



# Bifunctional Enzyme SpoT Is Involved in Biofilm Formation of *Helicobacter pylori* with Multidrug Resistance by Upregulating Efflux Pump Hp1174 (*gluP*)

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**ABSTRACT** The drug resistance of *Helicobacter pylori* is gradually becoming a serious problem. Biofilm formation is an important factor that leads to multidrug resistance (MDR) in bacteria. The ability of *H. pylori* to form biofilms on the gastric mucosa is known. However, there are few studies on the regulatory mechanisms of *H. pylori* biofilm formation and multidrug resistance. Guanosine 3'-diphosphate 5'-triphosphate and guanosine 3',5'-bispyrophosphate [(p)ppGpp] are global regulatory factors and are synthesized in *H. pylori* by the bifunctional enzyme SpoT. It has been reported that (p)ppGpp is involved in the biofilm formation and multidrug resistance of various bacteria. In this study, we found that SpoT also plays an important role in *H. pylori* biofilm formation and multidrug resistance. Therefore, it was necessary to carry out some further studies regarding its regulatory mechanism. Considering that efflux pumps are of great importance in the biofilm formation and multidrug resistance of bacteria, we tried to determine whether efflux pumps controlled by SpoT participate in these activities. We found that Hp1174 (glucose/galactose transporter [*gluP*]), an efflux pump of the major facilitator superfamily (MFS), is highly expressed in biofilm-forming and multidrug-resistant (MDR) *H. pylori* strains and is upregulated by SpoT. Through further research, we determined that *gluP* is involved in *H. pylori* biofilm formation and multidrug resistance. Furthermore, the average expression level of *gluP* in the clinical MDR strains (C-MDR) was considerably higher than that in the clinical drug-sensitive strains (C-DSS). Taken together, our results revealed a novel molecular mechanism of *H. pylori* resistance to multidrug exposure.

**KEYWORDS** *Helicobacter pylori*, SpoT, efflux pump, biofilm, antibiotic resistance, GluP

*Helicobacter pylori*, the rate of infection with which is over 50% throughout the world, is highly associated with a wide range of upper gastrointestinal diseases, especially gastric carcinoma (1). Contemporaneously, the most common method of treatment of infections caused by this bacterium is called triple therapy, which consists of a proton pump inhibitor and two antibiotics, namely, macrolides, nitroimidazoles, or  $\beta$ -lactams (2). However, in recent years, eradication of *H. pylori* has become increasingly difficult because the rate of antibiotic resistance acquisition by *H. pylori* has generally increased (3). In addition, with the extended use of antibiotics, the appearance of multidrug-resistant (MDR) *H. pylori* strains, which are resistant to multiple antibiotics, has been reported (4).

There are numerous molecular mechanisms that contribute to multidrug resistance in the bacteria, including decreased drug permeation, efflux pumps, alteration or bypass of the drug target, production of antibiotic-modifying enzymes, and other

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physiological states, such as the formation of biofilms (5). Biofilms are communities of microorganisms that are anchored to a surface and live in an extracellular matrix made up of extracellular polymeric substances (EPS); this matrix is produced by the microorganisms to form their immediate environment (6). The property that makes biofilms distinct from planktonic cells is their increased resistance to antimicrobial agents. It has recently become widely accepted that biofilms play an important role in the pathogenesis of some chronic human infections, as well as bacterial multidrug resistance (6, 7). Furthermore, *H. pylori* has the ability to form biofilms *in vitro* (8). In 2006, using scanning electron microscopy (SEM), Carron et al. first proposed that *H. pylori* could form biofilms *in vivo* (9). The formation of biofilms *in vivo* is an important cause of *H. pylori* resistance to multiple antibiotics (10), as is its stringent response to a stressful environment lacking nutrients (11).

The stringent response is a bacterial stress response that controls bacterial adaptation to stress signals, such as nutrient deprivation (12). In bacteria, the signal molecules guanosine 3'-diphosphate 5'-triphosphate and guanosine 3',5'-bispyrophosphate [(p)ppGpp], which are induced by diverse stresses, activate the stringent response (12, 13). The phenomenon of (p)ppGpp affecting bacterial multidrug resistance has already been reported for some other bacteria (14). Maisonneuve et al. reported that under antibiotic stress, *Escherichia coli* can produce rare cells that transiently become multidrug tolerant (15). In these rare cells, the level of (p)ppGpp was high (15). In addition, it has also been proven that (p)ppGpp can affect the formation of the bacterial biofilm. For example, Sugisaki et al. determined that the accumulation of (p)ppGpp accelerated the formation of biofilms in *Bordetella pertussis* (16), and Li et al. reported that the low level of (p)ppGpp contributed to the formation of biofilms in *Actinobacillus pleuropneumoniae* S8 (17). Nevertheless, there are still no reports certifying the relationship between (p)ppGpp and the formation of biofilms in *H. pylori*.

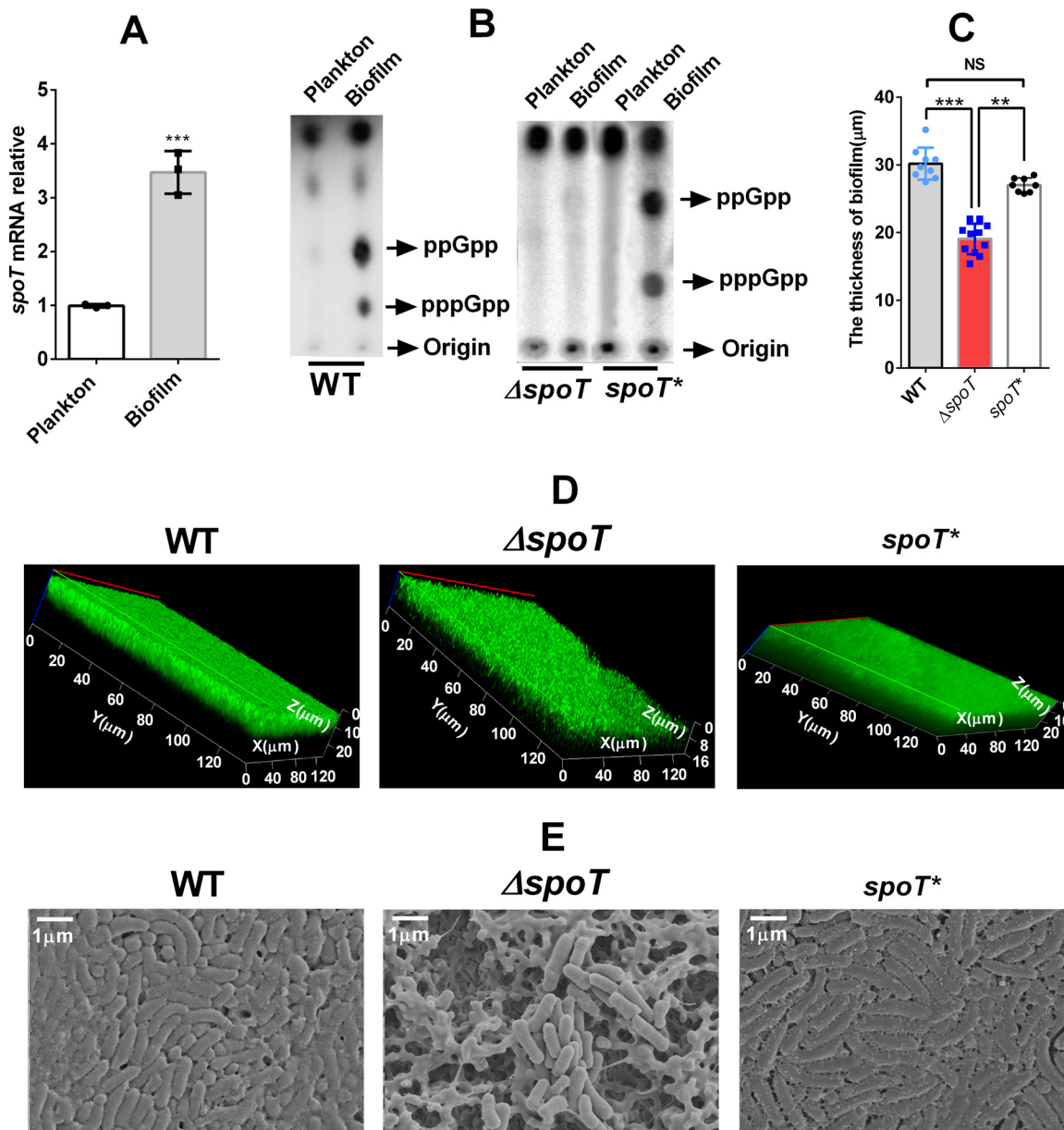
In many bacteria, such as *E. coli*, (p)ppGpp is synthesized by two enzymes, RelA and SpoT (18), and SpoT is a bifunctional enzyme with both (p)ppGpp synthetase and hydrolase activity (18). However, there is only one member of the RelA/SpoT family, SpoT, in the *H. pylori* genome (19–21), and it is also a bifunctional enzyme (22); therefore, in *H. pylori*, we focused on whether SpoT is involved in *H. pylori* biofilm formation and multidrug resistance.

