

Twin-Arginine Translocation System in *Helicobacter pylori*: TatC, but Not TatB, Is Essential for Viability

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ABSTRACT The twin-arginine translocation (Tat) system, needed to transport folded proteins across biological membranes, has not been characterized in the gastric pathogen *Helicobacter pylori*. Analysis of all *H. pylori* genome sequences available thus far reveals the presence of single copies of *tatA*, *tatB*, and *tatC* needed for the synthesis of a fully functional Tat system. Based on the presence of the twin-arginine hallmark in their signal sequence, only four *H. pylori* proteins appear to be Tat dependent: hydrogenase (HydA), catalase-associated protein (KapA), biotin sulfoxide reductase (BisC), and the ubiquinol cytochrome oxidoreductase Rieske protein (FbcF). In the present study, targeted mutations were aimed at *tatA*, *tatB*, *tatC*, or *queA* (downstream gene control). While double homologous recombination mutations in *tatB* and *queA* were easily obtained, attempts at disrupting *tatA* proved unsuccessful, while deletion of *tatC* led to partial mutants following single homologous recombination, with cells retaining a chromosomal copy of *tatC*. Double homologous recombination *tatC* mutants were obtained only when a plasmid-borne, isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible copy of *tatC* was introduced prior to transformation. These conditional *tatC* mutants could grow only in the presence of IPTG, suggesting that *tatC* is essential in *H. pylori*. *tatB* and *tatC* mutants had lower hydrogenase and catalase activities than the wild-type strain did, and the ability of *tatC* mutants to colonize mouse stomachs was severely affected compared to the wild type. Chromosomal complementation of *tatC* mutants restored hydrogenase and catalase activities to wild-type levels, and additional expression of *tatC* in wild-type cells resulted in elevated Tat-dependent enzyme activities. Unexpectedly, the *tat* strains had cell envelope defects.

IMPORTANCE This work reports the first characterization of the twin-arginine translocation (Tat) system in the gastric pathogen *Helicobacter pylori*. While *tatB* mutants were easily obtained, only single-crossover partial *tatC* mutants or conditional *tatC* mutants could be generated, indicating that *tatC* is essential in *H. pylori*, a surprising finding given the fact that only four proteins are predicted to be translocated by the Tat system in this bacterium. The levels of activity of hydrogenase and catalase, two of the predicted Tat-dependent enzymes, were affected in these mutants. In addition, all *tat* mutants displayed cell envelope defects, and *tatC* mutants were deficient in mouse colonization.

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The twin-arginine translocation (Tat) system is needed for protein export across the cytoplasmic membranes of bacteria and archaea, as well as for protein import into the thylakoids of chloroplasts. The key feature of the Tat pathway is its ability to transport folded proteins across biological membranes, while another translocation system, the Sec system, can transport only unfolded, nascent proteins that fold after they cross the membrane (1). The Tat system enables cofactors such as flavins or iron-sulfur clusters to be retained during transit across the membrane. The target proteins are often predicted to perform redox functions; therefore, such cofactor stability is crucial to translocated enzyme activity. In addition, some Tat-transported proteins are involved in metabolism, metal acquisition, or cell envelope maintenance (2, 3). Precursor proteins that are translocated through the Tat pathway contain a conserved, distinctive (S/T)-R-R-X-F-L-K motif, in which X can be any polar amino acid and the consecutive arginine residues are almost invariant (4). The minimal set of components

required for Tat translocation in *Escherichia coli*, the most extensively studied organism, consists of three integral membrane proteins: TatA, TatB, and TatC (5). Two other genes designated *tatD* and *tatE* can also be found in bacteria such as *E. coli*. While *tatD* has no apparent function in Tat-dependent protein transport (6), *tatE* is a cryptic gene duplication of *tatA*, and the proteins encoded by these genes are functionally interchangeable (7). In other bacteria, the TatB component does not seem to be essential for export, as some genomes (*Staphylococcus aureus* for instance) carry genes that encode only a single TatA and TatC (8).

The importance of the Tat system varies among microorganisms. It has been shown to be required for virulence in several animal, human, or plant pathogens, including *Salmonella enterica* serovar Enteritidis (9), *Yersinia pseudotuberculosis* (10), *Vibrio cholerae* (11), *Dickeya dadantii* 3937 (12), or *Campylobacter jejuni* (13). The last pathogen is of significance for the present study, because *C. jejuni* and *Helicobacter pylori* are closely related micro-

TABLE 1 Predicted Tat-transported proteins in *H. pylori*

Protein ^a	N-terminal sequence ^b	Description	Protein predicted by the following Tat signal prediction program ^c :		
			PRED-TAT	TATFIND	TatP
HP0407	MSIS RRS SILTKIPIALASANVLKA	Biotin sulfoxide reductase (BisC)	Yes	No	No
HP0631	MFYDEKKTQKIEERLDIVRSFNAHNEHKNLQDEFKAGI SRRDLK WAG MMSTALALPASFAPLTLKA	Hydrogenase small subunit (HydA)	Yes	Yes	No
HP0874	M KRRD FKTTLTGATGAVLGAQILQA	Catalase-associated protein (KapA)	Yes	Yes	Yes
HP1540	MADI QRRD FLGMSLASVTAIGAIAASLVAMKKTWDPLPSVSA	Ubiquinol cytochrome <i>c</i> oxidase, Rieske (FbcF)	Yes	Yes	Yes

^a HP numbers refer to *H. pylori* strain 26695 (21).

^b The signal sequence with the twin-arginine motif (the two arginines shown in boldface type) [consensus sequence (S/T)RRXFLK] is shown up to the cleavage site predicted by PRED-TAT (29). Conserved amino acids found in the Tat consensus sequence are underlined.

^c Prediction programs used in this study are PRED-TAT (29), TATFIND (30), and TatP (31). The proteins that were predicted by the programs are shown in boldface type.

organisms that belong to the same group of *Epsilonproteobacteria*. While the Tat system is dispensable in most microorganisms characterized so far (including *C. jejuni*), it has been shown to be essential in only a few bacterial or archaeal species, including *Sinorhizobium meliloti* (14), *Bdellovibrio bacteriovorus* (15), *Mycobacterium tuberculosis* (16), and the halophilic archaea *Halobacterium salinarum* and *Haloferax volcanii* (17, 18).

H. pylori, the causative agent of peptic ulcers, colonizes the gastric epithelium (19) in about 50% of the world's population (20); therefore, it is probably the most successful pathogenic bacterium in the world. So far, very little is known about the Tat system in *H. pylori*. Analysis of all *H. pylori* genomes available thus far suggests that there is a single copy of *tatA*, *tatB*, and *tatC* genes. Both *tatB* and *tatC* (*hp1060* and *hp1061*, respectively, in sequenced strain 26695 [21]) are part of the same six-gene operon, while *tatA* (*hp0320*) is located elsewhere on the chromosome and is part of a five-gene polycistronic unit (22). Based on a study by Sargent and coworkers who successfully complemented an *E. coli* *tatA* mutant with a plasmid expressing *H. pylori* *tatA* (23), *H. pylori* *tatA* appears to be functional, at least in *E. coli*. In the present study, we strived to determine the role of the Tat pathway in *H. pylori* and its importance for survival and pathogenesis by generating a set of mutations in *tatB* and *tatC* genes in various *H. pylori* parental strains. Our results reveal the essentiality of TatC, but not TatB, for viability of this gastric pathogen and an intriguing and unexpected role of TatC in cell envelope defects and host cell colonization.

