



# Transcriptomic and Functional Approaches Unveil the Role of tmRNA in Zinc Acetate Mediated Levofloxacin Sensitivity in *Helicobacter pylori*

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**ABSTRACT** Rapid increase in resistance of *Helicobacter pylori* (*H. pylori*) has hindered antibiotics-based eradication efforts worldwide and raises the need for additional approaches. Here, we investigate the role of zinc-based compounds in inhibiting *H. pylori* growth and modulating antibiotic sensitivities, interrogate their downstream transcriptomic changes, and highlight the potential mechanism driving the observed effects. We showed that zinc acetate inhibited *H. pylori* growth and increased *H. pylori* sensitivity to levofloxacin. Transcriptomic profiling showed distinct gene expression patterns between zinc acetate treated groups *versus* controls. In particular, we independently replicated the association between zinc acetate treatment and increased *ssrA* expression. Knockdown of *ssrA* restored levofloxacin resistance to levels of the control group. In this study, we first demonstrated the role of zinc acetate in *H. pylori* growth and antibiotic sensitivities. Additionally, we explored the transcriptomic perturbations of zinc acetate followed by functional knockdown follow-up of differentially expressed *ssrA*, highlighting the role of tmRNA and trans-translation in *H. pylori* levofloxacin resistance. Our results provide alternative and complementary strategies for *H. pylori* treatment and shed light on the underlying mechanisms driving these effects.

**IMPORTANCE** *Helicobacter pylori* (*H. pylori*) eradication plays an important role in gastric cancer prevention, but the antimicrobial resistance of *H. pylori* is fast becoming a growing concern. In this study, we investigated the role of zinc acetate in inhibiting *H. pylori* growth and modulating antibiotic sensitivities *in vitro*. Additionally, we explored the transcriptomic perturbations of zinc acetate followed by functional knockdown follow-up of differentially expressed *ssrA*, highlighting the role of tmRNA and trans-translation in *H. pylori* levofloxacin resistance. Our results open up a new horizon for the treatment of antibiotic-resistant *H. pylori*.

**KEYWORDS** *Helicobacter pylori*, zinc acetate, levofloxacin resistance, transcriptomics, *ssrA*

**H**elicobacter *pylori* (*H. pylori*) infection is one of the leading causes of chronic gastric inflammation, predisposing individuals to peptic ulcerations and gastric cancer (1–3). Approximately more than half of the worldwide population carry *H. pylori* within the gastric mucosa with prevalence in certain regions such as Asia exceeding 80%

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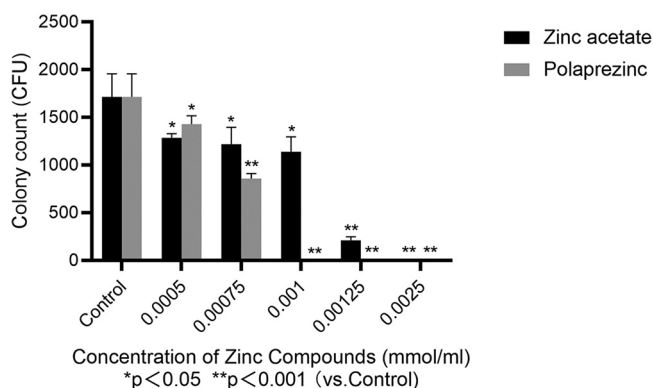
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**FIG 1** Zinc compounds inhibit the growth of *H. pylori* (NCTC 11637).

(4, 5). Eradication of *H. pylori* reduces the long-term risks of peptic ulcers and gastric cancer (6, 7) and is fast becoming an emerging global health issue (8).

Current first-line treatment for *H. pylori* in general consists of a proton pump inhibitor with a combination of three or four antibiotics, such as penicillin-based or fluoroquinolones combined with macrolides and metronidazole, depending on local resistance patterns (9, 10). The combination of antibiotic treatment reflects the challenges in *H. pylori* eradication (11), and rapidly increasing resistance to current antibiotic therapies (12) is a particular cause for concern. Therefore, additional means to inhibit *H. pylori* growth and increase their sensitivity to existing antibiotics are needed to tackle this increasingly difficult-to-treat endemic.

