*Hp*SecAN68. DMSO working concentration of 5% for all drug screening groups. The above reaction mixtures were incubated at 30 °C for 60 min, then 160 μL of color reagent was added for 1 min, and finally 20 μL of 34% sodium citrate solution was added to terminate the reaction. The absorbance values at 660 nm were measured.

The *Hp*SecAN68 protein ATPase activity assay system is shown in **Table 1**. The effects of the inhibitor on SecA activity were calculated based on the absorbance value at 660 nm.

To assess the inhibitory effect of the three inhibitors, we employed the micro broth method. The assay system consisted of 185 μL of MH broth medium, 10 μL of diluted bacterial solution ($1x10^7$ CFU/mL), 5 μL of various concentrations of diluted drug solution, and the control group was substituted with an equal volume of DMSO. This resulted in a total volume of 200 μL, ensuring a working concentration of 2.5% DMSO in each group. The mixtures were incubated at 37°C for 24 h, and detected OD_{600}. The inhibition was calculated according to the following formula, in which the OD_{600} of the positive control group was calculated according to the initial value.

$$\text{Inhibition rate (\%)} = \left(1 - \frac{\text{OD}_{660}\ \text{(sample)} - \text{OD}_{660}\ \text{(Positive control)}}{\text{OD}_{660}\ \text{(Negative control)} - \text{OD}_{660}\ \text{(Positive control)}}\right) \times 100\%$$

Table 1 The screening system of *Hp*SecAN68 ATPase activity

	Blank control	**Positive control**	**Negative control**	**Sample**
10× enzyme buffer	+	+	+	+
*Hp*SecAN68	-	+(thermal inactivation)	+	+
ATP	+	+	+	+
ddH_2O	+	+	+	+
SecA inhibitor	-	-	-	+

2 Partial results information

2.1 Construction of recombinant *Hp*SecAN68

The recombinant plasmid pET-28a(+)-*Hp*SecAN68 was analyzed by *BamH* I and *Xho* I double digestion. The size of the target DNA fragment matched with *Hp*SecAN68 (1830 bp) (Figure 1), with the vector fragment at 5230 bp. The recombinant DNA was sequenced by TBC. The result showed that the coding DNA fragment had been correctly inserted into the pET-28a(+) plasmid with the N-terminal fusion to the His-Tag. This indicates that the *Hp*SecAN68 recombinant plasmid has been successfully constructed and used for subsequent protein expression.

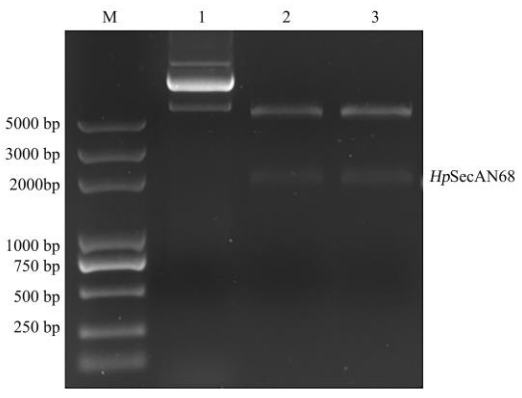

Fig.1 Digestion map of recombinant plasmids pET-28a(+)-*Hp*SecAN68. M: DNA Marker, 1: pET-28a(+)-*Hp*SecAN68, lane 2 and 3: pET-28a(+)-*Hp*SecAN68 digested with *BamH* I and *Xho* I.

2.2 Optimization of expression conditions of recombinant *Hp*SecAN68

Expression condition is essential for high levels of protein expression. The prokaryotic expression system of *Hp*SecAN68 was optimized under four conditions: IPTG concentration, initial OD_{600} of induction, induction temperature, induction time, and IPTG concentration to induce *Hp*SecAN68 expression.

