**Trans-Translation in *Helicobacter pylori*: Essentiality of Ribosome Rescue and Requirement of Protein Tagging for Stress Resistance and Competence**

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**Abstract**

**Background:** The ubiquitous bacterial trans-translation is one of the most studied quality control mechanisms. Trans-translation requires two specific factors, a small RNA SsrA (tmRNA) and a protein co-factor SmpB, to promote the release of ribosomes stalled on defective mRNAs and to add a specific tag sequence to aberrant polypeptides to direct them to degradation pathways. *Helicobacter pylori* is a pathogen persistently colonizing a hostile niche, the stomach of humans.

**Principal Findings:** We investigated the role of trans-translation in this bacterium well fitted to resist stressful conditions and found that both smpB and ssrA were essential genes. Five mutant versions of ssrA were generated in *H. pylori* in order to investigate the function of trans-translation in this organism. Mutation of the resume codon that allows the switch of template of the ribosome required for its release was essential in vivo, however a mutant in which this codon was followed by stop codons interrupting the tag sequence was viable. Therefore one round of translation is sufficient to promote the rescue of stalled ribosomes. A mutant expressing a truncated SsrA tag was viable in *H. pylori*, but affected in competence and tolerance to both oxidative and antibiotic stresses. This demonstrates that control of protein degradation through trans-translation is by itself central in the management of stress conditions and of competence and supports a regulatory role of trans-translation-dependent protein tagging. In addition, the expression of smpB and ssrA was found to be induced upon acid exposure of *H. pylori*.

**Conclusions:** We conclude to a central role of trans-translation in *H. pylori* both for ribosome rescue possibly due to more severe stalling and for protein degradation to recover from stress conditions frequently encountered in the gastric environment. Finally, the essential trans-translation machinery of *H. pylori* is an excellent specific target for the development of novel antibiotics.

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**Introduction**

*Helicobacter pylori* is a gram negative bacterial pathogen that infects the stomach of about half of the world population. *H. pylori* is mostly acquired during childhood and the infection persists during decades unless patients receive an eradication treatment. Persistent colonization is concomitant with a strong inflammation during decades unless patients receive an eradication treatment. Lifelong colonization of the gastric mucosa by duodenal and peptic ulcer, adenocarcinoma or MALT lymphoma [1]. Lifelong colonization of the gastric mucosa by *H. pylori* implies that this bacterium is well adapted to this hostile environment facing both permanent acid stress in the mucus layer and oxidative stress at the gastric epithelium due to the host’s immune response [2]. The mechanisms involved in the recovery from damages caused by the exposure to stress are critical in the adaptive response. These involve both active repair procedures (well studied for oxidative stress in *H. pylori* [3]) and quality control mechanisms. In the present study, we addressed the role of trans-translation in *H. pylori*. Trans-translation is one of the most studied quality control mechanisms that provides bacteria with a general surveillance of the flow of genetic information [4,5,6,7]. This mechanism rescues ribosomes sequestered on defective mRNAs lacking appropriate termination signals hence unable to efficiently resume the translation process. In addition, trans-translation promotes decay of these defective mRNAs and adds an amino acid tag to the truncated proteins to direct them to degradation pathways. Trans-translation relies on the properties of SsrA, a small stable RNA also called tmRNA, which shares features with a tRNA and an mRNA [5,6]. Studies mainly performed on the *E. coli* system established the following mechanism of trans-translation. First, alanylated SsrA forms a complex with essential protein partners SmpB and EF-Tu and acts as a tRNA by allowing the nascent polypeptide encoded by the defective mRNA to be transferred onto the tRNAAla-like domain of SsrA. Then, a short coding...
sequence within SsrA, referred to as the tag sequence behaves like a surrogate mRNA. This tag sequence provides the stalled ribosome with a new template for translation that is terminated by an in-frame stop codon; thus, allowing the release of recyclable ribosomal subunits, and the addition of a C-terminal tag to the nascent peptide. These tagged trans-translation polyepitides are specifically targeted to mainly ATP-dependent proteases [8,9] and the defective mRNAs are degraded by RNase R [10].