Various alternate treatment approaches have been investigated previously (reviewed in detail in [13]); however, many showed limited efficacy. Metal complexes, such as bismuth, are commonly added to *H. pylori* combination therapies in widespread clinical settings (14). Zinc-based complexes, in particular, such as the addition of polaprezinc (a chelate compound of zinc and L-carnosine) to combination therapies, have been shown to improve *H. pylori* eradication (15, 16). Currently, there have been limited studies examining effects of zinc acetate on *H. pylori* growth and sensitivities to antibiotics.

Here, we demonstrate (i) the inhibitory effect of zinc acetate on *H. pylori* growth *in vitro*, (ii) explore the transcriptomic changes in response to zinc acetate, and (iii) investigate the knockdown effects of the differentially expressed transfer-messenger RNA (*ssrA*) on antibiotic sensitivity.

## RESULTS

**Zinc acetate inhibits *H. pylori* growth.** We cultured *H. pylori* (NCTC 11637) with zinc acetate for 3 days at 37°C. *H. pylori* growth was inhibited at increasing concentrations of zinc acetate, which becomes apparent at 0.00125 mmol/mL, with complete inhibition observed at 0.0025 mmol/mL ( $P < 0.001$ , versus control). Similar effects were also observed with polaprezinc (Fig. 1). In addition to the standard strains, we also tested the resistance of various clinical *H. pylori* strains to zinc acetate (and polaprezinc) and found broadly comparable MICs across the various strains (Table S1).

**Zinc acetate increases *H. pylori* sensitivity to levofloxacin.** Next, we investigated changes in *H. pylori* (NCTC 11637) sensitivity to levofloxacin, clarithromycin, and tetracycline after culturing with zinc acetate or polaprezinc for 3 days. MICs of levofloxacin and clarithromycin decreased after *H. pylori* exposure to both zinc acetate and polaprezinc, although only the MIC of levofloxacin strictly remained below the resistance breakpoint ([https://www.eucast.org/clinical\\_breakpoints/](https://www.eucast.org/clinical_breakpoints/)) after exposure to zinc acetate (Table 1, Fig. S1). MICs of tetracycline remained unchanged in both conditions.

**Transcriptomic changes in response to zinc acetate.** To investigate the transcriptomic changes associated with decreased resistance of *H. pylori* to levofloxacin after exposure to zinc acetate, we used RNA-sequencing to assess gene expression changes after culturing *H. pylori* with zinc acetate for 3 days versus controls ( $n = 3$  each). The zinc acetate

**TABLE 1** Zinc compounds affect the MIC ( $\mu\text{g}/\text{mL}$ ) of antibiotics to *H. pylori* NCTC 11637<sup>a</sup>

Antibiotics	Control	Polaprezinc	Zinc acetate
Levofloxacin	1–1.5	0.75–1	0.5–0.75
Clarithromycin	0.5–1	0.25–0.5	0.25–0.5
Tetracycline	0.5–1	0.5–1	0.5–1

<sup>a</sup>Breakpoints of antibiotic sensitivity: Levofloxacin: MIC  $\leq 1 \mu\text{g}/\text{mL}$ ; Clarithromycin: MIC  $\leq 0.25 \mu\text{g}/\text{mL}$ ; Tetracycline: MIC  $\leq 1 \mu\text{g}/\text{mL}$ .

treated samples were separable from the control groups based on principal-component analysis (PCA) using the transcriptomic profiles (Fig. 2a) and displayed distinct gene expression profiles (Fig. 2b). In total, we found 227 nominally significant ( $P < 0.05$ ) differentially expressed genes (DEGs) between the treated and control groups, with 23 DEGs significant after adjustment for multiple correction (Fig. 2c, Table S2). Additionally, we tested whether DEGs were enriched for any GO processes or KEGG pathway, and we did not find any significant enrichment after multiple correction (Table S3, 4). Genome sequencing was also performed on *H. pylori* NCTC 11637 with zinc acetate treatment versus controls ( $n = 3$  each). Comparative genomics analyses, including indels, single nucleotide polymorphisms, structural variations, synteny analyses, did not reveal changes as a result of zinc acetate exposure (Table S5).