There are a series of transport proteins in bacteria that can acquire nutrients and extrude metabolic by-products, and some of these proteins are called efflux pumps (23). Efflux pumps have the ability to expel a broad range of antibiotics and have been recognized as significant components of multidrug resistance in many bacteria (24, 25), such as *H. pylori* (26–32). It has been demonstrated that efflux pumps work as one of the mechanisms that contribute to the antimicrobial resistance of biofilms (33), and evidence can be found in several microorganisms, such as *Pseudomonas aeruginosa* (34), *E. coli* (35), and *Candida albicans* (36). Moreover, Yonezawa et al. reported that in *H. pylori* clinical MDR strains (C-MDR), the expression of some efflux pump genes was elevated in biofilm cells compared to that in planktonic cells (37). While some efflux transporters have been detected in *H. pylori* 26695 (21), their functions, especially their functions in *H. pylori* biofilm formation, must be further studied.

Considering that SpoT is a global regulator, we suppose that SpoT can influence the formation of biofilms and multidrug resistance by regulating the expression of the efflux pump in *H. pylori*.

## RESULTS

**SpoT is involved in *H. pylori* biofilm formation and multidrug resistance.** As a global regulatory factor, SpoT has been proven to participate in the formation of bacterial biofilms (16, 17, 38), while no related studies regarding *H. pylori* have been done. Therefore, we analyzed the difference in *spoT* expression between biofilm-forming and planktonic cells by real-time PCR (RT-PCR). The *spoT* gene is highly expressed in the latter (Fig. 1A). The (p)ppGpp production assay showed that the *H. pylori* cells in the biofilm produced more (p)ppGpp than did the planktonic cells



**FIG 1** SpoT is involved in *H. pylori* biofilm formation. (A) mRNA expression of SpoT in biofilm-forming and planktonic cells determined by qRT-PCR. The levels of the signals were normalized to the 16S rRNA levels. Data are the means  $\pm$  standard errors of the means from three independent experiments. Significance was determined by the unpaired Student's *t* test. \*\*\*,  $P < 0.001$ . (B) (p)ppGpp was induced in the biofilm-forming cells (of the wild-type [WT] and *spoT*-complemented [*spoT*\*] strains) but not in the planktonic cells or the *spoT* mutant strain (the  $\Delta spoT$  strain).  $^{32}$ P-labeled nucleotides of formic acid extracts of *H. pylori* were detected by thin-layer chromatography. Planktonic *H. pylori* bacteria were grown to the exponential phase. (C) Comparison of the biofilm thickness of the WT,  $\Delta spoT$ , and *spoT*\* strains, using data from the assay whose results are presented in panel D. The data presented are the means  $\pm$  standard errors of the means from three independent experiments. Significance was determined by the paired Student's *t* test. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; NS, not significant. (D) Confocal laser scanning microscopy (CLSM) images of WT,  $\Delta spoT$ , and *spoT*\* strain biofilms. Cells stained with membrane-permeant SYTO 9 (green) and membrane-impermeant propidium iodide (PI) (red) were visualized by confocal microscopy. (E) Scanning electron microscopy (SEM) images of WT,  $\Delta spoT$ , and *spoT*\* strain biofilms. The biofilm used in this experiment is a mature biofilm grown on a nitrocellulose membrane for 3 days, and the planktonic bacteria were from early exponential phase ( $OD_{600}$ , approximately 0.4 to 0.5).

(Fig. 1B). By constructing an *spoT* mutant strain (the  $\Delta spoT$  strain) and an *spoT*-complemented strain (the *spoT*\* strain), we compared the biofilms of the wild-type (WT),  $\Delta spoT$ , and *spoT*\* strains by confocal laser scanning microscopy (CLSM) (Fig. 1C and D) and SEM (Fig. 1E). LIVE/DEAD cell viability assays showed that the  $\Delta spoT$  strain formed a lighter biofilm than did the WT strain and the *spoT*\* strain (Fig. 1C and D). The

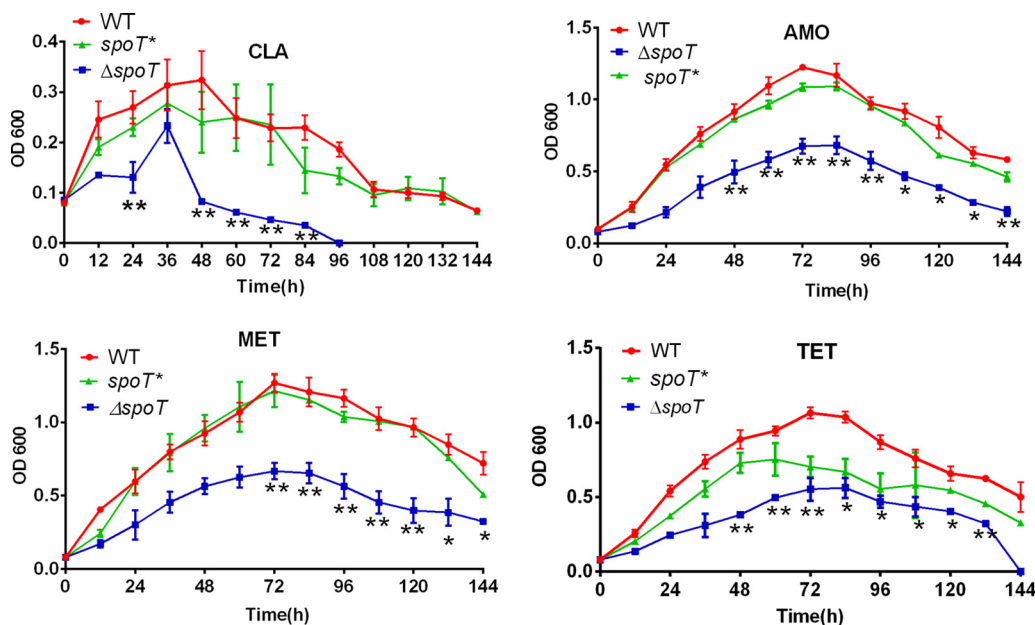
**TABLE 1** MICs determined for WT, MDR,  $\Delta spoT$ ,  $\Delta gluP$ ,  $spoT^*$ , and  $gluP^*$  strains in biofilm-forming and planktonic cells