RESULTS

Putative components and predicted targets of the Tat pathway in *H. pylori*. Analysis of several *H. pylori* genome sequences, including those of *H. pylori* strains 26695 (21), J99 (24), HPAG-1 (25), G27 (26), 98-10 (27), and B128 (27), reveals the presence of one copy of *tatA*, *tatB*, and *tatC*, the minimum set of genes required for a functional Tat translocase in Gram-negative bacteria (5, 28). For the well-characterized *E. coli* model system, *tatA*, *tatB*, and *tatC* genes are part of one unique *tatABC* operon; however, in *H. pylori*, the *tatA* gene is located on another locus (*hp0320* for strain 26695) unrelated to *tatBC* (*hp1060-hp1061* for strain 26695) (see Fig. S1 in the supplemental material). Another feature of the *H. pylori* Tat pathway is the absence of a *tatE* ortholog, which is present in *E. coli*. Similarities and differences between *E. coli* and

H. pylori Tat systems have been previously highlighted by the fact that *H. pylori* *tatA* could functionally complement an *E. coli* *tatA* mutant, whereas *H. pylori* *tatB* cannot complement an *E. coli* *tatB* mutant (complementation of *E. coli* *tatC* by *H. pylori* *tatC* was not tested) (23). These findings are supported by comparison analysis of *H. pylori* and *E. coli* Tat protein sequences: TatA homologs share 46% identity and 63% similarity, while TatB homologs share only 27% identity and 46% similarity; TatC homologs display 33% identity and 54% similarity.

Compared to most bacteria, *H. pylori* appears to have a surprisingly limited list of putative Tat targets (Table 1). Indeed, a search based on the presence of the conserved (S/T)-R-R-X-F-L-K motif (X is any polar amino acid) (the hallmark of the Tat system) in putative signal sequences using three different prediction programs—PRED-TAT (29), TATFIND (30), and TatP (31)—suggests that there are only four proteins believed to depend upon Tat for their translocation in the gastric pathogen: the catalase accessory protein KapA; the hydrogenase small-subunit protein HydA; a putative biotin sulfoxide reductase, BisC; and the cytochrome oxidase Rieske subunit protein FbcF. Only two of these (KapA and FbcF) are predicted to be Tat-dependent proteins by all Tat prediction programs.

Construction of *tatC*, Δ *tatC*, and Δ *tatA* mutants. Our initial attempt to inactivate the Tat system in *H. pylori* consisted of transforming several wild-type (WT) strains (26695, 43504, and SS1) with a suicide plasmid (pSLB129) that contained the *tatC* gene (*hp1061*) disrupted with a kanamycin resistance cassette (*aphA3*). Multiple attempts were mostly unsuccessful and led to only one kanamycin-resistant mutant (SLB1066), generated from strain 26695. This mutant was analyzed further but with caution because of the possibility of compensatory mutations. PCR analysis of this unique clone revealed a single homologous recombination (SHR) with (i) the entire plasmid inserted at the *tatC* locus, as detected by PCR using primers specific for the *bla* plasmid marker (data not shown), and (ii) the presence of the disrupted *tatC::aphA3* construct and an intact copy of *tatC* within the chromosome (see Fig. S2 in the supplemental material).

Given the possibility that a truncated TatC polypeptide (still synthesized from the *tatC::aphA3* construct) would be detrimental to *H. pylori*, we constructed an alternative suicide plasmid in which *tatC* was almost completely deleted (pSLB137 [see Table S1 and Fig. S1 in the supplemental material]). Transformation of the

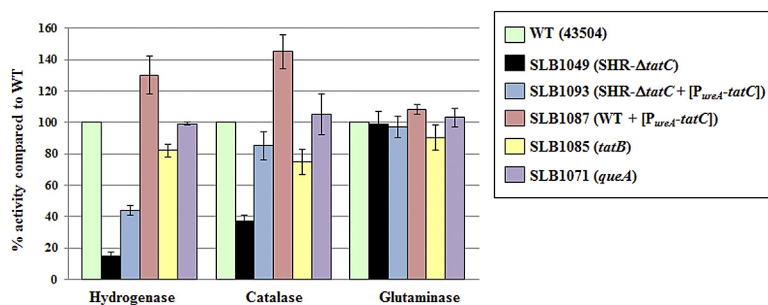


FIG 1 Hydrogenase, catalase, and glutaminase activities of wild-type and various mutant strains. Each enzyme activity was determined using live whole cells. The strains are shown in the symbol key to the right of the graph. Results shown are means \pm standard deviations (error bars) of activities relative to that of the wild type and represent 2 to 4 independent growth experiments, with at least three replicate assays.

three *H. pylori* strains described above either with pSLB137 or with a purified PCR product containing the same (Δ tatC::*aphA3*) sequence yielded dozens of Kan^r transformants, but only in strain 43504. PCR analysis revealed that none of the mutants was the result of double homologous recombination (DHR); instead, they all retained an intact copy of *tatC* while integrating Δ tatC::*aphA3* within their chromosome (Fig. S2). To increase our chances of obtaining DHR-*tatC* mutants, the *aphA3* marker was replaced by a chloramphenicol (Cm) acetyltransferase (*cat*) marker, previously shown to be a better tool to generate mutations in *H. pylori*, according to Gorrell et al. (32). Transformation with plasmids or PCR products containing Δ tatC::*cat* yielded Cm^r clones; however, once again, PCR analysis revealed that they resulted from SHR (data not shown). Even though all attempts at generating *tatC* and Δ tatC mutants led to SHR, we reasoned that recombination within the *tatBC* locus or in its vicinity might still yield useful strains (i.e., affecting the Tat system). These partial mutants (from here on referred to as “SHR-*tat* mutants”) were therefore analyzed further by (i) looking at Tat-dependent enzyme activities (hydrogenase and catalase) and (ii) complementing them with a chromosomal copy of *tatC* (see below). Finally, attempts at constructing Δ tatA mutants by introducing PCR products containing Δ tatA::*cat* in several parental strains were unsuccessful, suggesting that *tatA* is an essential gene in *H. pylori*.

Unlike *tatA* or *tatC*, the *tatB* gene can be inactivated by double homologous recombination. Since disruption of *tatA* or *tatC* proved to be a challenge, we targeted another component of the Tat machinery, TatB. The *tatB* gene (*hp1060*) was cloned into two different vectors, and a *cat* cassette was inserted into two unique restriction sites naturally present within *tatB* (see Fig. S1 in the supplemental material). Transformation of wild-type *H. pylori* with both plasmids yielded dozens of Cm^r clones, and PCR analysis of the mutants’ genomes revealed that recombination by double crossover had occurred in each case (Fig. S2), in contrast with what had been observed for SHR-*tatC* or SHR- Δ tatC mutants (Table S3). This result suggests that, unlike *tatA* or *tatC*, *tatB* can be successfully mutagenized. Therefore, *tatB* does not appear to be essential in *H. pylori*.