We independently tested 6 DEGs with the largest absolute fold expression changes (Table 2, Fig. 3a) that were nominally significant using qRT-PCR ( $n = 3$ ). We observed significant and consistent changes in expression for *ssrA* (DQL14\_RS05110, also known as transfer- and messenger-RNA, tmRNA), where expression increased by 2.6-fold ( $P = 0.02$ , Fig. 3b) after incubation with zinc acetate.

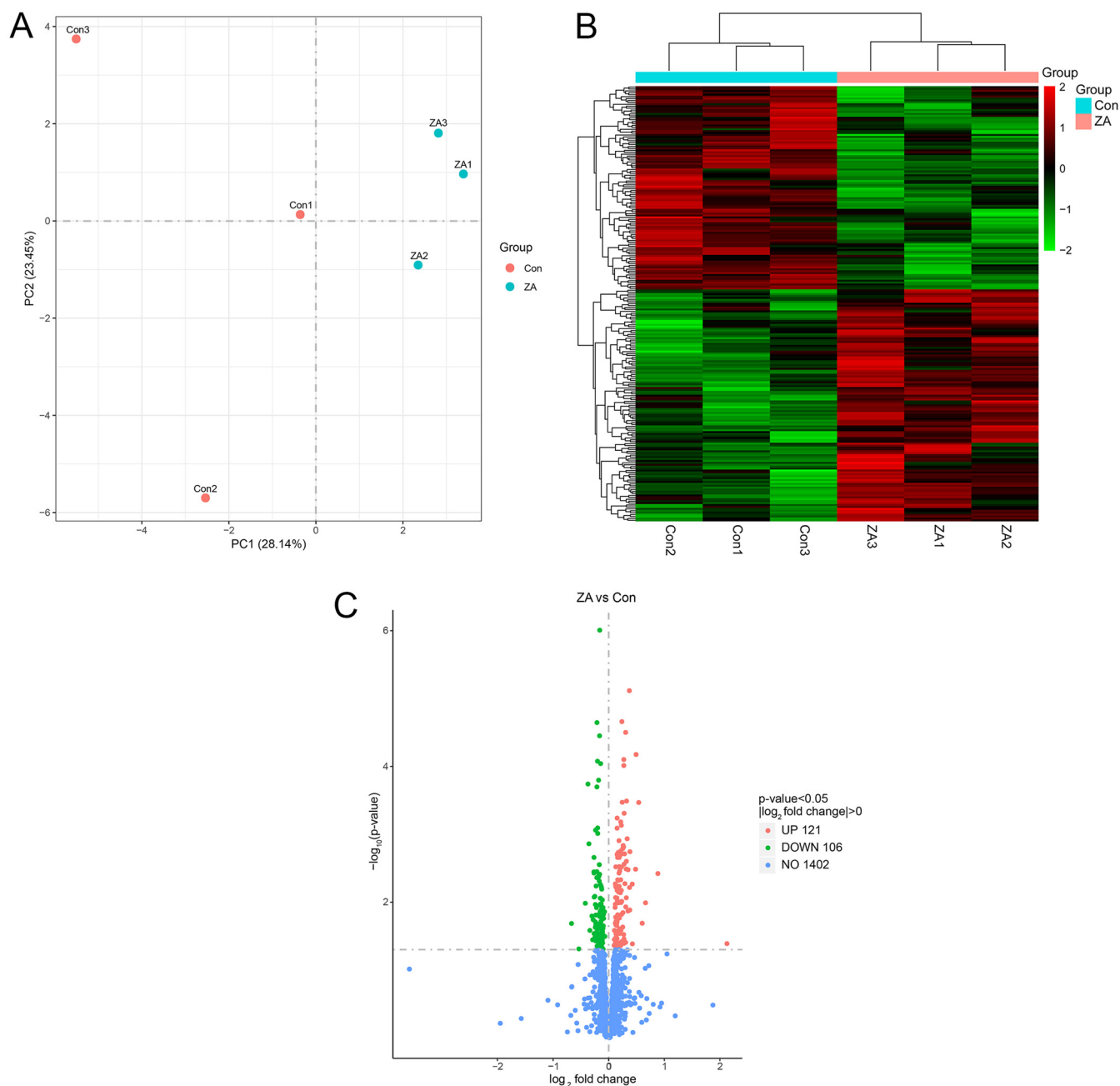
**Acidity affects the transcription of *ssrA*.** The pH of liquid cultures was adjusted by the addition of HCl to pH 6.0, 4.5 and 3.0 with 5 mM urea. After NCTC 11637 was incubated in acidity gradient liquid media for 4 h, the *ssrA* gene expression was evaluated by qRT-PCR. Compared with the control group (pH 7.4), NCTC 11637 incubated in acidic media (pH 4.5 and 3.0) has a significantly increased *ssrA* expression to 3.9 times ( $P < 0.05$ , Fig. S2).