While the overall mechanism of trans-translation and the origin of defective or broken mRNAs have been extensively studied, questions on the precise biological role of this system are only partially answered. It was shown that normally growing cells undergo frequent trans-translation events [11]. In addition, there appears to be some specificity in the proteins tagged by tmRNA under normal growth conditions. [12]. Situations favoring stalling of ribosomes which are shown to require trans-translation are typically use of miscoding antibiotics [13], premature transcription termination or ribonucleolytic cleavage by RNases. Although stress or starvation are thought to enhance the amount of defective mRNAs, little is known about the actual damages occurring to ribonucleic acids under these conditions. The general assumption is that these damages are similar to those of DNA molecule i.e. generation of base adducts upon alkylation [14] or single stranded-breaks due to ROS (Reactive Oxygen Species) [15].

Genes encoding SsrA and its protein co-factor SmpB are conserved among bacteria [4] and are generally dispensable. Surprisingly, despite this conservation no common physiological function of trans-translation was found in the different bacterial systems studied. Mutants defective in trans-translation exhibit a wide range of phenotypes related to regulation of cellular physiology, cell cycle timing, stress response or virulence [7,16]. The precise reason why trans-translation is associated with these functions is rarely understood. In E. coli, inactivation of ssrA leads to reduced growth rate, delayed recovery from carbon starvation and temperature sensitivity [17,18]. In Bacillus subtilis, tmRNA dependent growth was shown during temperature or chemical stress conditions that correlated with an increase of the cellular amounts of SsrA [19]. E. coli, Salmonella or Synchocystis strains defective in trans-translation were found to be hypersensitive to different antibiotics [20,21]. Deficiency in trans-translation affects the ability of Salmonella enterica serovar Typhimurium to colonize mice [22] and to survive within macrophages [23]. A ΔssrA-ΔsmpB mutant of Teresina pseudotuberculosis is avirulent in a mouse infection model, this is due to a loss in the induction of known virulence factors (motility, Type 3 secretion system) [24]. tmRNA also has a regulatory role for the correct timing of cell cycle regulation of C. crescentus [16]. Interestingly, SsrA with a protease-resistant SsrA tag does not restore motility or proper DNA replication in T. pseudotuberculosis and C. crescentus, respectively [24,25]. However, the role of the tag in stress response was never investigated.

The essentiality of the smpB gene has been deduced from systematic gene interruption studies performed in only three organisms Mycoplasma genitalium [26], Mycoplasma palmonis [27] and Haemophilus influenzae [28]. Essentiality of ssrA was only demonstrated in Neisseria gonorrhoeae [29]. Due to the difficulties in studying essential functions inside the cells, little is known about trans-translation in species in which it is required for in vitro growth. Only in N. gonorrhoeae this phenotype analyzed further. It was demonstrated that the essential function of trans-translation is the ribosome rescue whereas tagging activity was dispensable [29].

We decided to investigate the role of trans-translation in H. pylori because this bacterium is permanently subjected to stressful conditions that could increase the occurrence of premature transcription termination events. While the predicted H. pylori tmRNA structure and essential residues were conserved in comparison with those of the well-studied molecule of E. coli, the tag sequence of H. pylori presented some striking differences. This manuscript presents the demonstration of the essential character of trans-translation during in vitro growth of H. pylori and the investigation of its functional characteristics by site directed mutagenesis. We showed that residues necessary for ribosome rescue by SsrA are essential for H. pylori growth and that the tagging of trans-translationed proteins is required for its adaptation to stressful conditions and for competence.