Hydrogenase and catalase activities are affected in *tatB* mutants, SHR- Δ tatC mutants, and merodiploid cells. Hydrogenase and catalase are among the proteins whose location, and as a consequence, activity, is expected to be affected if the Tat machinery is impaired in *H. pylori*. Indeed, *H. pylori* possesses only one (hydrogen uptake) [Ni-Fe] hydrogenase, previously shown to be

membrane-bound (33), and there is a twin-arginine consensus in the sequence of the small-subunit HydA (Table 1). Catalase (KatA, HP0875) does not have a signal sequence; however, the catalase-associated protein (KapA, HP0874) possesses one signal sequence that includes a Tat motif (Table 1), and it is therefore hypothesized that KatA relies on KapA to be translocated on the periplasmic side of the membrane through a “hitchhiking” mechanism (34). This hypothesis is strengthened by the fact that KatA and KapA have been shown to interact (35) and that catalase activity is reduced in periplasmic fractions of *kapA* mutants (34). Therefore, whole-cell hydrogenase and catalase activities were determined on wild-type and various mutant strains (Fig. 1). While hydrogenase and catalase activities were only slightly decreased in *tatB* (SLB1085) mutants (approximately 80% of the level in the wild type), they were significantly reduced in SHR- Δ tatC (SLB1049) mutants. The latter suggests that the Tat machinery is indeed impaired in single-crossover Δ tatC mutants. By inserting a copy of *tatC* in an unrelated region of the chromosome of SHR- Δ tatC mutants, we generated a strain (SLB1093) in which hydrogenase and catalase activities were partially and fully restored, respectively (Fig. 1). Expression of the same P_{ureA}-*tatC* construct in wild-type cells (merodiploid cells, strain SLB1087) led to higher hydrogenase and catalase activities than those measured in the wild type (Fig. 1).

Hydrogenase and catalase activities in a *queA* strain (our control strain for assessing possible polar effects) were similar to the activities in the wild type (Fig. 1); this confirmed that the phenotype of *tat* mutant strains can be assigned to the absence or disruption of *tatB* or *tatC*. All mutant strains and the wild type were also assayed for glutaminase activity, our control enzyme for non-Tat-dependent periplasmic activity. Glutaminase (also known as γ -glutamyl transpeptidase) was previously shown to be detected in periplasmic and extracellular fractions (36, 37). Despite the presence of an almost perfect Tat-specific consensus (RRSFLK) in its N-terminal sequence, it is not predicted to be translocated by the Tat system by any of the programs; instead, the PRED-TAT program predicts that glutaminase is Sec dependent (29). Wild-type-like glutaminase activities were recorded for all mutants (Fig. 1), confirming that glutaminase is indeed not a Tat target and suggesting that cellular integrity of *tat* mutants is not compromised.

To confirm that the differences in catalase activities in these different strains were indeed due to cellular mislocalization rather than to differences in catalase activities *per se*, complementary

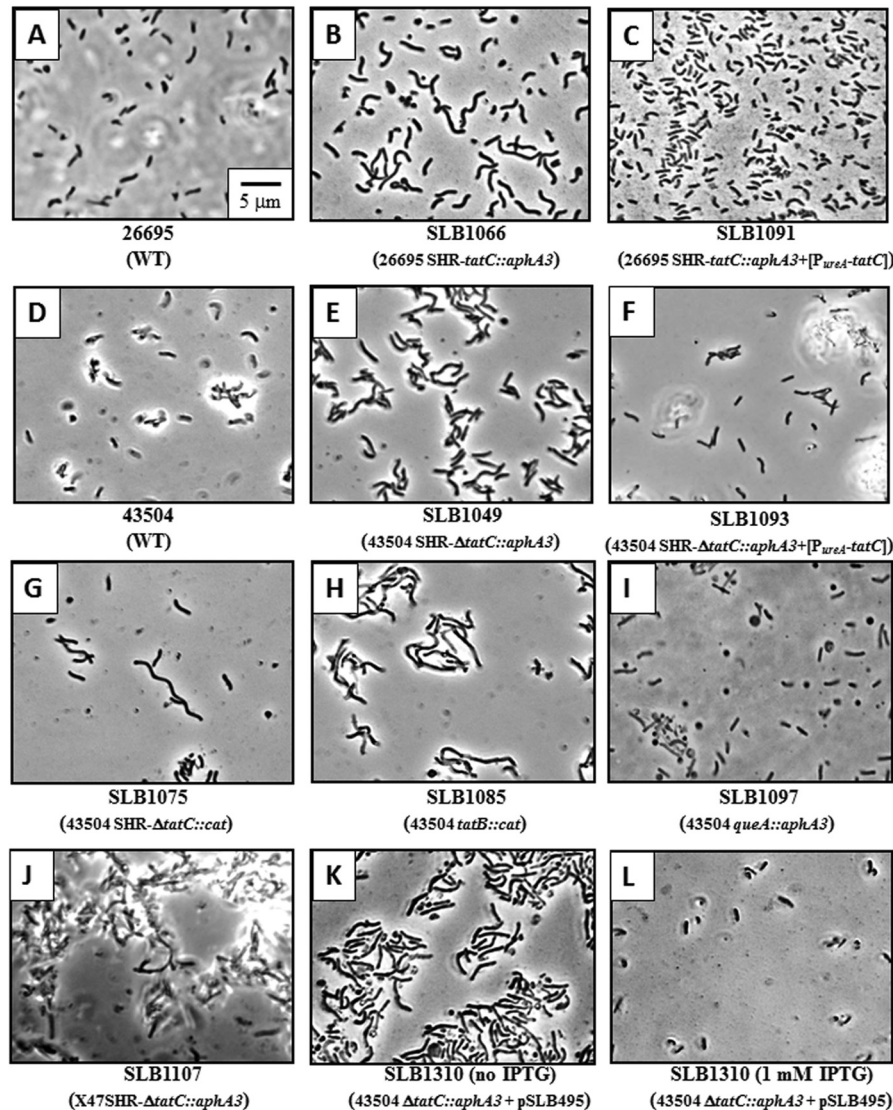


FIG 2 Phase-contrast microscopy of various wild-type, *tatB*, *tatC*, and *queA* mutant cells.

assays were carried out using cell-free protein extracts. As shown in Fig. 1, whole-cell catalase activity of SHR- Δ *tatC* mutants was only $37\% \pm 4\%$ of that of the WT; however, when cell-free extracts of the same SHR- Δ *tatC* mutant strain were used for the assay, there was no noticeable difference in catalase activity levels between SHR- Δ *tatC* and wild-type cells (data not shown). From this control experiment it is concluded that the observed decreased catalase activity in whole cells of *tatC* mutants is likely due to mislocalization of the catalase enzyme. Taken together, these results suggest that SHR-*tatC* mutants are indeed *tat* mutants and that hydrogenase and catalase, as previously hypothesized, rely on the Tat machinery to be transported to their final destination.

***H. pylori* *tat* mutants have cell division defects.** Phase-contrast microscopy analysis of various strains with mutations in genes encoding components of the Tat system revealed unusually long cells that appear to result from deficient cell division (Fig. 2). Indeed, cells from strain 26695 SHR-*tatC* (SLB1066 [Fig. 2B]) were longer than cells from the parental strain (26695 [Fig. 2A]),

while cells from strains 43504 SHR- Δ *tatC*::*aphA3* (SLB1049 [Fig. 2E]), 43504 SHR- Δ *tatC*::*cat* (SLB1075 [Fig. 2G]), and 43504 *tatB*::*cat* (SLB1085 [Fig. 2H]) were all longer than WT cells (43504 [Fig. 2D]). The other *tatB* mutant (strain SLB1086) also displayed the same phenotype (data not shown). Chromosomal complementation of SLB1066 and SLB1049 strains with a copy of *tatC* restored wild-type-like cellular morphology (SLB1091 and SLB1093 [Fig. 2C and F], respectively). The morphologies of mutant strains with mutations generated in the *queA* control gene in strain 43504 (SLB1097 [Fig. 2I]), 26695, or X47 (data not shown) were similar to that of the wild type, suggesting that the observed phenotype is not due to a polar effect on the *queA* gene; rather, it can be attributed to deficiencies of the Tat pathway in these different mutants.