Drug	MIC ( $\mu\text{g/ml}$ )											
	Planktonic cells					Biofilm-forming cells						
	WT	$\Delta spoT$	$spoT^*$	$\Delta gluP$	$gluP^*$	MDR-H	MDR-H ( $\Delta gluP$ )	WT	$\Delta spoT$	$spoT^*$	$\Delta gluP$	$gluP^*$
Ampicillin (AMP)	0.0625	0.03125	0.0625	0.0156	0.0625	0.3125	0.156	3.75	0.9375	3.75	0.6	3.75
Penicillin G (PEN)	0.125	0.0156	0.0625	0.0078	0.0625	0.625	0.3125	2.5	0.234	2.5	0.078	1.25
Amoxicillin (AMO)	0.0625	0.0156	0.0625	0.0039	0.0625	0.8	0.3125	2.5	0.486	1.25	0.312	1.25
Clarithromycin (CLA)	0.125	0.0625	0.125	0.125	0.125	0.75	0.625	5	1.56	2.5	2.5	3.125
Tetracycline (TET)	0.25	0.0625	0.125	0.125	0.125	5	2.5	10	1.875	5	1.25	6.25
Ciprofloxacin (CIP)	0.25	0.25	0.25	0.25	0.25	4	1	5	2	5	3.75	5
Metronidazole (MET)	0.5	0.125	0.5	0.125	0.25	4	1.5	5	3.125	5	2.5	5
Furazolidone (FUR)	1	0.5	1	0.25	2	6	2	8	2.5	10	2	10

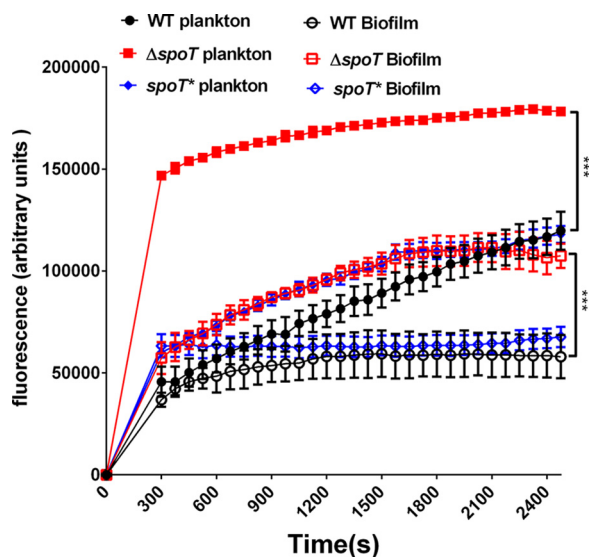
WT strain and the  $spoT^*$  strain could form complete and compact biofilms on the nitrocellulose (NC) membrane, whereas for the biofilm of the  $\Delta spoT$  strain, the bacteria were not packed tightly enough, the biofilm matrix was not complete, and some cavities could be seen (Fig. 1E).

According to the MIC of the WT strain and the  $\Delta spoT$  strain, for planktonic cells, the  $\Delta spoT$  strain was apparently more sensitive to various antibiotics, not including ciprofloxacin, than was the WT and  $spoT^*$  strain (Table 1). With regard to the biofilm-forming cells, after knocking out  $spoT$ , cell resistance to various antibiotics, especially that to penicillin G, was reduced (Table 1). Then, we treated the WT,  $\Delta spoT$ , and  $spoT^*$  strains with antibiotics (clarithromycin [CLA], amoxicillin [AMO], tetracycline [TET], and metronidazole [MET]) at their MICs. The growth inhibitory curve demonstrated that the growth ability of the  $\Delta spoT$  strain was obviously inhibited (Fig. 2).

**Comparing the efflux capacity of the wild-type,  $\Delta spoT$ , and  $spoT^*$  strains.** As SpoT is involved in the formation of the biofilm and multidrug resistance of *H. pylori*, along with an efflux pump (25, 33), we inferred that SpoT could possibly carry out those functions by regulating the expression of the efflux pump. Therefore, we compared the efflux activity of the WT,  $\Delta spoT$ , and  $spoT^*$  strains. The results revealed that the inactivation of SpoT, whether it was in biofilm-forming or planktonic cells, caused a



**FIG 2** Comparison of growth inhibition curve characteristics of the WT,  $\Delta spoT$ , and  $spoT^*$  strains with various antibiotics (AMO [0.125  $\mu\text{g/ml}$ ], CLA [0.125  $\mu\text{g/ml}$ ], MET [0.5  $\mu\text{g/ml}$ ], TET [0.25  $\mu\text{g/ml}$ ]). Data are the means  $\pm$  standard errors of the means from three independent experiments. The significance of the difference between the WT and  $\Delta spoT$  strains was determined using the paired Student's *t* test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .



**FIG 3** Comparison of the accumulation of Hoechst 33342 (2.5 M) in biofilm and planktonic cells of the WT,  $\Delta spoT$ , and  $spoT^*$  strains. The fluorescence intensity was recorded at excitation and emission wavelengths of 350 and 460 nm, respectively, over a 30-min incubation period. The data presented are the means  $\pm$  standard errors of the means from three separate experiments. The paired Student's *t* test was performed to compare the accumulation of Hoechst 33342 between the WT and  $\Delta spoT$  strains. \*\*\*,  $P < 0.001$ .

distinct increase in the accumulation of Hoechst 33342, notably for planktonic cells, and the fluorescence values of the  $\Delta spoT$  strain were >3-fold greater than those of the WT and  $spoT^*$  strains. These results demonstrate that the efflux activity of the  $\Delta spoT$  strain in response to Hoechst 33342 was weaker than that of the WT strain (Fig. 3), which suggests that SpoT likely regulates the efflux pump gene expression of *H. pylori*.

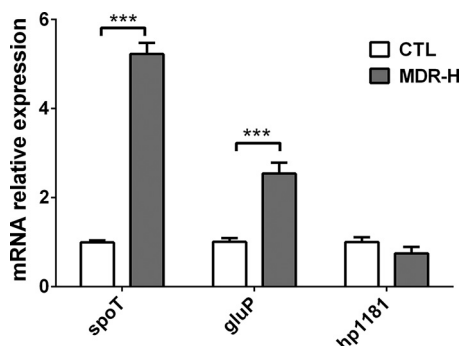
**Screening efflux pumps involved in biofilm formation and multidrug resistance in *H. pylori* by qRT-PCR.** We analyzed the difference in expression of some efflux pumps belonging to the major facilitator superfamily (MFS) and ATP-binding cassette (ABC) superfamily between biofilm-forming and planktonic cells by using quantitative real-time PCR (qRT-PCR) (Table 2). As seen in Table 2, the expression levels of two particular genes increased greater than 3-fold in biofilm-forming cells compared to those in planktonic cells: Hp1181 (multidrug transporter) and Hp1174 (*gluP*). Furthermore, we analyzed the difference in mRNA expression of these two genes in an artificially selected multidrug-resistant *H. pylori* strain (MDR-H), and only *gluP* was highly expressed compared to its expression in the sensitive strain (*H. pylori* 26695) (Fig. 4).

**TABLE 2** qRT-PCR analysis of efflux pump expression difference between biofilm-forming and planktonic *H. pylori* cells

Efflux pump family	Locus tag	Predicted function	Fold change in expression between biofilm and planktonic cells	<i>P</i> value for significance <sup>a</sup>
Major facilitator superfamily (MFS)	Hp0313	Nitrite	0.46 $\pm$ 0.14	*
	Hp0936	Proline/betaine	1.08 $\pm$ 0.17	
	Hp1091	Alpha-ketoglutarate	0.58 $\pm$ 0.03	**
	Hp1165	Multidrug efflux	2.66 $\pm$ 0.86	
	Hp1174	Glucose/galactose transporter	<b>5.75 <math>\pm</math> 0.68<sup>b</sup></b>	***
	Hp1181	Multidrug efflux	<b>3.34 <math>\pm</math> 0.31</b>	***
	Hp1185	Sugar efflux	0.45 $\pm$ 0.27	*
ATP-binding cassette (ABC) superfamily	Hp1220	Multidrug efflux	2.99 $\pm$ 0.32	**
	Hp1082	Multidrug efflux	2.02 $\pm$ 0.25	
	Hp1486	Multidrug efflux	1.11 $\pm$ 0.29	
	Hp1206	Multidrug efflux	1.93 $\pm$ 0.17	**

<sup>a</sup>Significance was determined using the paired Student's *t* test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