**Knockdown of *ssrA* restores *H. pylori* resistance to levofloxacin.** After we replicated and validated increased expression of *ssrA* after zinc acetate treatment, we hypothesized whether *ssrA* expression is a potential causal mechanism driving the observed zinc acetate mediated *H. pylori* sensitivity to levofloxacin. First, we performed *ssrA* knockout in *H. pylori*, which led to complete inhibition of *H. pylori* growth, suggesting *ssrA* is essential for *H. pylori* growth (Fig. S3). Subsequently, we performed knockdown of *ssrA* in *H. pylori*, whereby siRNA *ssrA* knockdown reduced *ssrA* expression by 46%. The time course of knockdown results after *ssrA* siRNA treatment indicated that *ssrA* mRNA expression decreased the most after 1 h (Fig. 4a). MICs of levofloxacin for the zinc acetate treated group after *ssrA* knockdown were restored to levels similar to controls, consistent with *ssrA* being a driver of zinc acetate associated levofloxacin sensitivity (Fig. 4b).

## DISCUSSION

Rising antibiotic resistance to *H. pylori* is fast becoming a widespread global health concern, driving the need for alternative and complementary treatment approaches. Here, in this study, we demonstrated the role of zinc acetate in inhibiting *H. pylori* growth and increasing the sensitivity of *H. pylori* to levofloxacin, a commonly used antibiotic in *H. pylori* treatment. In the process, we also reproduced previously observed effects of polaprezinc on *H. pylori* growth (16).

This is one of the first studies to examine the transcriptomic perturbations associated with zinc acetate treatment, which showed distinct gene expression profiles compared to controls. Widespread transcriptomic perturbations were also previously seen after changes in environmental conditions such as temperature (17, 18). This suggests significant transcriptomic changes occur in *H. pylori* in response to biophysical as well as chemical perturbations. While we did not find evidence of enrichment for any



**FIG 2** Differentially expressed genes (DEGs) identified by transcriptomic (RNA) sequencing. (A) Principal-component analysis (PCA) analysis of tested samples. (Con, control group; ZA, zinc acetate treated group). (B) Hierarchical clustered heatmap of differentially expressed genes ( $n = 227$ ) between control and treated group. (C) Volcano plot exhibition of DEGs. The vertical axis represents the  $-\log_{10}$  ( $P$ -value), the horizontal axis displays the  $\log_2$  fold change. Red points signify the upregulated transcripts ( $n = 121$ ), green points stand for downregulated transcripts ( $n = 106$ ). Vertical dashed lines indicate absolute  $\log_2$  fold change = 0 and horizontal dashed lines indicate  $P$ -value = 0.05.

specific pathways, we were able to test and validate the effects of the specific gene *ssrA* in detail via hypothesis-driven functional follow-up.