SHR- Δ *tatC* mutants are deficient in mouse colonization. Since hydrogenase and catalase activities are required for full colonization of the mouse stomach (38, 39), we hypothesized that the ability to colonize mice might be affected in *tatC* mutants. There-

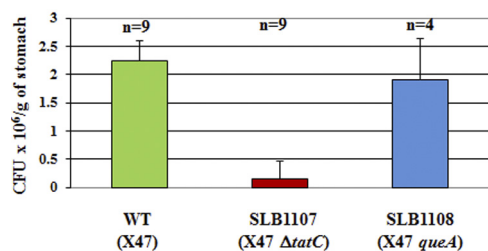


FIG 3 Mouse stomach colonization levels of wild-type, SHR-*tatC*, and *queA* mutant strains. Mice were orally given approximately 1.5×10^8 *H. pylori* cells of a strain. After 3 weeks, their stomachs were harvested, homogenized, diluted, and plated, and the number of CFU was determined 3 to 5 days after harvest. The number of mice used for each strain is shown above the columns.

fore, the mouse-adapted X47 strain was transformed with either plasmid pSLB137, containing Δ tatC::*aphA3*, or plasmid pSLB130, containing *queA*::*aphA3* (control). Kan^r transformants were isolated in both cases; however, PCR analysis revealed that there was double crossover for X47 *queA*::*aphA3* mutants (SLB1108 strain), but not for X47 Δ tatC::*aphA3* mutants (SLB1107 strain) as previously observed with other *H. pylori* strains in the current study (data not shown). Hydrogenase and catalase activities of SLB1107 mutants were approximately 80% and 50% compared to that of the wild-type X47 strain, respectively, and these were statistically significant differences. Also, SLB1107 mutant cells (Fig. 2J) were longer than their parental strain (X47), albeit less morphologically altered than other *tat* mutants. X47 (wild-type), SLB1107, and SLB1108 strains were orally given to mice, and colonization levels in the stomach were assessed 3 weeks later (Fig. 3). While the wild-type strain X47 colonized all 9 inoculated mice and the SLB1108 *queA* control strain colonized all 4 inoculated animals with colonization levels similar to that of the wild type, SLB1107 was severely deficient in its ability to colonize mice: most mice had lower colonization levels, and two of them had no detectable recovered CFU from stomach homogenates (Fig. 3). Therefore, a fully functional Tat system is required to efficiently colonize mouse stomachs.

Use of conditional *tatC* mutants reveals that *tatC* is an essential gene in *H. pylori*. The fact that the activities of predicted Tat-dependent enzymes (hydrogenase and catalase) were decreased in SHR- Δ tatC mutants (or increased upon the addition of chromosomal *tatC*) strongly suggests that TatC is indeed affected in SHR- Δ tatC strains. Nevertheless, we sought to complement these data by creating strains that contained inducible *tatC* and chromosomal inactivated *tatC*. Therefore, we constructed *tatC* conditional mutants by (i) cloning *tatC* under the control of a P_{tac}

promoter in a shuttle vector able to replicate in *H. pylori* (40), (ii) introducing this plasmid containing P_{tac} -*tatC* (pSLB495) into wild-type cells (strain SLB1308), and (iii) eventually targeting the chromosomal *tatC* locus in the presence of isopropyl- β -D-thiogalactopyranoside (IPTG). Using this approach, for the first time the chromosomal copy of *tatC* was inactivated by double homologous recombination (strain SLB1310 [see Fig. S2 in the supplemental material]). In the presence of Cm and IPTG, strain SLB1310 was able to grow as well as the wild-type strain (Table 2) and had similar cell morphology (Fig. 2L); however, when strain SLB1310 was diluted with brain heart infusion (BHI) broth and spread on blood agar (BA) medium without Cm or IPTG, it was unable to grow, indicating that *tatC* is indeed essential in *H. pylori* (Table 2).

Directly restreaking SLB1310 cells from selective medium (Cm-IPTG) to nonselective medium resulted in a mixed population of live and dying cells that had lower hydrogenase and catalase activity than the wild type, while retaining the same glutaminase activity (Fig. 4). These conditional mutants grown on nonselective plates displayed the same abnormal cellular morphology as SHR- Δ tatC or *tatB* mutants, confirming the link between Tat and cell morphology in *H. pylori* (Fig. 2K). Interestingly, the SLB1308 strain (the intermediate strain used to construct conditional *tatC* mutants), which possess chromosomal and plasmid *tatC* copies, had higher hydrogenase and catalase activities than the wild type when grown in the presence of Cm and IPTG (Fig. 4). Taken together, these results confirm that a fully functional Tat pathway is required for hydrogenase and catalase activities, as previously hypothesized based on signal peptide analysis. In addition, they highlight the unexpected link between the Tat system and cell division in *H. pylori*. Most importantly, they strongly suggest that the Tat pathway, or more specifically TatC, is required for *H. pylori*.

The requirement for *tatC* could be linked to FbcF. Among the four proteins expected to be translocated via the Tat pathway in *H. pylori*, two of these proteins, KapA (and its partner KatA) and hydrogenase have already been shown to be dispensable in *H. pylori*. Indeed, *kapA* can be successfully disrupted and even though *kapA* mutants cannot colonize mice anymore, they are viable under standard lab conditions (38). Likewise, a previous study revealed that strains with mutations in the large hydrogenase subunit HydB (encoded by *hp0632* in strain 26695 [21]) are viable, despite attenuated ability to colonize mice (39). To rule out the possibility that deletion of *hp0631*, the gene encoding the twin-arginine-containing subunit HydA, would be lethal, we deleted the entire *hyd* operon (*hp0631* through *hp0635*) in two *H. pylori*

TABLE 2 *tatC* is essential for *H. pylori* viability

Strain	Genotype	Medium	Growth ^a	OD ₆₀₀ ^b
43504	WT	BA	Yes	27 ± 3
SLB1308	WT carrying pSLB495 (P_{tac} - <i>tatC</i>)	BA	Yes	30 ± 2
		BA-Cm-IPTG	Yes	23 ± 2
SLB1310	Δ tatC mutant carrying pSLB495 (P_{tac} - <i>tatC</i>)	BA	No	ND
		BA-Cm-IPTG	Yes	21 ± 5

^a Cells were grown on BA (for WT) or BA-Cm-IPTG (for SLB1308 and SLB1310 strains) for 24 h. The cells were harvested and washed in BHI broth, and 0.2-ml suspension (OD₆₀₀ of 0.1) was plated on either BA or BA-Cm-IPTG medium. Cells from each plate were harvested after 48 h and resuspended in 1 ml of PBS, and OD₆₀₀ was determined.

^b Results shown are the means ± standard deviations for 4 independent experiments. ND, not determined.