**Results**

smpB and ssrA are essential genes in H. pylori

Attempts to inactivate the smpB and ssrA genes encoded by hp1444 and hp0784, respectively, in several H. pylori backgrounds were repeatedly unsuccessful suggesting that these genes and the trans-translation process are essential for in vitro growth of H. pylori. In parallel, gene hp1240 predicted to encode RnaseR was deleted showing that this function is dispensable in H. pylori. To formally demonstrate the essentiality of ssrA and smpB, H. pylori strain N6 was first transformed by stably replicating plasmids pILL788 and pILL786 expressing ssrA or smpB, respectively, under control of an IPTG inducible promoter derived from pILL2150 [30] (Tables 1 and S1). Deletions of the ssrA chromosomal copy of strain N6 pILL788 and the smpB chromosomal copy of strain N6 pILL786 were obtained after transformation by suicide plasmids in the presence of IPTG as illustrated in Figure 1. These suicide plasmids carried a kanamycin resistance cassette flanked by DNA regions situated immediately upstream and downstream of the genes to be inactivated, thereby forcing homologous recombination outside the coding sequences of ssrA or smpB and thus specifically targeting allelic exchange into the chromosomal gene copy. When N6 carrying the empty vector pILL2150 was transformed with either of the two suicide plasmids, we obtained either no kanamycin resistant clones or a couple of clones that were either non viable or had undergone illegitimate recombination (Fig. 1). This demonstrated that ssrA and smpB are essential genes in H. pylori and prompted the investigation of their roles in this pathogen.

SmpB depletion in H. pylori results in growth arrest

Construction of strain N6ΔsmpB pILL786 and strain N6ΔssrA pILL786 provided us with valuable H. pylori smpB or ssrA conditional mutants. The impacts of SmpB or SsrA depletion on H. pylori growth was measured. Notably, SsrA stability has been shown to be diminished in the absence of SmpB [31]. After approx. two doubling times in liquid medium without inducer, the conditional smpB mutant stopped dividing as a consequence of SmpB depletion (Fig. 2). Interestingly, we observed that SmpB depletion stops bacterial division but does not cause cell death growth. Indeed, the SmpB conditional mutant could be rescued if plated in the presence of the inducer IPTG (until 24 h growth, data not shown). In contrast, after 24 h, growth rescue was not possible suggesting that SmpB-depleted bacteria had undergone a physiological switch irreversibly directing them towards bacterial death.

Similar experiments with the SsrA conditional mutant did not result in observable bacterial growth arrest. We concluded that the number of bacterial divisions occurring between the inoculation and the entry of the cells into stationary growth phase was not sufficient to dilute the intracellular concentration of SsrA to a level critical for cell growth. Indeed, northern blots revealed significant SsrA over-expression in H. pylori strain N6ΔssrA pILL788 (Fig. 3A). In agreement with this interpretation is the documented high stability of SsrA molecules [18].
Upon IPTG induction, both SmpB and SsrA conditional mutants presented a slightly higher growth rate when compared with the wild type strain for SmpB, see Fig. 2). This suggested that enhancing the trans-translation process improves the fitness of *H. pylori* under these conditions.

**Site directed mutagenesis of *H. pylori ssrA***

To investigate the different functions of SsrA in the trans-translation process, five different mutations were introduced in the *ssrA* gene on plasmid pILL788. Figure 4 illustrates a model of the *H. pylori* SsrA (tmRNA) molecule based on the predictions of the tmRNA web site (http://www.indiana.edu/~tmrna/). The residues for which a defined function in ribosome rescue was assigned in the well-studied *E. coli* tmRNA presented strong conservation (Fig. 4). Interestingly, the tag sequence from *H. pylori* showed several differences when compared with that of the previously studied tmRNAs from *E. coli*, *N. gonorrhoeae* or *C. crescentus*. The positions of the mutations analyzed in the present study have been emphasized in Fig. 4. The first two mutations targeted residues that were identified to be required for the interaction of SsrA with factors involved in the trans-translation process in *E. coli*. First, the predicted SmpB interaction site of SsrA [32] was modified by the introduction of three consecutive mutations G19U-A20U-C21A, and this mutant was designated SsrA<sup>mpB</sup>. Second, the G-U mismatch in the tRNA<sub>Met</sub>-like domain of SsrA was targeted. Recognition of this mismatch by the alanyl-tRNA synthetase is mandatory for the addition of Ala at the 3′ end of SsrA [33]. This mutant designated SsrA<sup>mpk</sup> carried a U390C modification. Next, by substituting the resume codon GUA (positions 84-85-86) by a stop codon (UAA) the restart of translation was abolished. This type of mutant designated SsrA<sup>mpk</sup> was not tested before in *vivo* for essentiality in another organism. To specifically study the role of the tmRNA-dependent protein tagging in *H. pylori*, two different mutations in the tag region of *ssrA* were introduced. These mutations would uncouple the two functions of tmRNA, ribosome rescue and protein tagging for degradation. In one mutant, the two terminal codons of the tag region coding for Alanine were changed into Aspartate codons (SsrA<sup>Ada</sup> in Fig. 4). In *E. coli*, non polar residues in the C-terminus part of the tag (ALAA) are critical for recognition by cellular proteases [8,9] and their mutation causes stabilization of the trans-translated peptides. We also wanted to examine the behavior of the *H. pylori* essential tmRNA under conditions in which a minimal tag was appended to the truncated peptides generated by trans-translation events. Therefore, the second and the third codons of the tag sequence were replaced by two stop codons (UAA-UGA), the mutant was designated SsrA<sub>STOP</sub>.