Through siRNA knockdown of *ssrA*, we were able to restore *H. pylori* resistance to zinc acetate induced levofloxacin sensitivity, suggesting an important role of *ssrA* in driving these observed effects. *SsrA* codes for the highly conserved tmRNA, a specialized RNA with properties of both a tRNA and an mRNA. tmRNA is one of the key components of trans-translation, a ubiquitous bacterial mechanism that resolves ribosome stalling caused by nonstop mRNAs, which is diversely implicated in many processes such as responding to stress, pathogenesis, and differentiation (19). Trans-translation is

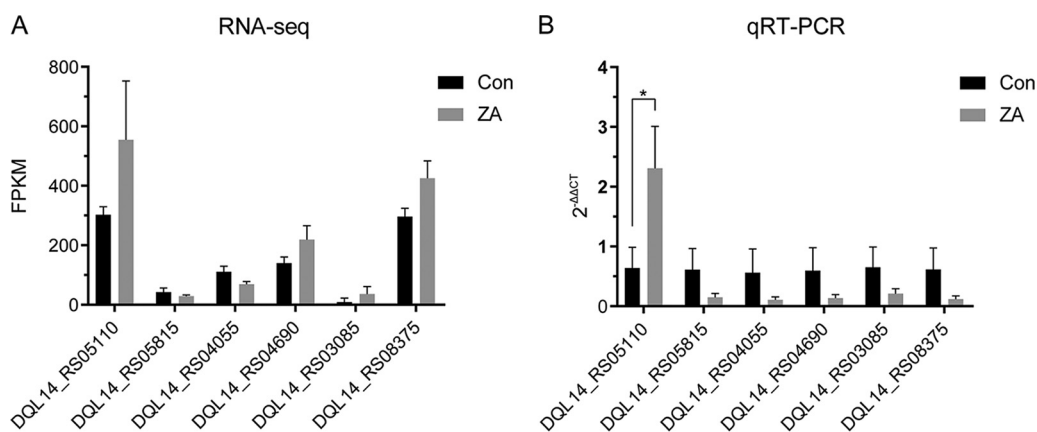
**TABLE 2** Six selected differentially expressed genes measured by qRT-PCR

Gene ID	Gene name	Gene description	ZA versus Con Log <sub>2</sub> (Fold Change)	ZA versus Con P value
DQL14_RS05110	ssrA	Transfer-messenger RNA	0.874	3.81E-03
DQL14_RS05815	hopJ	Hop family outer membrane protein HopJ/HopK PF01856:Helicobacter outer membrane protein	-0.548	4.89E-02
DQL14_RS04055		Hypothetical protein	-0.679	2.06E-02
DQL14_RS04690		Hypothetical protein	0.652	1.03E-02
DQL14_RS03085	rpmG	50S ribosomal protein L33	2.116	4.11E-02
DQL14_RS08375		Cag pathogenicity island protein	0.530	3.40E-04

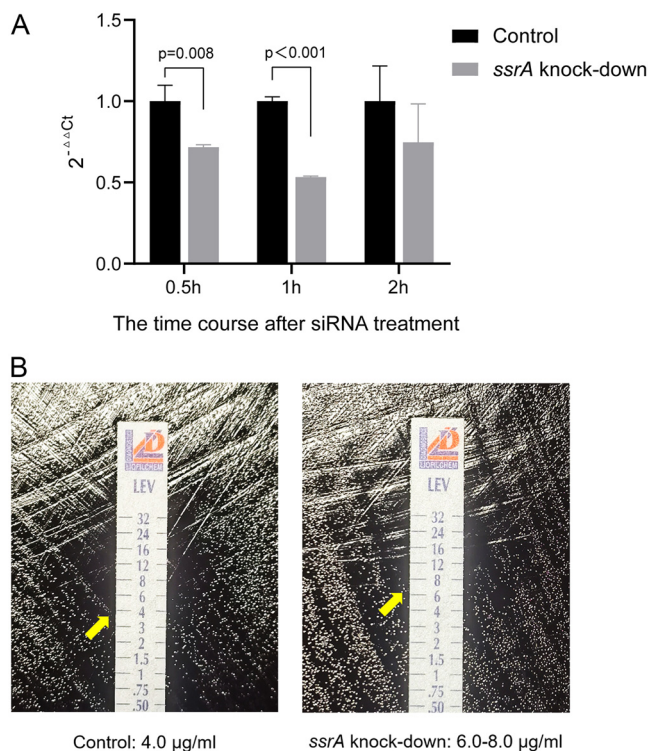
an essential function in bacteria (20), and plays regulatory roles in organisms such as *H. pylori* (21), *Escherichia coli* (22), *Streptomyces coelicolor* (23), etc. We showed that complete *ssrA* knockout inhibits *H. pylori* growth completely, suggesting tmRNA system is essential for *H. pylori* growth, consistent with similar reports in other bacterial species (24). Previous literature suggests that deletion of the tmRNA system reduces trans-translation and results in increased susceptibility to oxidative stress and antibiotics that inhibit protein synthesis and transcription (25). However, we demonstrated that zinc acetate treatment led to increased *ssrA* expression and sensitivity to levofloxacin and knockdown of *ssrA* led restored resistance. Our results are consistent with *Streptococcus pneumoniae* containing *ssrA* deletion becoming more resistant to levofloxacin and other fluoroquinolones (25). The exact mechanism by which this effect occurs in *H. pylori* remains to be elucidated.