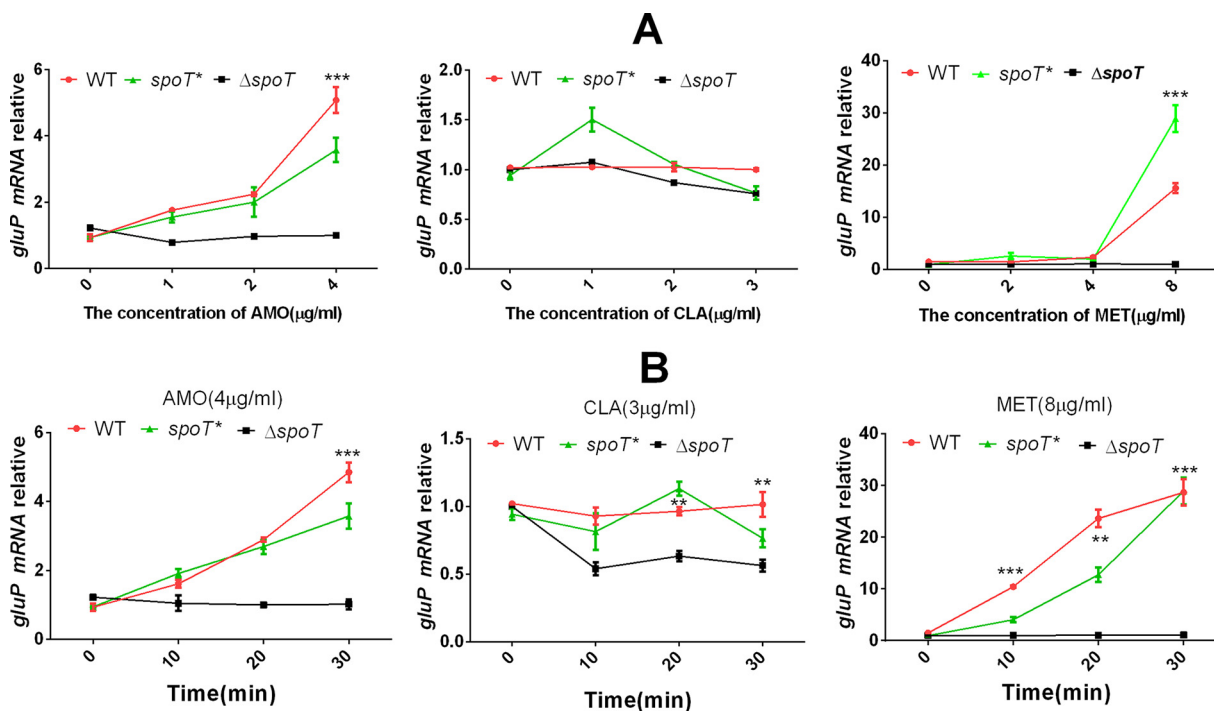
<sup>b</sup>The boldface data indicate that the expression levels of the two genes increased greater than 3-fold in biofilm-forming cells compared to those in planktonic cells.



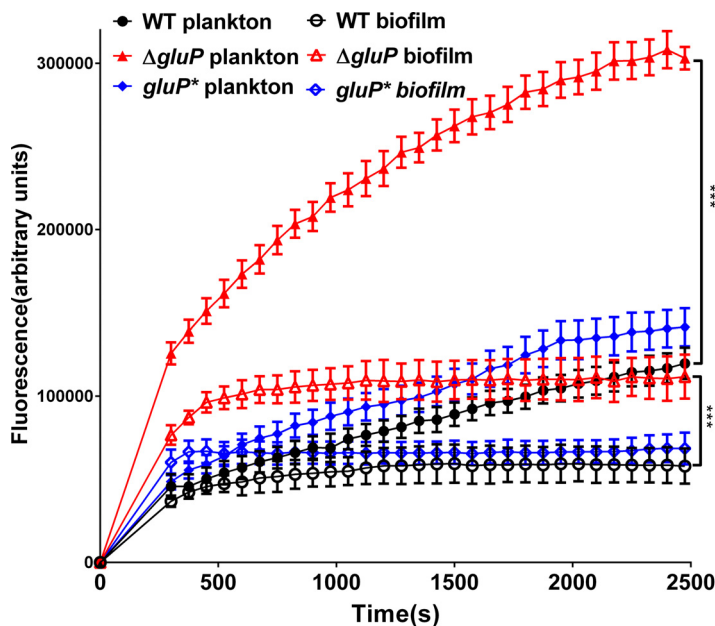
**FIG 4** qRT-PCR analysis of the mRNA levels of *spoT*, *gluP* (Hp1174), and Hp1181 in the MDR-H strain (selected artificially) compared to those in the WT. The signals were normalized to the 16S rRNA levels. Data are the means  $\pm$  standard errors of the means from three independent experiments. Significance was determined by the paired Student's *t* test. \*\*\*,  $P < 0.001$ . CTL, control.

Considering that SpoT is highly expressed in MDR-H (Fig. 4) and biofilm-forming cells, we proposed that SpoT may participate in the biofilm formation and multidrug resistance of *H. pylori* by upregulating *gluP*.

**SpoT regulates GluP expression.** To determine whether GluP is regulated by SpoT, we chose three antibiotics (CLA, AMO, and MET) with which to treat the WT,  $\Delta spoT$ , and *spoT*<sup>\*</sup> strains. First, we applied the three antibiotics at different concentrations to the WT,  $\Delta spoT$ , and *spoT*<sup>\*</sup> strains for 10 min; then, we applied a specific concentration of the three antibiotics along a time gradient. The qRT-PCR results showed that both AMO and MET could induce the expression of *gluP* in the WT and *spoT*<sup>\*</sup> strains but not in the control group (the WT strain without antibiotic treatment), doing so in a concentration- and time-dependent manner, while in the  $\Delta spoT$  strain, GluP could barely be induced by these two antibiotics (Fig. 5). However, CLA could hardly induce the expression of



**FIG 5** qRT-PCR analysis of the mRNA levels of *gluP* in the WT,  $\Delta spoT$ , and *spoT*<sup>\*</sup> strains. The WT,  $\Delta spoT$ , and *spoT*<sup>\*</sup> strains were exposed to different drug concentrations for 10 min (A) and exposed to specific drug concentrations for different time periods (B). The results were compared to those for the WT without drug treatment (control). The signal was normalized to the 16S rRNA levels. Data are the means  $\pm$  standard errors of the means from three independent experiments. Significance was determined by the paired Student's *t* test between the WT and  $\Delta spoT$  strains. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .



**FIG 6** Comparison of the accumulation of Hoechst 33342 (2.5 M) in biofilm and planktonic cells of the WT, *ΔgluP*, and *gluP\** strains. The fluorescence intensity was recorded at excitation and emission wavelengths of 350 and 460 nm, respectively, over a 30-min incubation period. The data presented are the means ± standard errors of the means from three separate experiments. The paired Student's *t* test was performed to compare the accumulation of Hoechst 33342 between the WT and *ΔgluP* strains. \*\*\*, *P* < 0.001.