**Identification of essential residues in the *H. pylori* tmRNA***

Plasmids carrying mutated *ssrA* were introduced into *H. pylori* strain N6 (Table 1). To evaluate the impact of these mutations on *H. pylori* viability, these strains were transformed with the suicide plasmid pILL796 designed to delete the chromosomal copy of *ssrA* and the number of transformants on selective medium were counted (Fig. 1). The frequency of transformation was determined by calculating the number of transformants for a given amount of viable cells (5x10<sup>8</sup> bacteria) with 1 μg DNA of the suicide plasmid (pILL796). The transformation frequency of the chromosomal deletion of *ssrA* in a recipient strain N6 carrying the wild type *ssrA* plasmid (pILL788) was estimated to be 2x10<sup>−3</sup>. Identical transformation frequencies of strains carrying pILL791 with SsrA<sup>Ada</sup> and pILL2328 with SsrA<sub>STOP</sub> were obtained that were similar to that of N6 with SsrA<sub>WT</sub> (Fig. 1 B). The frequency of transformation was at least four orders of magnitude lower for the inactivation attempts of strains carrying one of the three mutations affecting the ribosome rescue process, SsrA<sup>rescue</sup> (pILL792), SsrA<sup>mpB</sup> (pILL793) and SsrA<sub>STOP</sub> (pILL794) (Fig. 1 B). This data showed that each of these essential steps of the trans-translation process is essential in *H. pylori*. In contrast, the mutations affecting the tag do not impact bacterial viability. Importantly, viability of the SsrA<sub>STOP</sub> mutant appending a minimal tag (Ala from tmRNA and Val from the resume codon) suggests that one round of translation is sufficient to rescue the stalled ribosomes. This latter mutant allowed us to evaluate the role of protein tagging in *vivo* under conditions that were more drastic than the point mutations affecting tag recognition described in previous studies.

**Mutations in the tag of the tmRNA are viable in *H. pylori* and do not affect in *vivo* colonization***

To analyze the phenotype of *H. pylori* mutants with a modified tmRNA tag, SsrA<sup>Ada</sup> and SsrA<sub>STOP</sub> mutations were introduced by
allelic exchange into the chromosome replacing the wild type ssrA alleles in three different H. pylori backgrounds N6, X47-2AL and 26695 (Table S1). N6 is a strain in which the shuttle plasmid replicates in a stable manner, X47-2AL is a mouse-adapted strain and 26695 is a strain from which the entire genome has been sequenced. The expression level and stability of the mutated versions of SsrA in H. pylori strain 26695 were identical to that of the wild type SsrA (Fig. 3A).

Mutants were obtained in every strain as expected (Table S1) and their growth under normal conditions was not affected. These strains were used to evaluate the role of tagging under several conditions relevant to the gastric niche of H. pylori such as growth at pH 5.5 (mutants of strain 26695), motility and colonization of a mouse model (mutants of strain X47-2AL) (data not shown). The mutants behaved like the corresponding isogenic wild type strains under the conditions tested. It was concluded that the tagging process of trans-translation is not essential for in vivo survival and motility of H. pylori.