Our study revealed the effect of zinc acetate on *ssrA* gene expression in *H. pylori in vitro*. However, we have not explored the changes in the bacterial transcriptome *in vivo* condition. Previous studies found that the gastric acid environment *in vivo* was pH 1.0–6.0 (26, 27), whereas acidic conditions influenced *H. pylori* on the transcription of different genes related to adaptation to acidity, antioxidant response, mobility, adherence, and pathogenicity (T4SS) (28). What's more, acidity influenced the expression of genes related to the bactericidal effect of antibiotics (29). We investigated the transcriptional change on *ssrA* under acidic conditions and found *ssrA* was significantly up-regulated at pH 4.5 and pH 3.0. This implied that the transcription of *ssrA* may also be upregulated in the gastric environment *in vivo*.

In summary, our results suggest that zinc acetate reduces *H. pylori* growth and increases sensitivity to levofloxacin through upregulating tmRNA system along with other distinct transcriptomic changes and modulating the trans-translational system.



**FIG 3** Validation of selected candidate genes using qRT-PCR. (A) Expression of 6 selected genes measured by RNA sequencing and assessed by Fragments Per Kilobase of exon model per Million mapped fragments (FPKM). Error bars indicate standard deviation. (B) Expression of 6 selected genes measured by qRT-PCR.  $2^{-\Delta\Delta C_t}$  method was applied to evaluate gene expression differences. Error bars indicate standard deviation. Con, control group; ZA, zinc acetate treated group. (\*  $P < 0.05$ ).



**FIG 4** (A) The levels of *ssrA* expression after knockdown evaluated by qRT-PCR.  $2^{-\Delta\Delta C_t}$  was used to evaluate the difference of gene expression ( $P$ -value by independent samples  $t$  test). (B) The impact of *ssrA* knockdown on levofloxacin sensitivity. Left: *H. pylori* electroporation at 2500 V for 5 ms as control; the MIC of levofloxacin was 4.0  $\mu\text{g/ml}$ . Right: *H. pylori* electroporation at 2500 V for 5 ms and transformed by siRNA to *ssrA* for 1 h; the MIC of levofloxacin was 6.0–8.0  $\mu\text{g/ml}$ .

Our findings support zinc acetate as a useful adjunct in fluoroquinolone-based antibiotic therapies against resistant *H. pylori*.

## MATERIALS AND METHODS

**Bacteria strains and culture conditions.** *H. pylori* NCTC 11637, Sydney strain 1 (SS1), and clinical strains used in this study have been identified using 16S rRNA gene sequencing. *H. pylori* strains were cultured under microaerophilic condition (5%  $\text{O}_2$ , 10%  $\text{CO}_2$ , and 85%  $\text{N}_2$ ) on solid culture media composed of Karmali Agar Base (CM0935, Oxoid, UK) and 5% sterile defibrinated sheep blood (XLF Medical Sales Co., Beijing, China). Liquid culture medium was brain heart infusion (BHI) supplemented with 10% fetal calf serum (FBS), 0.25% yeast extract, and selective supplement (SR0147E, Oxoid, UK).