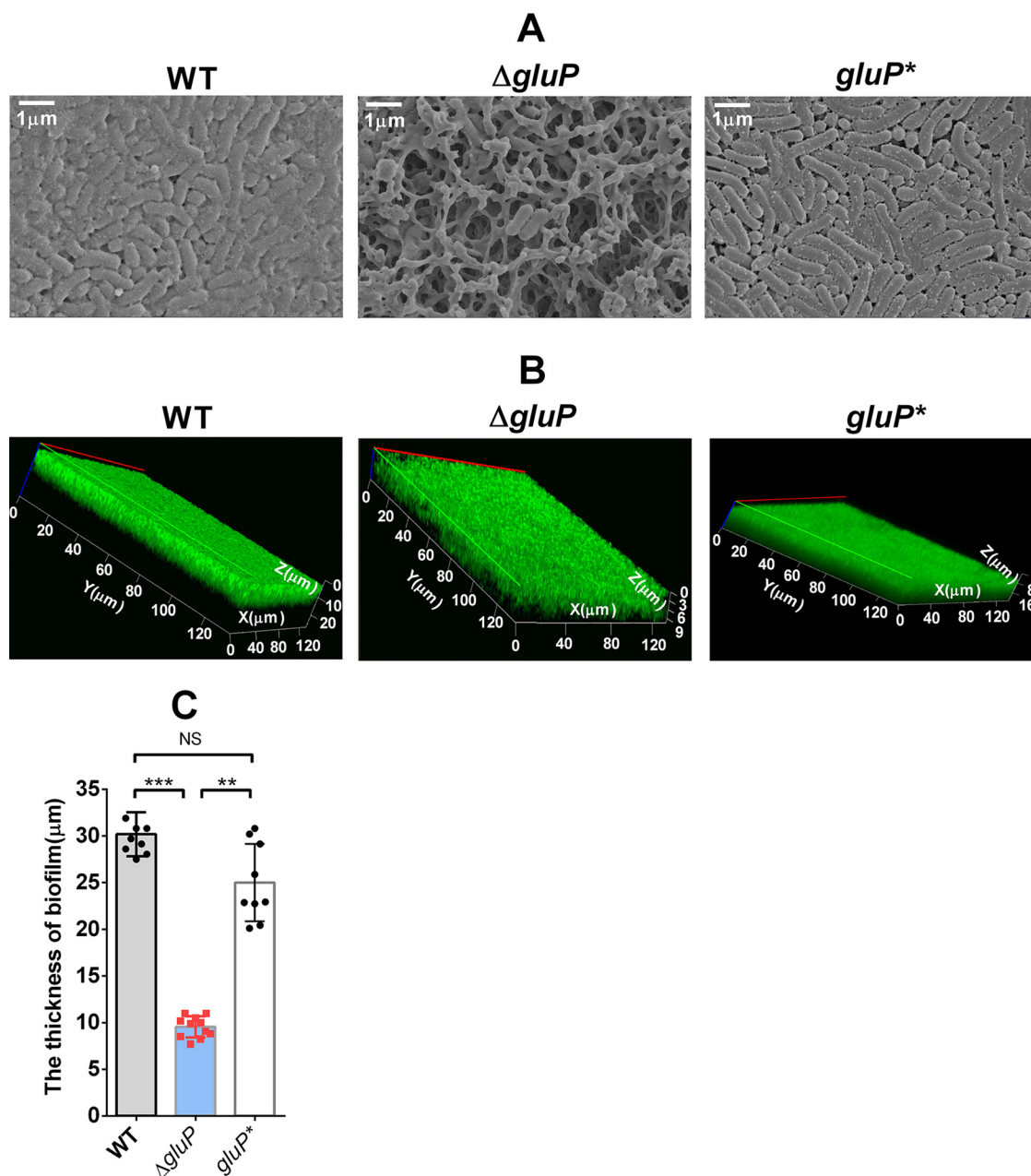
*gluP*, and the sensitivity of the WT and *ΔspoT* strains to CLA was similar according to the MIC data. This suggests that *gluP* may not be involved in the resistance of *H. pylori* to CLA. In short, the results presented above indicate that SpoT upregulates GluP to cope with specific antibiotic stress.

**GluP is involved in *H. pylori* efflux.** GluP, a glucose transporter, is responsible for glucose transport in *H. pylori* (21, 39). In addition, structural analysis demonstrates that GluP is an efflux pump belonging to the MFS, which suggests that GluP likely functions in drug efflux in *H. pylori*. Therefore, we successfully constructed a *gluP* mutant strain (the *ΔgluP* strain) and a *gluP*-complemented strain (the *gluP\** strain) and compared the efflux capacity of the WT, *ΔgluP*, and *gluP\** strains. These results revealed that the inactivation of GluP caused a distinct increase in the accumulation of Hoechst 33342, whether it was in biofilm-forming or planktonic cells but especially in planktonic cells, and the fluorescence values of the *ΔgluP* strain were >3-fold greater than those of the WT and *gluP\** strains. These results showed that the *ΔgluP* strain had a lower efflux capacity for Hoechst 33342 than the WT strain in both planktonic and biofilm-forming cells (Fig. 6).

**GluP is involved in *H. pylori* biofilm formation and multidrug resistance.** Studies have reported that efflux pumps participate in bacterial biofilm formation (33), so we compared the biofilms of the WT, *ΔgluP*, and *gluP\** strains by SEM. The images showed that compared with the bacteria in the biofilms of the WT and *gluP\** strains, the bacteria in the biofilm of the *ΔgluP* strain were not tightly packed, and the biofilm matrix was incomplete and showed more cavities (Fig. 7A). By CLSM, LIVE/DEAD cell viability assays showed that the *ΔgluP* strain formed a thinner biofilm (Fig. 7B and C).

According to the MIC for planktonic cells of the WT strain and the *ΔgluP* strain (Table 1), the *ΔgluP* strain was apparently more sensitive to various antibiotics than the WT strain, except for its sensitivity to CLA and ciprofloxacin. After knocking out *gluP* in the MDR-H strain, its resistance to drugs, especially its resistance to AMO, also decreased significantly (Table 1). In addition, for those biofilm-forming cells, after knocking out *gluP*, their sensitivity to various antibiotics increased (Table 1).

We treated the WT, *ΔgluP*, and *gluP\** strains separately with three antibiotics (CLA, AMO, and MET) at the MIC (for the WT strain) and then observed their growth inhibition

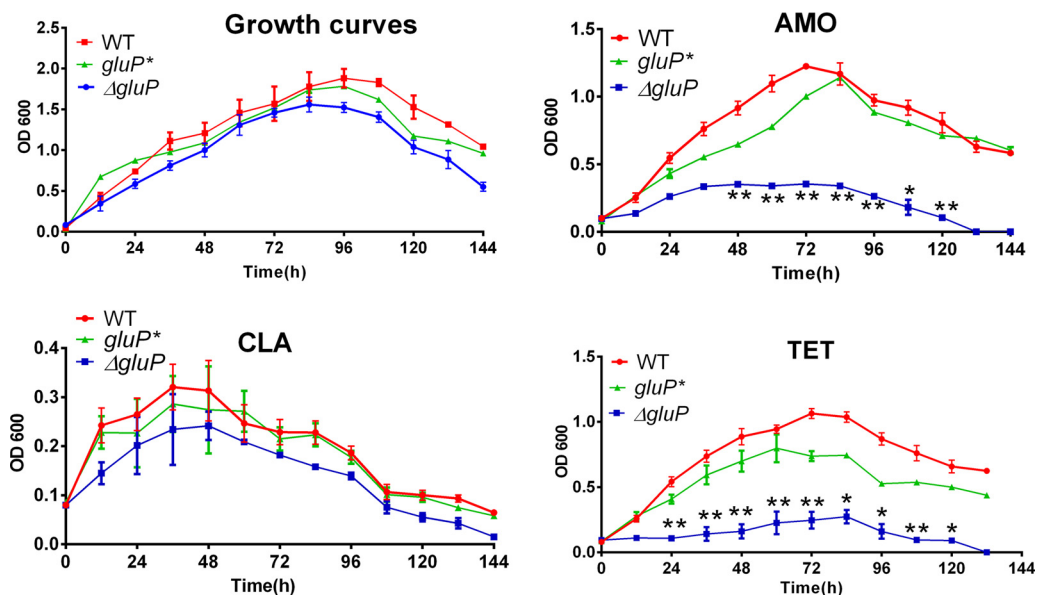


**FIG 7** GluP is involved in *H. pylori* biofilm formation. (A) Scanning electron microscopy (SEM) images of WT,  $\Delta$ *gluP*, and *gluP*\* strain biofilms. (B) Confocal laser scanning microscopy (CLSM) images of WT,  $\Delta$ *gluP*, and *gluP*\* strain biofilms. Cells stained with membrane-permeant SYTO 9 (green) and membrane-impermeant propidium iodide (PI) (red) were visualized by confocal microscopy. (C) Comparison of the biofilm thickness between the WT,  $\Delta$ *gluP*, and *gluP*\* strains, using data from the assay whose results are presented in panel B. The data presented are the means  $\pm$  standard errors of the means from three independent experiments. Significance was determined by the paired Student's *t* test. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . The biofilm used in this experiment is a mature biofilm grown on a nitrocellulose membrane for 3 days, while the planktonic *H. pylori* bacteria were from the early exponential phase ( $OD_{600}$  approximately 0.4 to 0.5).

curves. The results showed that compared with the growth of the WT and *gluP*\* strains, the growth of the  $\Delta$ *gluP* strain was significantly inhibited by AMO and MET but not by CLA (Fig. 8). The results presented above suggest that GluP may not be involved in the tolerance of planktonic *H. pylori* bacteria to CLA, which is consistent with the results of MIC testing.