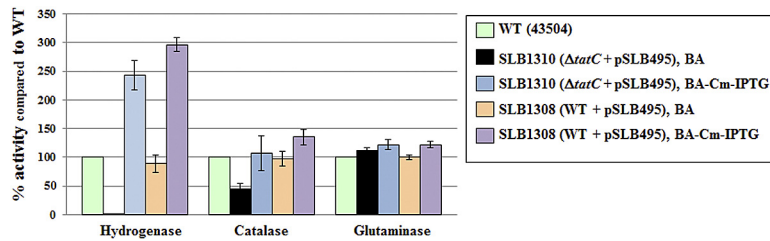


FIG 4 Hydrogenase, catalase, and glutaminase activities of WT and conditional *tatC* mutants. Each enzyme activity was determined using whole cells. Strains and growth conditions are indicated in the symbol key to the right of the graph. BA is blood agar, a noninducible medium. BA-Cm-IPTG is blood agar with Cm and IPTG, an inducible medium. Results shown are means \pm standard deviations of activities relative to that of the wild type and represent 2 to 4 independent growth experiments, with at least three replicate assays.

parental strains, 26695 and 43504 (data not shown). As expected, these $\Delta hydABCDE$ mutants were devoid of hydrogenase activity; however, their growth and morphology were similar to those of the wild type (data not shown). Therefore, HydA, or more broadly hydrogenase, is not an essential enzyme in *H. pylori*. Besides, mutations in the *hp0407* (*bisC*) gene were also generated in three different parental strains in the present study. These mutants were viable and microscopically similar to the wild type (data not shown). Finally, the fourth putative Tat-dependent protein, FbcF, was targeted. This time, multiple attempts at constructing *fbcF* mutants were unsuccessful, suggesting that *fbcF* is essential in *H. pylori*. Albeit indirect, this result links the essentiality of the Tat system at least in part to an incorrect localization of the essential cytochrome oxidase Rieske subunit.

DISCUSSION

In the present study, we showed that *H. pylori* *tatB* (*hp1060* in strain 26695 [21]) can be inactivated while *tata* (*hp0320*) or *tatC* (*hp1061*) appear to be essential in *H. pylori*. Indeed, using two independent suicide plasmids containing part of the *hp1060* DNA sequence disrupted by a chloramphenicol resistance cassette, we were able to obtain dozens of chloramphenicol-resistant clones in each case. Genetic analysis of the mutants unambiguously confirmed that in both cases *tatB* was disrupted by insertion of the marker, following double homologous recombination. Since it appears there is no *tatB* homolog (duplicated copy) in *H. pylori*, *tatB* does not seem to be essential in *H. pylori*. Mutations in *tatB* appear to have limited effect on hydrogenase and catalase activities in *H. pylori*, with cells retaining approximately 80% of activity in each case. Following the same mutant construction strategy, we were able to successfully disrupt *hp1062* (*queA*), the gene located downstream of *tatC* (see Fig. S1 in the supplemental material). In contrast, multiple attempts to disrupt *tata* were unsuccessful, and attempts at deleting *tatC* were also unsuccessful or led to partial mutants retaining an undisrupted copy of *tatC* in their chromosome (Table S3). These gene disruption results occurred regardless of the source of DNA (circular or linear suicide plasmid or PCR), the marker used (chloramphenicol or kanamycin resistance), the *H. pylori* recipient strain (SS1, 26695, 43504, or X47) or the DNA treatment (methylation with *H. pylori* cell extracts). The occurrence of single homologous recombination within the *tatBC* locus is expected to have a wide range of effects on Tat(B)C expression and stability and on the assembly of the final TatABC complex; this could include decreased *tatC* levels or the formation of truncated nonfunctional Tat chimeras that would compete

with intact Tat machinery, etc., and these phenotypes have yet to be characterized.

Results from a global transposon mutagenesis study aimed at determining essential genes in *H. pylori* (41) showed that there was no transposon insertion in *hp1061* (*tatC*), while there was one insertion in *hp1062* (*queA*), suggesting that *tatC* is essential, while *queA* is not, in agreement with results from the present study. However, these results have to be taken with caution, as the same study reported no transposon insertion in *hp1060* (*tatB*) and one transposon “hit” in *hp0320* (*tata*) (41); we now know from the current study that *tatB* mutants can be constructed, while *tata* mutants cannot. The fact that *tatB* appears to be dispensable (while *tata* and *tatC* are not) suggests that one or several Tat-dependent targets (yet to be determined) can be translocated in a TatB-independent manner. It also suggests that TatAC, rather than TatABC, constitutes the minimum translocase set in *H. pylori*. This would differentiate *H. pylori* from other Gram-negative bacteria, whose vast majority rely on an integral TatABC complex (5). However, this has to be stated with caution, given the fact that *tatB* mutants showed the same abnormal microscopic morphology as partial or conditional *tatC* mutants (Fig. 2). To determine whether the phenotypes observed for *tatB* mutants were due to a possible polar effect on *tatC* expression levels, we used quantitative reverse transcription-PCR (qRT-PCR) to compare *tatC* mRNA levels in *tatB* mutants (SLB1085 and SLB1086) to that of the wild-type strain. The levels of expression of *tatC* standardized to *gyrA* (see Text S1 in the supplemental material) were comparable in *tatB* mutant and wild-type strains (data not shown). Therefore, phenotypes observed for *tatB* mutants (decreased hydrogenase and catalase activities, abnormal morphology) are not the consequence of decreased *tatC* expression but rather suggest that TatB plays an important role, albeit one that is probably less critical than *Tata* or *TatC*, among the Tat machinery in *H. pylori*.

More generally, the finding that the Tat system—or more precisely TatAC—appears to be essential in *H. pylori* is surprising considering the limited number (four) of predicted target proteins in the gastric pathogen. The *H. pylori* Tat pathway is not the first bacterial pathway shown to be essential; however, the three other bacterial species requiring Tat for survival, *M. tuberculosis* (16), *S. meliloti* (14), and *B. bacteriovorus* (15), have significantly higher numbers of predicted Tat-dependent substrates with 31, 94, and 21 substrates, respectively (5, 42). In addition, the Tat system has been found to be also essential in halophilic archaea, such as *H. salinarum* and *H. volcanii* (17, 18). Most haloarchaea rely almost exclusively on Tat (instead of Sec) because the proteins need

to be properly folded in a timely manner (prior to translocation) before being exposed to the high-salinity environment. For instance, *H. salinarum* and *H. volcanii* appear to have 60 and 68 Tat-dependent substrates, respectively (5).

The search for Tat-specific signal peptides in *H. pylori* was done on translated DNA sequences from the sequenced strain 26695 (21). In order to account for possible start codon misannotation that would hamper our quest for Tat signal peptides, alternate start codons (still resulting in valid reading frames) were identified upstream and downstream of existing annotated start codons (Govind Chandra and Dave Widdick, personal communication). This search found only three proteins believed to be Tat targets in *H. pylori*, HP0874 (KapA), HP1031 (HydA), and HP1540 (FbcF), in agreement with previously published genome mining data (42). The HP0407 (BisC) protein was not found in this search because only TatP (31) and TATFIND (30) programs were used and none of them predicts HP0407 to be a Tat-translocated protein (Table 1). However, the most recent prediction program, PRED-TAT (29), categorizes HP0407 as Tat dependent. HP0407 is annotated as biotin sulfoxide reductase (based on sequence homology), but its role has yet to be determined. When expressed in *E. coli*, recombinant HP0407 localized to the membrane, consistent with its expected final localization (S. Benoit, unpublished data). In addition, a selenocysteine codon and a molybdopterin-guanine dinucleotide (MGD) binding motif can be identified in the sequence of HP0407 (J. Craig Venter Institute [JCVI] Comprehensive Microbial Resource [CMR] at <http://cmr.jcvi.org>). These features are shared by various reductases, such as formate dehydrogenases, dimethyl sulfoxide (DMSO) or trimethylamine N-oxide (TMAO) reductases, all of which are known to be translocated by the Tat system (3); therefore, HP0407 appears to be a prime candidate for Tat dependency in *H. pylori*. In the current study, we report the construction of viable *hp0407* mutants (by double homologous recombination) in three different *H. pylori* strains (43504, X47, and 26695), indicating that the *hp0407* gene is not essential in *H. pylori*.