Using the pET-28a(+) empty vector as the control, the results of SDS-PAGE showed that the recombinant plasmid pET-28a(+)-*Hp*SecAN68 induced by IPTG showed an obvious band at 69 kDa position Figure 2**A**, indicating that *Hp*SecAN68 was successfully expressed in BL21 (DE3). The moderate concentration of 0.75 mM IPTG was selected for induction of expression. The starting OD_{600} of bacteria hosting *Hp*SecAN68 has a certain influence on protein expression, inducing protein expression during the early logarithmic growth period may impose a metabolic burden, leading to slower bacterial growth. When the OD_{600} reaches approximately 1.0, although the bacterial population is high, it is more likely to form inclusion bodies, which are not favorable for subsequent purification experiments. As shown in Figure 2**B**, when starting OD_{600} is 0.4, the target band was narrower, and the protein expression was highest when starting OD_{600} is 0.6, so the bacteria at starting OD_{600} of 0.6 were chosen as initial induction bacteria.

Temperature is the major influential factor for *Hp*SecAN68 soluble expression, in which *E. coli* proliferates fast at 37 °C, and the protein expression rate is fast accordingly. However, the exogenous expressed *Hp*SecAN68 is prone to misfolding and forming inclusion bodies, leading to insoluble expression. As shown in Figure 2**C**, the soluble expression was the highest, and the expression was also the maximum, indicating that 20 °C was the most suitable induction temperature. Though the protein expression rate decreased, it effectively improves the correct folding and also avoids the appearance of inclusion bodies, resulting increases in the soluble expressed *Hp*SecAN68. As shown in Figure 2**D**, when the expression was induced for 16 h, the expression level of *Hp*SecAN68 was the highest. Collectively, the optimal expression condition of soluble *Hp*SecAN68 was 0.75 mM IPTG, 20 °C, an initial induction OD_{600} of 0.6 and 16 h induction time.