The relative mRNA expression levels of *gluP* were assessed by qRT-PCR in the clinical MDR strains (C-MDR) and clinical drug-sensitive strains (C-DSS). The average expression levels of *gluP* in the C-MDR were significantly higher than those in the C-DSS (Fig. 9).

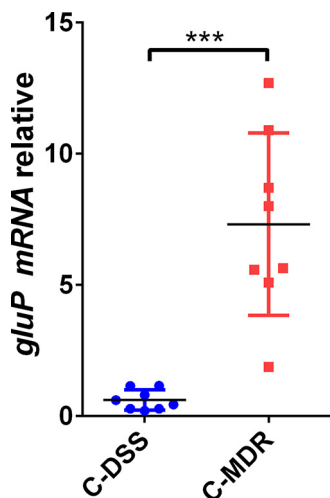




**FIG 8** Comparison of growth curves and growth inhibition curve characteristics of the WT,  $\Delta gluP$ , and *gluP\** strains. (A) Growth curves of the WT and  $\Delta gluP$  strains. (B, C, and D) Growth inhibition curves of the WT,  $\Delta gluP$ , and *gluP\** strains exposed to AMO (0.125  $\mu\text{g/ml}$ ), CLA (0.125  $\mu\text{g/ml}$ ), and MET (0.5  $\mu\text{g/ml}$ ). Data are the means  $\pm$  standard errors of the means from three independent experiments. Significance was determined by the paired Student's *t* test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

## DISCUSSION

Recently, the antibiotic resistance acquired by *H. pylori* has generally increased, and the formation of biofilms *in vivo* is an important cause leading to this multidrug resistance (10). In this research, we found that SpoT is involved in biofilm formation in *H. pylori*. While it has been reported that the efflux pump functions in the formation of biofilms and multidrug resistance (33), we found that *gluP* is involved in the biofilm formation and multidrug resistance of *H. pylori*. Further analysis showed that the expression of *gluP* is upregulated by SpoT. Considering that SpoT is a global regulator,



**FIG 9** Analysis of *gluP* expression differences in clinical drug-sensitive strains (C-DSS) ( $n = 8$ ) and clinical multidrug-resistant strains (C-MDR) ( $n = 8$ ) by qRT-PCR. The results were compared to the value for *gluP* in the WT. The signal was normalized to the 16S rRNA levels. Data are the means  $\pm$  standard errors of the means from three independent experiments. Significance was determined by the paired Student's *t* test. \*\*\*,  $P < 0.001$ .

our study partly explains the molecular mechanism by which SpoT is involved in the biofilm formation and multidrug resistance of *H. pylori*.

Antibiotics, as signaling molecules (40), can induce bacteria to produce (p)ppGpp and lead to a stringent response (14). Studies have shown that (p)ppGpp is involved in bacterial tolerance to various antibiotics, such as penicillin (41), vancomycin (42), and ampicillin (43); in *H. pylori*, (p)ppGpp is synthesized from SpoT (21). Our previous studies discovered that (p)ppGpp modulates the expression of *H. pylori* efflux transporters (44), accompanied by the importance of the efflux pump to multidrug resistance (24); thus, we wondered whether (p)ppGpp participates in the multidrug resistance of *H. pylori*. This research confirmed that inference.

Biofilm formation is another important factor that causes multidrug resistance in bacteria (7). Biofilms are communities of microorganisms anchored to a surface and live in an extracellular matrix that is made up of EPS produced by the organisms to form their immediate environment (7). Bacteria with biofilms have strong resistance to antibiotics and host immune defenses (6). It has been reported that (p)ppGpp is involved in the formation of biofilms in various bacteria, but the function of (p)ppGpp during this process varies from species to species. Several previous studies have shown that the lack of (p)ppGpp resulted in decreased biofilm formation in bacteria, such as *Enterococcus faecalis* (38), *Vibrio cholerae* (45), and *Bordetella pertussis* (16). However, other studies have proven that (p)ppGpp synthase deletion mutants of *Actinobacillus pleuropneumoniae* (17) and *Francisella novicida* (46) can produce significantly more biofilms than can the WT. Since *spoT* is the only gene that plays a part in the synthesis of (p)ppGpp in *H. pylori*, its ability to synthesize (p)ppGpp is totally lost after the knockout of *spoT*, consequently lowering its ability to adapt to stressful environments (19, 20, 22). In this study, we induced *H. pylori* to form a biofilm through nutrient starvation because nutrition deficiency is a significant factor that induces bacteria to form biofilms. After *spoT* knockout, the ability of *H. pylori* to form biofilms was greatly reduced.

Biofilm formation is an important cause of multidrug resistance in bacteria, and biofilms lead to continuous chronic infections, which add to the difficulty in providing a clinical cure (6). Drug resistance mechanisms include the following: poor diffusion of antibiotics through the biofilm polysaccharide matrix, physiological changes due to the low growth rate and starvation responses that form persistent cells, phenotypic changes of the cells forming the biofilm, and the expression of efflux pumps (7). Drug efflux is a key mechanism of drug resistance in Gram-negative bacteria (24). Microorganisms regulate the internal environment to adapt to their outer circumstances by excluding poisonous substances (antimicrobial agents, metabolites, and quorum-sensing chemical signals) via efflux pumps (25). There are six families of bacterial efflux pumps: (i) the ABC superfamily, (ii) the MFS, (iii) the multidrug and toxic compound extrusion (MATE) family, (iv) the small multidrug resistance (SMR) family, (v) the resistance-nodulation-division (RND) superfamily, and (vi) the drug metabolite transporter (DMT) superfamily (33). It is generally agreed that the ABC, MFS, and RND families play important roles in Gram-negative bacteria (33). As extensive studies regarding the RND family in *H. pylori* have been conducted (8, 28, 32, 37), we focused on the function of the MFS and the ABC superfamily.

Normally, the expression of efflux pumps in biofilm-forming bacteria is higher than that in planktonic cells (33). For example, in *P. aeruginosa*, the mechanism for tolerance to colistin is the upregulation of the MexAB-OprM efflux pump in biofilms (47). Moreover, in *E. coli* cells grown in biofilms and exposed to several antibiotics, the genes encoding the AcrAB-TolC efflux pump are upregulated (48). In addition, the expression levels of the *acrA* and *acrB* genes have been observed to increase in *Salmonella* biofilm cells (49). It has been reported that the RND family of efflux pumps is highly expressed in *H. pylori* biofilms (37), and recent studies have shown that Hp1165 and *hefA* are highly expressed in clinically isolated *H. pylori* biofilms (50). In our research, the expression of several efflux pumps was high in *H. pylori* biofilms. Some further studies of *gluP* found that efflux pump expression functioned in the formation of *H. pylori*

biofilms. After *gluP* knockout, the *H. pylori* biofilm matrix is damaged and the bacteria are unable to form a well-structured biofilm. Some studies confirmed that the extremely low level of biofilm formation observed in the WT can be observed in *E. coli* mutants without *emrD*, *emrE*, *emrK*, *acrD*, *acrE*, and *mdtE* efflux pump-encoding genes (51). Efflux pumps are involved in biofilm formation, possibly due to the export or import of some substances related to biofilm formation. *gluP* is a glucose/galactose transporter belonging to the MFS (21), which is mainly responsible for the physiological uptake of sugars, such as D-glucose, into *H. pylori* (39). D-Glucose is involved in the synthesis of bacterial exopolysaccharides, while polysaccharides account for a major fraction of the biofilm matrix (52). Therefore, the deletion of *gluP* may affect the synthesis of the *H. pylori* biofilm matrix. We found that the matrix of the *H. pylori* biofilm was incomplete after *gluP* knockout, even if the concentration of glucose in the medium was increased (see Fig. S1 in the supplemental material). In addition, the biofilm matrix can limit the transport of some antimicrobial agents to cells within the biofilm (7).