KapA (HP0874) is one of the other proteins predicted to be dependent on Tat (Table 1). A previous two-hybrid study revealed that KapA can interact with KatA (HP0875), the catalytic catalase subunit (35). Through a mechanism commonly described as “hitchhiking” (43), KatA would bind to KapA, and the heterologous protein complex would be translocated by the Tat system through the cytoplasmic membrane (using KapA signal peptide) to reach the periplasm. In agreement with this model, Harris and Hazell showed that there was 5.5-fold-less catalase activity in periplasmic fractions of *kapA* mutants compared to periplasmic fractions of wild-type cells (34). Results from the present study also support this model: we found less catalase activity in whole cells of *tatB* mutants, SHR- Δ *tatC* partial mutants, or *tatC* conditional mutants grown in the absence of IPTG compared to whole-cell activity in the parental strain, suggesting that less catalase was effectively transported toward the periplasm. The decrease in catalase activity observed in SHR- Δ *tatC* mutants was not due to decreased overall activity in these mutants, because catalase activity in cell-free extracts (total protein) of mutant and wild-type cells was shown to be almost identical. Besides, increasing the expression of *tatC* in mutant or wild-type cells, either through expression of a P_{ureA} -*tatC* chromosomal copy or a P_{tac} -*tatC* plasmid copy in the presence of IPTG, led to increased levels of whole-cell catalase activity, indicating a correlation between *tatC* levels and cat-

alase distribution within the cell. The construction of *kapA* and *kata* mutants in *H. pylori* has been previously reported (34); therefore, none of these genes appear to be essential under lab conditions. Hence, it is unlikely that the lethality observed for conditional *tatC* mutants is linked to mislocalization of KapA and KatA.

Whole-cell enzyme assays were also used as an indirect way to assess hydrogenase distribution in *H. pylori* *tat* mutants. There is only one hydrogenase in *H. pylori*, previously shown to be the H₂ uptake type and membrane bound (33). The heterotrimeric HydABC complex is expected to rely on HydA and its Tat signal peptide (Table 1) to be translocated to the cytoplasmic membrane. If the Tat machinery is absent or nonfunctional, the hydrogenase complex will still be synthesized but unable to reach the membrane and electron transport components, and H₂ oxidation coupled to cytochrome oxidases will probably be decreased. Indeed, a decrease of hydrogenase activity was observed in whole cells of SHR- Δ *tatC* partial mutants or uninduced conditional *tatC* mutants and, to a lesser extent, in *tatB* mutants. In contrast, the addition of *tatC* (complemented SHR- Δ *tatC* partial mutants, merodiploid strain, or induced conditional *tatC* mutants) led to increased whole-cell hydrogenase activities. These results strongly suggest a correlation between *tatC* levels and hydrogenase distribution within the cell. HydB-deficient or hydrogenase-negative (Δ *hydABCDE*) mutants are viable (reference 39 and our current study); therefore, it is not expected that mislocalization of the hydrogenase complex (due to Tat deficiency) would lead to cellular death.

The fourth hypothesized target is FbcF (HP1540). Based on protein sequence analysis, it is a [2Fe-2S]-containing Rieske subunit of a cytochrome *b* oxidase. Rieske subunits from a variety of microorganisms, such as *Synechocystis*, *Paracoccus denitrificans*, or *Legionella pneumophila*, have been shown to be Tat dependent (44–46). Mislocalization of FbcF would lead to impaired (oxygen) respiration and aerobic growth defects, as recently shown with Rieske protein homologs in the obligate aerobe *Streptomyces coelicolor* or the facultative anaerobe *Shewanella oneidensis* (47, 48). *H. pylori* is microaerophilic and has very limited respiration capacity, with no formate dehydrogenase, nitrate reductase, or DMSO or TMAO reductase; as stated above, the role of the HP0407 protein is still unknown. In addition, the gastric pathogen appears to possess only one *cbb3*-type cytochrome *c* terminal oxidase (21, 49). The closely related epsilonproteobacterium *C. jejuni* also possesses a Tat-dependent ubiquinol cytochrome *b* oxidase (PetA or Cj1186c [50]); however, it has a branched respiratory chain with two different terminal oxidases, a *cbb3*-type cytochrome *c* oxidase and a *bd*-type quinol oxidase (50). Therefore, mislocalization of FbcF is expected to have a bigger impact on *H. pylori* respiration (and viability) than mislocalization of PetA on *C. jejuni*. This difference could explain why *C. jejuni* *tatC* (13) mutants are viable while *H. pylori* *tatC* mutants are not. To determine whether FbcF is important for *H. pylori*, we attempted to generate *fbcF* mutants; however, this approach was unsuccessful, suggesting that *fbcF* is essential in *H. pylori*. Therefore, mislocalization of FbcF, along with the mislocalization of the other Tat-dependent proteins and their associated complex, HydA-hydrogenase, KapA-catalase, and HP0407, probably accounts for the lethality of conditional *tatC* mutants, as well as the deficiency in mouse stomach colonization observed for X47 SHR- Δ *tatC* partial mutants.

Finally, another phenotype was observed for all the *tat* mutants: long cells that appeared to be unable to properly divide and had envelope defects were observed for *tatB*, *SHR-tatC*, *SHR-ΔtatC*, and conditional *tatC* mutants grown under uninduced conditions. Complementation with chromosomal *tatC* or plasmid-borne *tatC* in the presence of IPTG resulted in wild-type-like cell morphology. This phenotype was not completely unexpected because *E. coli* *tat* mutants have been reported to exhibit similar abnormal cellular morphology (51). The cause of this phenotype was attributed to mislocalization of two cell wall hydrolases, AmiA and AmiC, both of which possess a signal peptide with a twin-arginine motif (51). *H. pylori* possesses only one AmiA protein (HP0772 in strain 26695 [21]), which shares 28% identity with *E. coli* AmiA and 32% identity with *E. coli* AmiC. Analysis of the protein sequence of *H. pylori* AmiA (*HpAmiA*) reveals the presence of a signal sequence (see Fig. S3 in the supplemental material); however, there is no twin-arginine motif, and it is predicted to be Sec dependent by programs such as PRED-TAT (29) or SignalP-4.1 (52). Therefore, AmiA is not expected to be mislocalized in *H. pylori* *tat* mutants, and it is probably not the reason for the observed abnormal cell morphology. Since none of the (four) proteins predicted to be Tat dependent are supposed to play an active role in cell envelope synthesis or cell division in *H. pylori*, this phenotype suggests one of the following. (i) There are more Tat-dependent targets yet to be discovered than the four described in this article. (ii) The mislocalization of some of the Tat-dependent proteins, especially hydrogenase and cytochrome oxidase, might introduce global changes in the cell redox state that in turn will have effects such as the one observed. Further work is currently under way to elucidate which of these two hypothesis account for the observed phenotype in *tatB* or *tatC* mutants.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* and *H. pylori* strains and plasmids used in this study are listed in Table S1 in the supplemental material. Genomic DNA from *H. pylori* strain 26695 was used as the template for all PCR amplifications. All DNA plasmids or PCR products used to generate mutants were sequenced on both strands at the Georgia Genomics Facility, University of Georgia, Athens, GA.