Assessment of SsrA mediated protein tagging in H. pylori strains expressing mutant SsrA versions

To examine the actual protein tagging activities in H. pylori, we engineered a pair of artificial trans-translation target proteins (Fig. 5 A) composed of a fusion between the non-essential gene hypB (coding for H. pylori hydrogenase accessory protein) and a sequence encoding protein A from Staphylococcus aureus that could easily be

![Inactivation strategy and measurements of relative transformation frequency of H. pylori strain N6 harboring different plasmids: A) pILL786 carrying wild-type smpB, or B) pILL788 carrying wild-type ssrA or different plasmids with mutagenized versions of ssrA (short names of the mutations are indicated) by suicide plasmids designed to create chromosomal deletions of smpB or ssrA, respectively. A strain carrying the empty vector pILL2150 served as a negative control. The transformation frequency is calculated as the number of transformants obtained for 5×10⁸ cells and 1 μg of DNA and expressed as a percentage of that by plasmids with wild-type smpB or ssrA.](image_url)
detected by western blotting. This gene fusion designated hypB-TAP is described in Stingl et al. [34]. Our aim was to evaluate the fate of these target proteins when expressed in *H. pylori* mutants defective in tagging activity. Therefore, two constructions were generated, one was terminated by a translational stop codon and the other devoid of a stop codon, both were followed by a

![Figure 2. The effect of SmpB depletion on the growth kinetics of *H. pylori* strains.](image)

The following strains N6 pILL2150 (empty vector), N6ΔsmpB pILL786 (vector expressing the SmpB protein under control of an inducible promoter P_{tac}) were grown with the inducer IPTG 1 mM (+I) or without inducer (-I). An arrow indicates the arrest of growth of strain N6ΔsmpB pILL786. The standard deviations for 5 different measurements are shown by error bars.

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![Figure 3. Northern blot analysis of total RNA extracted from *H. pylori* 26695 strain or different isogenic mutants using a ssrA riboprobe. Panel A: *H. pylori* expressing wild type SsrA, different mutant versions of SsrA or over-expressing wild type SsrA from plasmid pILL788. Panel B: *H. pylori* wild type strain incubated at different pH values. Normalization was performed with 5S rRNA probes. The ladder corresponds to DNA of pBR322 plasmid digested by MspI, labeled and denaturated.](image)

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transcriptional terminator. Western blots in *E. coli* (Fig. 5 B) indicated that these constructs behaved like efficient trans-translation tagging target proteins. In *E. coli* MG1655 wild-type strain, the protein fusion with stop (expressed by pILL2332) was expressed in large amounts while that without stop (pILL2333) was less present indicating that protein degradation had occurred (Fig. 5 B). Involvement of trans-translation in the degradation of HypB-TAP fusion without stop was demonstrated by the strong stabilization of this protein in an *E. coli* ΔssrA strain (Fig. 5 B) in contrast to the amounts of the fusion with stop that were unchanged. Given the large Molec Mass (50 kDa) of the HypB-TAP fusion, addition of the 1.5 kDa tag by trans-translation was not visible on an acrylamide gel.

We then decided to introduce by natural transformation the two reporter genes hypB-TAP with or without stop either expressed from plasmids (pILL2332 and pILL2333) or directly by recombination on the chromosome of *H. pylori* N6 wild type strain and of each of the two tag mutants. The wild type strain was transformed by the constructs at expected frequencies. In contrast, transformation efficacy was repeatedly diminished in both SsrA mutants; SsrA<sup>STOP</sup> strain presented a three fold lower efficacy and no transformants were obtained in the SsrA<sup>STOP</sup> background (three independent experiments). Similar observations were made when a suicide plasmid targeting allelic exchange into ureA-B (described in [35]) was used as a control. The loss of competence of the SsrA<sup>STOP</sup> mutant was unexpected and suggested that trans-translation dependent tagging is required for natural transformation in *H. pylori*.

As in *E. coli*, we found that in *H. pylori* wild type strain, HypB-TAP without stop expressed from the chromosome was heavily degraded (Fig. 5B) as compared to HypB-TAP with stop suggesting that it was indeed targeted by trans-translation. The SsrA<sup>STOP</sup> mutant only marginally stabilized the HypB-TAP without stop protein (about two fold) indicating that it was still subject to proteolysis.