The results of the growth inhibition curve (Fig. 8) and MIC (Table 1) analyses showed that knocking out *gluP* did not affect the sensitivity of *H. pylori* to CLA, suggesting that *gluP* may not be involved in the tolerance of *H. pylori* to CLA. In fact, although the efflux pump is an important weapon for multidrug resistance, it has a certain specificity for antibiotic substrates (53); for example, the AcrAB-TolC pump is not involved in telithromycin efflux in *Enterobacter aerogenes* or *Escherichia coli* (54). Our results suggest that CLA is not a good substrate for *gluP*.

Extensive studies concerning the regulatory mechanism of efflux pumps, such as two-component signal (TCS) transduction systems, local repressors, and global response regulators, have been conducted (55). In *E. coli*, (p)ppGpp can also regulate the expression of the efflux pump of Yojl (56). There is one possible mechanism that explains how (p)ppGpp indirectly mediates global changes at the transcriptional level, such as by reducing the RpoD competitiveness for the core RNA polymerase. (p)ppGpp releases RpoD from the RNA polymerase and shifts the use of the alternative sigma factors (57). It is known that the *H. pylori* genome includes only two alternative sigma factors ( $\sigma^{54}$  and  $\sigma^{28}$ ) (21). According to the research of Niehus and coworkers, the promoter sequence of  $\sigma^{54}$ -dependent genes is TTTGCTT (58). By analyzing the upstream sequence of the putative ATG start codon of the open reading frame of *gluP*, we discovered a possible conserved sequence (TTTGCAT) that was recognized by  $\sigma^{54}$  (Fig. S2), which suggests that *gluP* could be regulated by  $\sigma^{54}$ . In further studies, antibiotic treatment could not induce high *gluP* expression in the  $\sigma^{54}$  mutant strain (Fig. S3). Therefore, SpoT may upregulate the expression of *gluP* by  $\sigma^{54}$ -dependent transcription, but detailed mechanisms require more studies.

In conclusion, this research has discovered a new mechanism regarding biofilm formation and multidrug resistance in *H. pylori*. Further analysis is required to identify the specific mechanisms by which SpoT regulates *H. pylori* biofilm formation. On account of the present data, our research provides evidence and clues for the clinical treatment of patients infected with drug-resistant strains and epidemiological investigation.

## MATERIALS AND METHODS

**Bacterial strain, media, growth conditions, and clinical isolation of *H. pylori*.** *H. pylori* 26695, which was kindly provided by Zhang Jianzhong (Chinese Disease Control and Prevention Center), was used in this study. The strain was maintained at  $-80^{\circ}\text{C}$  in brucella broth (BB; Difco, Detroit, MI) with 20% (vol/vol) glycerol and 10% fetal bovine serum (FBS). The strain was cultured under a microaerobic environment (5%  $\text{O}_2$ , 10%  $\text{CO}_2$ , 85%  $\text{N}_2$ ) at  $37^{\circ}\text{C}$  on brucella agar plates containing 7% lysed sheep blood. The liquid culture medium for *H. pylori* consisted of BB containing 10% FBS, and the cells were incubated in a shaker set at 120 rpm at  $37^{\circ}\text{C}$ . The mutant strains were cultured on agar plates with kanamycin (Sigma-Aldrich, St. Louis, MO) at  $30\ \mu\text{g}/\text{ml}$ . The *E. coli* strain was TOP10 (TransGen Biotech, Beijing, China) and was grown in Luria-Bertani medium at  $37^{\circ}\text{C}$ .

Eight MDR clinical isolates and eight sensitive clinical isolates were obtained from patients, including those with gastritis, gastric ulcers, duodenal ulcers, and gastric cancer, at Qiannan People's Hospital (Guizhou Province). All the patients provided informed consent before examination. The study was approved by the ethics committee of School of Medicine, Shandong University. MDR *H. pylori* strains from the patients could not be killed with repeated standard triple-therapy treatment. Isolated *H. pylori* strains

were identified using universally accepted phenotypic tests: typical morphology on Gram-stained smears; and positive urease, oxidase, and catalase tests. The names of the C-DSS ( $n = 8$ ) and C-MDR ( $n = 8$ ) are listed in Table S1 in the supplemental material.

**Assessment of susceptibility to antibiotics.** The MICs of various antibiotics for all the clinical and standard strains were determined by the Etest and the agar dilution method as reported by Osato et al. (59). The bacteria (optical density at 600 nm [ $OD_{600}$ ], 0.8) were inoculated on an agar plate containing 2-fold dilutions of antibiotics. All the plates were incubated at 37°C under microaerobic conditions, and MIC values were determined. The method used to determine the MICs for biofilm-forming bacteria was similar to that used for the planktonic bacteria. The biofilm, which was attached to a nitrocellulose (NC) membrane, was incubated in a liquid medium containing different concentrations of various antibiotics for 12 h. After being washed three times with phosphate-buffered saline (PBS), the biofilm bacteria were suspended in liquid culture medium, and this liquid was inoculated on an agar plate to determine the MIC values.

**Construction of biofilm.** The biofilm could be cultivated under two kinds of conditions: the static condition or the continuous-flow condition. There are many ways to cultivate biofilms under the static condition; we used the colony biofilm (described in previous articles [60, 61]) with slight modification. To allow the adherence of *H. pylori* at the interface, 20  $\mu$ l of bacteria was inoculated at  $5 \times 10^7$  cells  $ml^{-1}$  onto an NC membrane (approximately 1 by 1  $cm^2$ ), which was placed on an agar plate. The agar plate was cultured in a microaerobic environment (5%  $O_2$ , 10%  $CO_2$ , 85%  $N_2$ ) at 37°C for 3 days.

**SEM.** To observe the *H. pylori* biofilm, scanning electron microscopy (SEM) was used. The samples for SEM were prepared using the following standard procedures. Planktonic bacteria were fixed with glutaraldehyde after centrifugation. For the biofilm-forming bacteria, the samples were gently washed with autoclaved PBS three times to remove the planktonic bacteria. The biofilms were fixed in 2.5% glutaraldehyde at 4°C for 2 h and then washed three times with cacodylate buffer and dehydrated through a series of graded ethanol solutions (25%, 50%, 75%, 95%, and 100%). Subsequently, the samples were freeze-dried, sputter coated with gold, and observed by SEM.

**CLSM.** To determine bacterial shape and viability, planktonic *H. pylori* bacteria were stained with membrane-permeant and membrane-impermeant fluorescent dyes from LIVE/DEAD BacLight bacterial viability kits (Molecular Probes, Invitrogen, USA). Then, they were observed by confocal microscopy, which was performed as described in a previous study (44). For biofilm-forming cells, the NC membrane with the biofilm was removed from the agar plate and then gently washed three times with PBS. Subsequently, the NC membrane was stained in a 12-well plate with 1 ml of SYTO 9 dye for 20 min under dark conditions and gently washed three times with PBS. After that, the NC membrane was fixed on a slide, covered with a coverslip, and subsequently analyzed by confocal laser scanning microscopy (CLSM; Leica TCS SP5; Leica Microsystems GmbH, Wetzlar, Germany). SYTO 9 is a green fluorescent membrane-permeant dye that labels all bacteria by staining nucleic acid, whereas propidium iodide (PI) is a red fluorescent membrane-impermeant dye that labels only bacteria with damaged membranes.