Growth conditions. *E. coli* cells were grown aerobically in Luria-Bertani (LB) medium or plates at 37°C. Ampicillin (100 μg/ml), chloramphenicol (25 μg/ml), or kanamycin (30 μg/ml) was added as needed. *H. pylori* was routinely grown on brucella agar (BA) plates supplemented with 10% defibrinated sheep blood at 37°C under microaerophilic conditions (5% CO₂, 4% O₂, and 91% N₂). Brain heart infusion (BHI) broth was used to resuspend the cells for microscopy analysis. Chloramphenicol (8 or 25 μg/ml) or kanamycin (20 μg/ml) was added as needed. BA medium was supplemented with amphotericin B (10 μg/ml), vancomycin (10 μg/ml), and bacitracin (50 μg/ml) for mouse colonization experiments.

Construction of *H. pylori* Δ*tatA* mutants. Attempts at generating Δ*tatA* mutants were done using a splicing by overlap extension PCR (SOE PCR) method. Briefly, primers TASOE1 and TASOE2 (see Table S2 in the supplemental material) were used to amplify a 460-bp-long DNA sequence located upstream of *tata* (*hp0320*). Primers TASOE3 and TASOE4 were used to amplify a 410-bp-long sequence located downstream of *tata*. The final amplification step included each purified PCR product, a *cat* cassette, and primers TASOE1 and TASOE4. The resulting 1,670-bp-long PCR product was introduced into *H. pylori* strains X47, 43504, and 26695. The same procedure was carried out two more times without success.

Construction of *H. pylori* *tatB* mutants. Two different *tatB* mutants were generated using two restriction sites naturally present within the *tatB* (*hp1060*) gene, SspI and HindIII, located 164 bp and 324 bp downstream

of the *tatB* start codon, respectively. Primers TB1 and TB2 (see Table S2 and Fig. S1 in the supplemental material) were used to PCR amplify a 720-bp-long DNA sequence containing the 482-bp-long *hp1060* gene and flanking sequences from *hp1059* (*ruvB*) and *hp1061* (*tatC*) genes. The PCR product was either cloned directly into a pGEM-T vector to generate plasmid pSLB208 or digested with BamHI and ligated into BamHI-cut pSLB112 plasmid (SspI-free pUC19 derivative [Table S1]) to yield plasmid pSLB209. Plasmids pSLB208 and pSLB209 were digested with HindIII and SspI, respectively. Following digestion, each plasmid was subsequently blunt ended with T4 polymerase before being ligated with a blunt-ended 800-bp-long *cat* cassette (chloramphenicol resistance [Cm^r]). Finally, each newly generated plasmid, pSLB210 or pSLB212 (Table S1), was introduced into various *H. pylori* strains by natural transformation or electroporation to generate *tatB::cat-I* or *tatB::cat-II* mutants, respectively. *H. pylori* cells were transferred after 16 h onto BA plates supplemented with 25 μg/ml chloramphenicol. When using strain 43504 as the recipient strain, hundreds of clones appeared after 3 to 5 days of incubation. The disruption of the *tatB* gene and the insertion of the *cat* cassette (by double crossover) were confirmed by PCR using genomic DNA from each mutant as a template and primers TB1 and TB2 (Fig. S1 and Fig. S2).

Construction of *H. pylori* *tatC* and Δ*tatC* mutants. Two different approaches were followed to either disrupt or delete the *tatC* (*hp1061*) gene in *H. pylori*. First, a unique SspI site present within *tatC* was used to insert an *aphA3* (Kan^r) cassette. Briefly, primers TC3 and TC4 (see Table S2 and Fig. S1 in the supplemental material) were used to amplify a 717-bp-long DNA fragment containing part of *tatC*. The PCR product was digested with HindIII and cloned into similarly digested plasmid pSLB112 to yield plasmid pSLB113. A 1.3-kb-long, blunt-ended *aphA3* cassette was cloned into *tatC* (SspI; 415 bp downstream of the *tatC* start codon), generating plasmid pSLB129 (Table S1). This plasmid was introduced by natural transformation or electroporation into several *H. pylori* strains (26695, 43504, or X47). *H. pylori* cells were transferred after 16 to 24 h onto BA plates supplemented with either kanamycin or chloramphenicol. After numerous attempts, only one kanamycin-resistant clone (SLB1066) could be recovered using 26695 as the recipient strain. This mutant was analyzed by PCR using internal primers TC3 and TC4 and external primers TC1 and TC6 (Fig. S1 and Fig. S2).

In order to generate Δ*tatC::aphA3* or Δ*tatC::cat* deletion mutants, the following sequential method was used. First, a 416-bp-long DNA sequence containing part of *tatB* and the first four codons of *tatC* was amplified by PCR using primers TC1 and TC2 (see Table S2 and Fig. S1 in the supplemental material). The PCR product was digested with BamHI and EcoRI and cloned into similarly digested vector pBS-KS to generate plasmid pSLB135. This plasmid was subsequently digested with EcoRI and ligated with an EcoRI-cut *aphA3* cassette, yielding plasmid pSLB136. Next, a 410-bp-long PCR product that contained the last three codons of the *tatC* gene and the first 397 bp of the *queA* gene (obtained by using primers TC5 and TC6) was digested with SalI and XhoI and ligated with similarly cut pSLB136 plasmid to generate pSLB137 (Fig. S1). Alternatively, the *aphA3* cassette from pSLB137 was excised using EcoRI and replaced by a *cat* cassette, yielding plasmid pSLB196 (Table S1). Plasmids pSLB137 and pSLB196 were used to generate kanamycin-resistant or chloramphenicol-resistant *tatC* deletion mutants in various *H. pylori* parental strains, respectively. No Kan^r or Cm^r clone could be obtained in *H. pylori* strain 26695 despite numerous attempts, including DNA methylation with *H. pylori* cell extracts prior to transformation. In contrast, dozens of Kan^r or Cm^r transformants were isolated when strain 43504 or X47 was used. Mutants were genetically analyzed by PCR using internal primers TC3 and TC4 or external primers TC1 and TC6 (Fig. S1 and Fig. S2).

Chromosomal complementation of *SHR-tatC* or *SHR-ΔtatC* mutants. Primers TC7 and TC8 (see Table S2 and Fig. S1 in the supplemental material) were used to amplify a 763-bp-long DNA sequence containing the *tatC* coding sequence and to introduce an NdeI restriction site (at the ATG start codon) and an XhoI restriction site, respectively. The PCR

product was digested with NdeI and XhoI and ligated into similarly digested plasmid pPA (53) to place the *tatC* gene under the control of the 200-bp-long *H. pylori* P_{ureA} promoter. The plasmid generated, pSLB217, was subsequently digested with BglII and XhoI (to release the 963-bp-long P_{ureA} -*tatC* construct) and blunt ended using T4 polymerase before being ligated into EcoRV-digested plasmid pEU39Cm (54), yielding plasmid pSLB218 (Table S1). This suicide plasmid was introduced into *H. pylori* wild-type strain 43504 and into 26695 *tatC::aphA3* (SLB1066) or 43504 Δ *tatC::aphA3* (SLB1049) mutant cells, and Cm^r mutants were isolated following homologous recombination of the 1.76-kb-long P_{ureA} -*tatC*-*cat* construct within the *hp0405* gene in the chromosome, as confirmed by PCR, using genomic DNA from each mutant as a template and primers HP405 and Cat2 (data not shown).