Minimal trans-translation-dependent protein tagging leads to increased sensitivity of *H. pylori* to antibiotics and oxidative stress

The role of the trans-translation dependent protein tagging in *H. pylori* strain 26695 after exposure to two types of stresses was addressed (Fig. 6). First, susceptibility to sub-lethal doses of two antibiotics was examined (i) chloramphenicol, a peptidyl transfer-
ase inhibitor that targets the translation machinery and, (ii) amoxicillin that irreversibly binds to the active site of penicillin-binding proteins (PBPs) involved in cell wall biosynthesis. Amoxicillin is one of the recommended components of the triple therapy employed in anti-*H. pylori* treatment. Second, the response of the mutants to oxidative stress was tested by measuring the sensitivity to paraquat (methyl viologen) that generates superoxide radicals. Superoxide radicals are among the molecules synthesized during the oxidative burst of immune cells. The SsrADD mutant behaved like the wild type strain during exposure to chloramphenicol, amoxicillin and paraquat (Fig. 6). In striking contrast, SsrASTOP presented an enhanced sensitivity to chloramphenicol stress for doses of 2.0 and 2.5 μg.ml⁻¹ and to amoxicillin with lethality at 0.6 μg.ml⁻¹ (Fig. 6). In addition, the SsrASTOP mutant that has a minimal tag sequence presented higher sensitivity to oxidative stress upon exposure to paraquat (Fig. 6).

**Acid stress causes induction of both ssrA and smpB**

The association of trans-translation with the response to stress and the continual exposure of *H. pylori* to the acidity of its gastric niche lead us to ask whether this mechanism could provide the cell with a rapid adaptive response to stressful conditions. In a previous transcriptomic study, we detected *smpB* gene induction upon acid exposure of *H. pylori* strain 26695 [36]. Acid activation of *smpB* was validated with RT-PCR [36] and more recently by Northern blotting analysis (data not shown), *ssrA* messenger RNA was examined by Northern blots on total RNA extracted from exponential growing *H. pylori* cells (strain 26695) incubated for
30 min at pH 7, pH 4.5 or pH 2. We observed a band that corresponded to a molecule of 386 nt which is the length expected for a mature SsrA (as predicted from the 26695 genome sequence) (Fig. 3B). For bacteria exposed to pH 2 and pH 4.5, this band was significantly more intense than for bacteria exposed to pH 7 (Fig. 3B). This suggests that in *H. pylori*, SsrA amounts are increased at low pH.

**Discussion**

While the mechanistic and structural aspects of trans-translation and of tmRNA have been extensively studied, several questions remain concerning the biological role of this system. It was established that under normal growth conditions, a specific pattern of proteins are targeted by tmRNA [12]. Yet, the function of this process in the cell is not clear. The role of trans-translation in ribosome rescue under stress conditions has been demonstrated, although the importance of tagging truncated proteins was not known. In addition, the essentiality of trans-translation in some bacterial species is not understood. The two latter issues and the role of this quality control mechanism in the pathogen *H. pylori* was addressed due to its exceptional ability to persist in a harsh environment.

Essentiality of trans-translation in *H. pylori* and in other organisms

Both *ssrA* and *smpB* were demonstrated to be essential in *H. pylori*. Using a conditional expression system, SmpB depletion in *H. pylori* cells resulted in growth arrest that was not associated with immediate cell death, that only occurred after 24 h depletion. This suggested that no irreversible process or toxic product accumulation occurred when trans-translation was inactivated. The reason why trans-translation is essential in some organisms is still not understood but several hypothesis were raised. Essentiality of trans-translation has been proposed to be associated with small genomes or with the necessity to accurately manage a restricted pool of ribosomes expressed by a limited number of rRNA operons [29]. Table 2 summarizes the available data on trans-translation essentiality or dispensability in several bacteria with their genome size, the number of rRNA operons and the duplication time. It can be concluded that there is no correlation between any of these criteria and trans-translation essentiality. In particular, the proposed correlation between trans-translation essentiality and a reduced number of rRNA operons [29] has not been confirmed by this analysis. Slow growth rates that are associated with a reduced number of rRNA can also be excluded as a cause of trans-translation essentiality (Table 2). While essentiality was originally thought to be associated with small genomes, this notion is contradicted by the recent example of *Shigella flexneri* [7] rendering unlikely the hypothesis of the absence of an alternative mechanism for mRNA quality control in bacteria with reduced coding capacity. Other interpretations of trans-translation essentiality during normal growth conditions can be proposed. The accumulation of truncated proteins or mRNAs may be lethal or tmRNA-dependent tagging of a specific protein could be essential for bacterial survival. This was shown not to be the case in *H. pylori* since (i) tagging is not the essential function of trans-translation in *H. pylori* and, (ii) RnaseR, a conserved ribonuclease likely to be responsible for the degradation of defective messengers is dispensable.