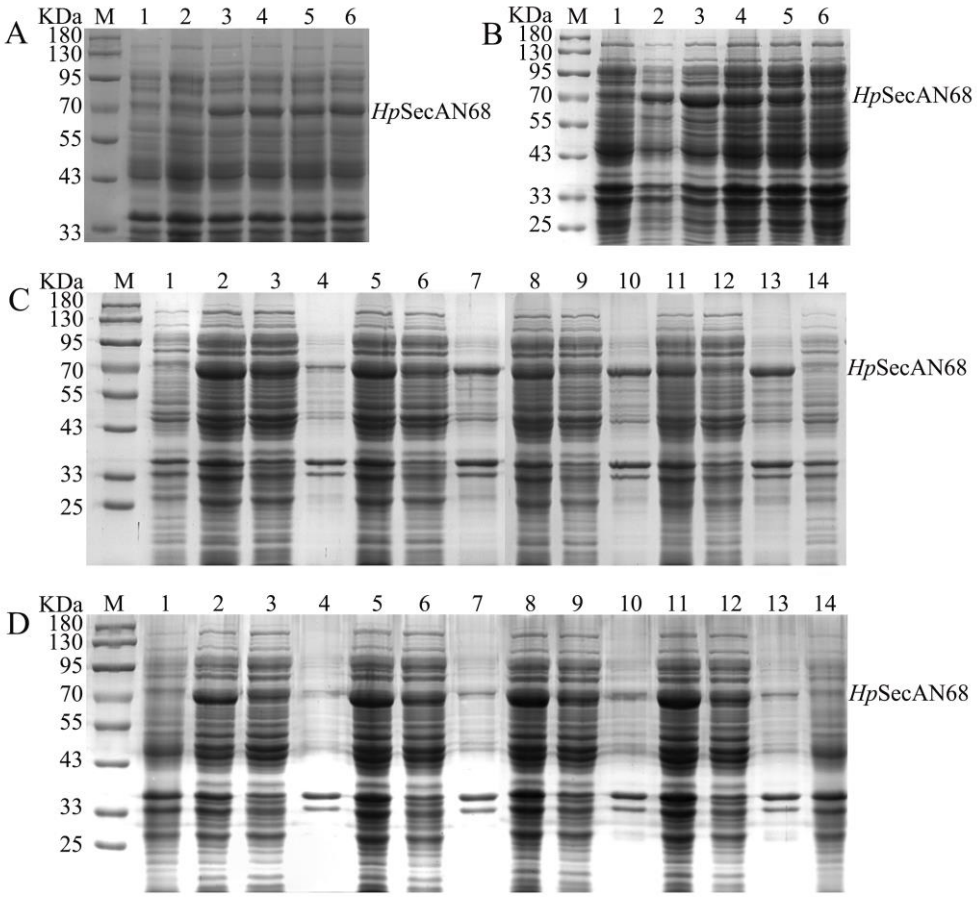

Fig.2 Effects of IPTG, starting OD_{600}, temperatures, and induction time on *Hp*SecAN68 expression. **(A) IPTG.** M: Protein Marker, 1: Cells with (pET-28a(+)), 2: 0 mM IPTG, 3: 0.1 mM IPTG, 4: 0.25 mM IPTG, 5: 0.5 mM IPTG, 6: 0.75 mM IPTG. **(B) Starting OD_{600}.** M: Protein Marker, 1: Cells with (pET-28a(+)), 2: OD_{600}=0.4, 3: OD_{600}=0.6, 4: OD_{600}=0.8, 5: OD_{600}=1.0, 6: Uninduced *Hp*SecAN68. **(C) Temperatures.** M: Protein Marker, 1: Cells with (pET-28a(+)), 2: 20 °C Whole bacterial protein, 3: 20 °C Supernatant, 4: 20 °C Sediment, 5: 25 °C Whole bacterial protein, 6: 25 °C Supernatant, 7: 25 °C Sediment, 8: 30 °C Whole bacterial protein, 9: 30 °C Supernatant, 10: 30 °C Sediment, 11: 37 °C Whole bacterial protein, 12: 37 °C Supernatant, 13: 37 °C Sediment, 14: Uninduced *Hp*SecAN68. **(D) Induction time.** M: Protein Marker, 1: Cells with (pET-28a(+)), 2: 4 h Whole bacterial protein, 3: 4 h Supernatant, 4: 4 h Sediment, 5: 10 h Whole bacterial protein, 6: 10 h Supernatant, 7: 10 h Sediment, 8: 16 h Whole bacterial protein, 9: 16 h Supernatant, 10: 16 h Sediment, 11: 24 h Whole bacterial protein, 12: 24 h Supernatant, 13: 24 h Sediment, 14: Uninduced *Hp*SecAN68.

2.3 Purification of recombinant *Hp*SecAN68

Expression of *Hp*SecAN68 protein was induced under optimal conditions, and the cells were broken after ultrasonication and centrifugation. The supernatant was subjected to a nickel affinity chromatography column for

purification of His-tag *Hp*SecAN68. The impurity proteins were eluted with 20 mM and 60 mM imidazole buffer until there was no impurity protein band. At the imidazole concentration of 200 mM, the *Hp*SecAN68 protein showed a single major band with good purity (>90%) and was used for subsequent experiments (Figure 3).

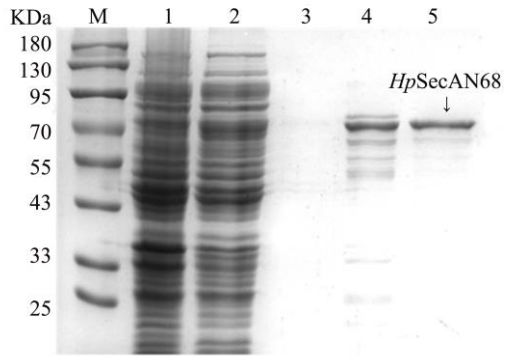

Fig.3 Purification of *Hp*SecAN68 protein. M: Protein Marker, 1: Empty (pET-28a(+)), 2: Purified through-liquid, 3: 20 mM Imidazole 4th eluate product, 4: 60 mM Imidazole eluate product, 5: 200 mM Imidazole eluate product.

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Conflicts of Interest: There are no conflicts of interest to declare.

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