Construction of a conditional *tatC* mutant. The NdeI- and BamHI-digested *tatC* PCR product obtained with primers TC7 and TC8 (see above and see Table S2 and Fig. S1 in the supplemental material) was ligated into similarly digested plasmid pILL2150 (40) to generate plasmid pSLB495. In this plasmid, the *tatC* gene is under the control of a (IPTG-inducible) P_{tac} promoter. Plasmid pSLB495 was methylated in the presence of *S*-adenosylmethionine (New England Biolabs, Ipswich, MA) and cell-free extracts from *H. pylori* parental strain 43504, as described by Boneca et al. (40). Methylated plasmid pSLB495 was then introduced into strain 43504, and chloramphenicol-resistant mutants were isolated. The resulting strain, SLB1308, was transformed with plasmid pSLB137 (suicide vector harboring Δ *tatC::aphA3* [see above and Fig. S1]) in the presence of Cm (8 μ g/ml) and IPTG (1 mM). Kanamycin-resistant transformants were isolated on plates supplemented with kanamycin (20 μ g/ml) as well as chloramphenicol (8 μ g/ml) and 1 mM IPTG. The disruption of *tatC* by double crossover was confirmed by PCR using external primers TC1 and TC6 (Fig. S1 and S2). These mutants were viable only in the presence of 1 mM IPTG.

Construction of *H. pylori queA* mutant. Primers QA1 and QA2 (see Table S2 and Fig. S1 in the supplemental material) were used to PCR amplify a 937-bp-long DNA sequence containing part of the 1,038-bp-long *queA* gene (*hp1062*). The PCR product was digested with EcoRI and inserted within similarly cut pBS-KS vector to generate plasmid pSLB114. A unique AfeI restriction site located within *queA* was used to introduce a 1.3-kb-long *aphA3* cassette. The resulting plasmid (pSLB130) was introduced into various *H. pylori* strains by natural transformation to generate *queA::aphA3* chromosomal mutants. Kanamycin-resistant transformants were obtained from strain 26695, 43504, or X47, generating strain SLB1071, SLB1097, or SLB1108, respectively (Table S1). The concomitant chromosomal disruption of the *queA* gene and the insertion of *aphA3* were confirmed by PCR using genomic DNA from each mutant as a template and primers QA1 and QA2 (Fig. S1 and Fig. S2).

Whole-cell enzyme assays. Hydrogenase, catalase, or glutaminase activity was determined on whole cells. Cells were grown on BA plates (with Cm and IPTG as reported) for 24 to 48 h. While the mutant to wild-type enzyme activity ratio was similar between experiments, there was significant variation in the net values in independent experiments due to the use of whole cells and growth condition variables (blood batches, gas conditions, etc.). To minimize this natural variability while still reflecting the respective enzyme activity of each mutant compared to the wild type, all enzyme activities are given as means \pm standard deviations of percentages of each activity relative to the wild-type value. One unit of optical density at 600 nm (OD₆₀₀) corresponds to approximately 1×10^9 *H. pylori* cells per ml.

(i) Hydrogenase assays. Cells were grown in presence of H₂ (in sealed anaerobic jars with CampyPak Plus microaerophilic envelopes, Becton Dickinson, Sparks, MD), harvested and resuspended in phosphate-buffered saline (PBS), cell density (OD₆₀₀) was measured and hydrogen uptake was followed using a previously described amperometric method (33). Hydrogenase activity of wild-type (43504) cells ranged from 0.5 to 1.6 nmol of H₂ used per min per 10⁸ cells. Results represent 2 to 4 independent growth experiments, each with at least 3 assay replicates.

(ii) Catalase assays. Cells were washed and resuspended in phosphate-buffered saline (PBS) to a final OD₆₀₀ of 1.0. Five microliters of whole cells was mixed with 495 μ l of PBS containing 15 mM H₂O₂, and the initial H₂O₂ disappearance (decrease in OD₂₄₀) was monitored for up to 1 min. Catalase activity of wild-type (43504) cells ranged from 20 to 110 μ mol H₂O₂ per min per 10⁸ cells depending on the experiment. Results are averages \pm standard deviations from 2 to 4 independent growth experiments, each with 5 to 10 assay replicates. For a control, catalase activities were also determined on cell-free protein extracts. In this case, cells were broken by sonication and spun down, and total protein concentration was determined using the bicinchoninic acid (BCA) kit (Thermo, Fisher Pierce, Rockford, IL). Catalase assays were carried out as described above, using 0.5 μ g total protein.

(iii) Glutaminase assays. Glutaminase activity was monitored by measuring glutamine-dependent ammonium production by whole cells, a combination of two previously published methods (55, 56). Cells were harvested, washed, and resuspended to a final OD₆₀₀ of 1.0 in 50 mM HEPES (pH 7.5) and 25 mM NaCl. The reaction was started by the addition of 10 mM glutamine, and ammonium production was determined (after 15 to 30 min) using the phenol-sodium hypochlorite method of Weatherburn (56). Glutaminase activity of wild-type (43504) cells ranged from 15 to 80 nmol NH₃ produced per min per 10⁹ cells between independent growth experiments. Results are averages \pm standard deviations from 2 to 4 growth independent experiments, each with at least 3 assay replicates.

Microscopy analysis. *H. pylori* cells grown for 24 to 48 h on BA plates were resuspended in a drop of BHI broth and examined with a phase-contrast microscope (Leica Microsystems, DM55008). Digital images were obtained at a magnification of \times 1,000 using a QIQCAM Fast 1394 camera (Compix Inc.).

Mouse colonization experiments. *H. pylori* X47 (mouse-adapted, parental strain), X47 SHR- Δ *tatC*, and X47 Δ *queA* mutant strains were grown on BA plates, harvested in sterile PBS, and resuspended (in PBS) to a final OD₆₀₀ of 1.7. Each mouse ($n = 9$ for the WT, $n = 9$ for the SHR- Δ *tatC* mutant, and $n = 4$ for the *queA* mutant) was orally given 0.15 ml of bacterial suspension (approximately 1.5×10^8 cells). Mice were sacrificed 3 weeks postinoculation. Their stomachs were quickly removed, weighed, and gently homogenized in 5-ml phosphate-buffered saline using a Dounce hand homogenizer. The homogenates were diluted in PBS and plated (0.1 ml) in duplicate on plates supplemented with bacitracin, amphotericin B, and vancomycin. The plates were incubated for 5 to 7 days at 37°C in a 4% O₂ partial pressure atmosphere for colony counting. Data are expressed as CFU recovered per gram of stomach.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01016-13/-/DCSupplemental>.

Text S1, DOCX file, 0.1 MB.

Figure S1, TIF file, 1.7 MB.

Figure S2, PDF file, 0.4 MB.

Figure S3, DOCX file, 0.1 MB.

Table S1, DOC file, 0.1 MB.

Table S2, DOC file, 0.1 MB.

Table S3, DOC file, 0.1 MB.

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