Interestingly, we observed that over-expression of either SsrA or SmpB enhances the *in vitro* growth rate of *H. pylori* suggesting an increase in the fitness of the bacterium under these normal conditions. In *B. subtilis*, while trans-translation is not essential under normal growth conditions, cells grew depending on the expression level of SsrA under stress conditions such as high oxidative stress.

![Figure 6. Increased susceptibility to sub-lethal doses of antibiotics, chloramphenicol (A) and amoxicillin (B) and high sensitivity to oxidative stress generated by paraquat (C) of *H. pylori* SsrA mutants defective in trans-translation tagging.](doi:10.1371/journal.pone.0003810.g006)
temperature [19]. Therefore, it can be proposed that (i) *H. pylori* cells grown in vitro are submitted to some type of stress that produces damaged RNAs at a high occurrence and/or causes frequent ribosome pausing and, (ii) that in this bacterium, trans-translation components represent a limiting factor for normal growth. This could be related to the fact that *H. pylori* has intrinsically an elevated mutation rate compared to most other bacteria [37].

Ribosome rescue with an intact resume codon is an essential function of trans-translation in *H. pylori*

The essentiality of several point mutations in ssaI was tested in *H. pylori*. Mutations in the SsrA tag sequence of *H. pylori* were viable. The lethality of SsrA mutations affecting the tRNAαL-like domain (wobble), the interaction with SmpB and the resume codon for the restart of translation after ribosome stalling indicated that, in *H. pylori*, rescue of stalled ribosomes by trans-translation is essential. The two latter mutations were particularly interesting, since they were never tested in vivo for essentiality. In vivo studies showed that resume of the translation is mandatory for the dissociation of the stalled ribosome [38]. However, here we show that a single ribosomal translocation step is sufficient to allow its recycling since the mutant carrying stop codons instead of the second and third codons of the tag (SsrA<sup>STOP</sup>) is viable. In *N. gonorrhoeae*, the essential function of trans-translation was also associated with ribosome rescue and not with protein tagging [29].

Viability of *H. pylori* mutants with a minimal tmRNA tag sequence

While mutation of the resume codon was lethal in *H. pylori*, introduction of two stop codons immediately after this position that restricted the added tag to only two amino acids did not affect *H. pylori* growth under normal conditions. This provided us with a valuable tool to examine in vivo the role of tagging of truncated peptides generated under conditions of functional trans-translation. Mutations in the tag sequence are expected to stabilize these peptides by preventing their recognition by specific proteases well defined in *E. coli* [8] and *C. crescentus* [39] and conserved in *H. pylori* [40]. The two last Ala codons of the tag (Fig. 4) have been reported to be critical for this recognition in several organisms. *H. pylori* SsrA<sup>DD</sup> strain carrying such a mutation only weakly stabilized the artificial trans-translation target protein (HypB-TAP). We concluded that in contrast to what was described in *E. coli* or *B. subtilis*, these two conserved codons of the tag are not central for protease recognition in *H. pylori*. In addition, the *H. pylori* tag sequence presents two striking differences with those of *E. coli*, *B. subtilis*, *N. gonorrhoeae* and *C. crescentus* that could reflect differences in the degradation process. This includes the presence of a polar residue at the antepenultimate position in the last four amino acids of the proteolysis tag (ACKA in *H. pylori* instead of AL/VAA, Fig. 4) and the absence of a SspB recognition motif, a proteolytic adaptor predicted to be absent in *H. pylori*.

Role of the trans-translation dependent tagging under stress conditions and for efficient DNA transformation

An original outcome of this study came from our observation that under conditions of functional ribosome rescue, the tagging of trans-translation protein was necessary for stress resistance and competence. Till now, in other organisms only mutants carrying deletions of the entire tmRNA or of smpB (deficient in both trans-translation functions) were examined for stress sensitivity.