**Bacteria growth at different concentrations of zinc compounds.** Culture mediums containing different concentrations (0, 0.0005 mmol/mL, 0.00075 mmol/mL, 0.001 mmol/mL, 0.00125 mmol/mL, 0.0025 mmol/mL) of zinc acetate or polaprezinc (Broadwell Pharmaceutical, China) were prepared. *H. pylori* NCTC 11637 was resuspended to  $10^5$  CFU/mL, and 100  $\mu\text{L}$  of bacteria suspension was evenly coated on culture medium and colonies were counted after incubating at 37°C for 3 days.

**MICs of zinc compounds to *H. pylori*.** *H. pylori* strains—two standard strains (NCTC 11637 and SS1) and eight clinical strains (19004, 20050, 27054, 28038, 827, L1, L2, L3)—were resuspended in sterile PBS to 2 McFarland. One hundred  $\mu\text{L}$  bacteria suspension was evenly coated on culture medium with 0.0005 mmol/mL, 0.001 mmol/mL, 0.0025 mmol/mL, 0.005 mmol/mL, 0.01 mmol/mL of zinc compounds. After incubating at 37°C for 3 days, concentration of the zinc compounds in the plate with no bacterial growth was regarded as MIC.

**Zinc compound effects on MICs of antibiotics to *H. pylori*.** *H. pylori* NCTC 11637 strain was used. Culture mediums containing half MIC of zinc compounds (0.0005 mmol/mL for polaprezinc and 0.00125 mmol/mL for zinc acetate) were prepared. *In vitro* MICs of three antibiotics, levofloxacin (0.002 to 32  $\mu\text{g/ml}$ ), clarithromycin (0.016 to 256  $\mu\text{g/ml}$ ), and tetracycline (0.016 to 256  $\mu\text{g/ml}$ ), were tested using the Epsilon test (Etest, Liofilchem, Italy). The MIC value was read at the point where the bacteriostatic ring intersects the Etest strip. The clinical breakpoints for levofloxacin, clarithromycin, and tetracycline were defined as:  $>1$   $\mu\text{g/ml}$ ,  $>0.5$   $\mu\text{g/ml}$ , and  $>1$   $\mu\text{g/ml}$ , respectively, as per European Committee on Antimicrobial Susceptibility Testing Breakpoints ([https://www.eucast.org/clinical\\_breakpoints/](https://www.eucast.org/clinical_breakpoints/)).

**Genome DNA extraction and analyses.** *H. pylori* NCTC 11637 was divided into two groups. The experimental group was incubated on medium contains 0.00125 mmol/mL zinc acetate for 3 days and the

control group was incubated on blank medium for 3 days. DNA was extracted using sodium dodecyl sulfate (SDS) method (30). Qubit RNA assay kit in Qubit 2.0 Fluorometer (Life Technologies, USA) was used to quantify the extracted DNA, and its purity and integrity was determined by agarose gel electrophoresis. Eligible DNA samples were broken into fragments of about 350 bp using Covaris ultrasonic crushing instrument, and NEBNext Ultra DNA Library Prep kit for Illumina kit (NEB, USA) was used for sequencing library preparation. Library quality control was performed using Agilent 2100 Bioanalyzer (Agilent Technologies, Beijing, China), and the sequencing procedure was completed by Illumina NovaSeq PE150. Library construction, sequencing, and comparative analysis between control and experimental groups were performed by Novogene Tech (Beijing) Co., Ltd.

**RNA preparation and transcriptomic analyses.** *H. pylori* NCTC 11637 strain grouping was the same as Genome DNA analysis and each group were run using three replicates. Total RNA was extracted with RNAPrep Pure Cell/Bacteria kit (DP430, TIANGEN, China) and quality control was assessed using agar gel electrophoresis, NanoPhotometer spectrophotometer, and Agilent 2100 bioanalyzer for integrity and purity. Eligible RNA sample was treated with Ribo-Zero Magnetic kit (Bacteria) (Epicentre, USA) to remove rRNA. The cDNA library was constructed according to the Strand-specific approach (31). For the quality of the cDNA library, Qubit2.0 Fluorometer was used for preliminary quantification, and then qRT-PCR was used for accurate quantification to ensure its effective concentration was above 2 nM. Library construction and sequencing were conducted by Novogene Tech (Beijing) Co., Ltd. Raw reads were filtered and mapped to the *H. pylori* NCTC 11637 genome using Bowtie2. Gene expression counts were quantified using HTSeq. Differentially expressed genes (DEGs) were analyzed using DESeq2. Genes with absolute  $\log_2$ -fold changes  $>0.5$  and multiple  $P$ -value  $<0.05$  were considered DEGs.