**Detection of (p)ppGpp accumulation patterns.** (p)ppGpp production was assayed according to the method described in a previous study with slight modification (44). The treatment of the planktonic bacteria was the same as previously described (44). The biofilm bacteria were washed with PBS, diluted to an  $OD_{600}$  of 0.2, and incubated for an additional 2 h. When all the strains reached an  $OD_{600}$  of approximately 0.3, samples from each culture plate were centrifuged at 10,000 rpm for 5 min and resuspended in 250  $\mu$ l of liquid culture medium.  $^{32}P$  (Amersham) was added to 100  $\mu$ Ci  $ml^{-1}$ , and the cultures were labeled for 2 h at 37°C. Then, 50  $\mu$ l of each sample was added to an equal volume of 2 M formic acid. Afterward, at least four freeze-thaw cycles were conducted. The acid extracts were briefly centrifuged, and the supernatants were spotted onto polyethyleneimine-cellulose plates (Sigma-Aldrich), dried, and developed in 1.5 M  $KH_2PO_4$  (pH 3.4) for approximately 2.5 h. The results were obtained under phosphor screen scanning (Bio-Rad).

**Extraction of RNA and quantitative real-time PCR (qRT-PCR).** Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA). To reverse transcribe the total RNA, a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa) was used. The primers used are shown in Table 3. The 20- $\mu$ l quantitative PCR mixtures contained 5  $\mu$ l of the resulting cDNA, which was already diluted; 10  $\mu$ l of SYBR Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan); 0.8  $\mu$ l of the primer mixture; and 4.2  $\mu$ l of double-distilled water. Then, RT-PCR was performed using an ABI Prism 7000 sequence detection system (Applied Biosystems, Carlsbad, CA) for 1 cycle at 95°C for 30 s and 40 cycles at 95°C for 5 s and 60°C for 31 s. Dissociation curve analysis was performed to verify the product homogeneity. The 16S rRNA amplicon was used as an internal control for data normalization. Changes in the transcript level were determined by applying the relative quantitative ( $\Delta\Delta C_T$ ) method. The threshold cycle ( $C_T$ ) values for all three biological replicates for each strain were compiled.

**Construction of Hp1174 (*gluP*) and *spoT* mutants and complemented strains.** In our previous studies, we successfully constructed an *spoT* mutant strain (the  $\Delta spoT$  strain) (62) and an *spoT*-complemented strain (the *spoT\** strain) (44). The plasmids (pILL570 and pUC18K2) used to construct the mutant strain were kindly provided by Agnès Labigne (Unité de Pathogénie Bactérienne des Muqueuses, Institut Pasteur). The construction of the *gluP* mutant strain was identical to the construction of the  $\Delta spoT$  strain, as described in a previous study (62). Briefly, the *gluP* gene from the genome of *H. pylori* 26695 was destroyed with the insertion of the nonpolar *aphA-3* gene, encoding a kanamycin resistance cassette. The construction of the *gluP*-complemented strain was similar to the construction of the *spoT\** strain. Briefly, the *gluP*-complemented strain (the *gluP\** strain) was constructed using the chloramphenicol resistance cassette from pMcagA, which was kindly presented by Wei Hong (Department of Microbiology, Guizhou Medical University, China). Full-length *gluP* was cloned into pMcagA, and the resulting

**TABLE 3** Primers used in this study<sup>a</sup>

Forward primers		Reverse primers	
Name	Sequence (5'–3')	Name	Sequence (5'–3')
hp1174SF	CGGGATCCTATTATTGGGGAGGCGCGAT	hp1174SR	CCATCGATAGCTCTGCCTCATCAAATACTG
hp1174XF	AACTGCAGACTTCTAACTCTGGCGCT	hp1174XR	CGGAATTCACCCGCAAGCCCAATAAG
hp1174CF	CGGAATTCATGCAAAAACTTCTAACAC	hp1174CR	CGGGATCCTTAGGAGTTTTCTTCTTGCT
hp0313	CGCATGCTTTTTACCCATTT	hp0313	AGAAAACACCACCCACGAAG
hp0936	ACGCGCCAAGTTTAGTCAAT	hp0936	CAGGCGTGCTATAGCTTTT
hp1091	TGCGTTCCTAGCGCCTTATT	hp1091	GCGCCATAAGGATAATGGAA
hp1165	AGGGAGTCTTTGGGATCGT	hp1165	AAGACGGCGTAATCAAATG
hp1174	CCGCTGGTAATCCCTTTGTA	hp1174	CTTGCAATATCGCCATTTT
hp1181	GGGGTGGCGTTTTTCTTTAT	hp1181	CCCCAAATAAGCCCTAAAGC
hp1185	ATCTCTGGGCATTTACCAC	hp1185	CCATCGCAAAAGCGATAAAT
hp1220	CAAAAGGCATGAGGGAAAAA	hp1220	TTGCGTTTTGGCTAAATTC
hp1082	TGCCGTTAGCTGCTATTCT	hp1082	ACGGCGATGTTTTTGATACC
hp1486	AAATGAAGCCACACCACTC	hp1486	TAAATCCGCATGCATTGTA
hp1206	TTTTCTGCTTGCTGATG	hp1206	CCCACCAAGCAAAAATAA
hp0714	GGGTTTTCCCATAAGCAT	hp0714	AGAGGCGATGTTGAGCAGTT

<sup>a</sup>Underlining indicates nucleotides that were added at the 5' end to create a restriction site.

plasmid was inserted into the middle of the Hp0547 (*cagA*) gene, which provided homologous recombination sites in *H. pylori*. The vector-transformed *gluP* mutant strain was constructed by electroporation to obtain the *gluP*-complemented strain (the *gluP\** strain). The genotype of the complemented *gluP\** transformant was verified by PCR and sequencing of the genomic loci. The primers used in these studies are listed in Table 3.

**Determination of growth curves and growth inhibition curves.** The growth curves were determined as described in a previous study (44). The growth profiles in BB with a preliminary OD<sub>600</sub> of 0.08 were monitored, and then the bacteria were cultured for another 144 h at 37°C with shaking. Records were taken every 12 h by determining the OD<sub>600</sub> of the test strain. The values stated are the mode values from at least three biological replicates performed on at least three independent occasions.

To analyze the growth inhibition curve, the *H. pylori* strains were inoculated into BB, which also contained different antibiotics (at the MIC for the WT strain), with a preliminary OD<sub>600</sub> of 0.08, and then the bacteria were cultured for another 144 h at 37°C with shaking. Each experiment was repeated at least three times.

**Hoechst 33342 accumulation assay.** For the planktonic bacteria, the accumulation assay was performed as described previously (44). The biofilm bacteria were first rinsed with PBS and subsequently suspended in PBS, with the final OD<sub>600</sub> being 0.1. Then, 180 μl of this liquid and 20 μl of Hoechst 33342 (25 μM; Sigma-Aldrich) were added to each well of a 96-well plate. Recording began 5 min after the addition of Hoechst 33342. Excitation and emission were measured at 355 nm and 460 nm, respectively, using a FLUOstar Optima microplate reader (BMG LABTECH, Aylesbury, UK). Readings were taken every 75 s for 30 cycles, and the raw data were analyzed by use of the Excel program. Each experiment was repeated at least three times.

**Generation of MDR-H.** In order to obtain artificially selected multidrug-resistant *H. pylori* (MDR-H), *H. pylori* 26695 was cultivated on an agar plate containing 0.5× MIC of chloramphenicol for 48 to 72 h under a microaerobic environment at 37°C, as described in a previous study (63). The resistant colonies were incubated with repeated doubling of the chloramphenicol concentration until no colony was seen. Colonies were maintained on agar plates containing 4× MICs of TET, ampicillin, penicillin G, and erythromycin. Then, the colonies were incubated for 48 to 72 h under a microaerobic environment.

**Statistical analysis.** Data are presented as the means ± standard errors of the means. Statistical significance was determined using the unpaired Student's *t* test, and the *P* values were corrected by the Sidak-Bonferroni method for multiple comparisons. *P* values of <0.05 were considered statistically significant. The results were analyzed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00957-18>.

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

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We declare that we have no conflicts of interest.

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