**Gene expression measurement by qRT-PCR.** Quantitative real-time PCR (qRT-PCR) assays were applied to detect the targeted gene expression. The same samples as in the RNA-sequencing transcriptomic analyses were used, including 3 controls incubated on blank medium and 3 incubated on medium contains 0.00125 mmol/mL zinc acetate. Based on transcriptomic analyses, 6 differentially expressed genes were selected for qRT-PCR validation. Gene-specific primers were designed using Primer Premier 5.0 and first strand cDNA was synthesized using HiFiScript cDNA Synthesis kit (Cowin Bio, China). qRT-PCRs were performed in technical triplicates using an ABI StepOne real-time PCR system (Perkin-Elmer Applied Biosystems, Foster City, CA). Reaction component and process are shown in Table S6. Gene expression and  $\log_2$ -fold changes were analyzed using the  $2^{-\Delta\Delta CT}$  algorithm.

**GO and KEGG pathway analyses.** First we mapped genes to Entrez Gene symbols, and then gene sets were annotated by GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) databases (32, 33). GO enrichment analysis (cellular component [CC], molecular function [MF], and biological process [BP]) and KEGG pathway enrichment analysis of DEGs were performed using clusterProfiler R package (v3.4.4), while the *H. pylori* 11637 reference strain annotated by Pfam (protein families database) was used as background, and Benjamini–Hochberg adjusted  $P$ -value  $<0.05$  was considered significantly enriched.

**Construction of *ssrA* gene knocking-out strain.** *ssrA* gene knockout in *H. pylori* NCTC 11637 was constructed according to the published protocol (34) and summarized in Table S7. The plasmid PILL570, which contains a kanamycin resistance gene, was kindly provided by Yundong Sun from Shandong University, China. The recombinant plasmid for knocking out *oipA* gene (used as positive control in this study) was kindly provided by Yong Xie, Nanchang University, China. Briefly, we amplified the upstream and downstream homologous arms of *ssrA* and then inserted them into PILL570 and transformed the reconstructed plasmid into the bacteria. The targeted genes (*ssrA* and *oipA*) would be replaced by the kanamycin resistance gene.

**Knockdown of *ssrA* by siRNA.** Levofloxacin-resistant *H. pylori* NCTC 11637 strain with a MIC of 4–6  $\mu\text{g}/\text{mL}$  induced by gradient levofloxacin exposure *in vitro* was used for analysis. siRNA against *ssrA* was designed and synthesized by Saisoft Biotech (Jiangsu) Co., Ltd., China. The sequence is shown in Table S8. siRNA transfection was performed following the published protocols (35). *H. pylori* strains were harvested from plates and washed three times with sterile 10% ice glycerol solution. One hundred  $\mu\text{L}$  mixture was blended with 10  $\mu\text{g}$  of siRNA, and  $10^9$  CFU bacteria was transferred to 0.1 cm electroporation cups (Bio-Rad, USA). Transfection was performed by electroporation using 2500V for 5 ms. At 30 min, 1 h, and 2 h after electro-transformation, bacterial RNA was extracted and reverse transcribed according to manufacturer's protocols (Vazyme, China). The qRT-PCR components and processes are detailed in Table S9. MICs of levofloxacin to *H. pylori* after *ssrA* was knocked down at different time points were tested using the E-strip method.

**Data availability.** The original contributions presented in the study are publicly available. These data can be found here: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA822664>.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.4 MB.

**SUPPLEMENTAL FILE 2**, XLS file, 0.3 MB.

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We have no conflicts of interest to declare.

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