The *H. pylori* SsrA<sup>STOP</sup> mutant presented a multifaceted phenotype including (i) increased susceptibility to sub-lethal doses of chloramphenicol, (ii) hypersensitivity to amoxicillin, and (iii) deficient natural transformation capacity. In agreement with our previous conclusions, these phenotypes were not or only very marginally displayed by the SsrA<sup>DD</sup> mutant.

In *E. coli*, sub-lethal concentrations of miscoding antibiotics such as kanamycin are known to enhance SsrA protein tagging activity due to translational read-through at normal stop codons, however read-through rarely occurs with chloramphenicol [13]. Alternatively, translation velocity reduction by chloramphenicol might increase the amount of cleaved mRNAs and thus the recruitment of tmRNA [5]. Bacterialid antibiotics such as amoxicillin targeting the cell wall synthesis are obviously not directly interfering with

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**Table 2.** Trans-translation essentiality or dispensability in bacteria grown under normal conditions.

| Organism                        | Size of the genome in Mb | Number of rRNA operons per genome | Duplication time in hours (Fast/Slow) | Status of trans-translation | Reference |
|--------------------------------|--------------------------|-----------------------------------|-------------------------------------|-----------------------------|-----------|
| *Helicobacter pylori*           | 1.65                     | 2                                 | 2.4 (F)                             | Essential                   | This work |
| *Mycoplasma genitalium*         | 0.58                     | 1                                 | 6 (S)                               | Essential                   | [26]      |
| *Mycoplasma pulmonis*           | 0.58                     | 1                                 | 2 (F)                               | Essential                   | [27]      |
| *Neisseria gonorrhoeae*         | 1.8                      | 4                                 | 1.1 (F)                             | Essential                   | [29]      |
| *Haemophilus influenzae*        | 2.2                      | 6                                 | 0.5 (F)                             | Essential                   | [28]      |
| *Shigella flexneri*             | 4.6                      | 7                                 | 0.35 (F)                            | Essential                   | Cited as non-published data in [7] |
| *Escherichia coli* K12          | 4.6                      | 7                                 | 0.35 (F)                            | Non-essential               | [50]      |
| *Salmonella enterica* serovar Typhimurium | 4.8                      | 7                                 | 0.4 (F)                             | Non-essential               | SmpB [23] |
| *Bradyrhizobium japonicum*      | 8.7                      | 1                                 | 20 (S)                              | Non-essential               | [51]      |
| *Caulobacter crescentus*        | 4.0                      | 2                                 | 1.5 (F)                             | Non-essential               | [52]      |
| *Streptomyces lividans*         | 8.6                      | 6                                 | 4.2 (S)                             | Non-essential               | [53]      |
| *Yersinia pseudotuberculosis*   | 4.8                      | 7                                 | 1.25 (F)                            | Non-essential               | [24]      |
| *Bacillus subtilis*             | 4.2                      | 10                                | 0.43 (F)                            | Non-essential               | [19]      |

The genome size, number of copies of rRNA operons, duplication time (information kindly provided by E. Rocha) and relevant references are also indicated. doi:10.1371/journal.pone.0003810.t002
transmission. Increased sensitivity to ampicillin for an E. coli AssA
mutant has been reported [21] while mutants of Synchocystis or Y.
pseudotuberculosis did not display this phenotype [24,41]. This class
of antibiotics have recently been shown to stimulate production of
hydroxyl radicals that damage nucleic acids including mRNA and
therefore might indirectly require trans-translation [42]. The
continual oxidative stress encountered by H. pylori at its
colonization site represents a major challenge despite H. pylori
being well-equipped to protect itself from ROS [43]. The
SsrA<sup>STOP</sup> mutant exhibits a striking hypersensitivity to ROS.
Importantly, these results demonstrate for the first time that the
tagging process is by itself important for the response to stress
conditions. Stress could enhance the amount of truncated mRNAs
either directly or through ribosome pausing and, as a consequence
produce toxic accumulation of truncated untagged peptides.
Alternatively, recovery from stress conditions might require trans-
translation of specific proteins.

We found a novel role of trans-translation in natural transfor-
mation competence that, in H. pylori, depends on the comB Type
IV secretion system [44]. Our results point to the need of trans-
translation tagging of a specific protein required for efficient
activity of this system. Noteworthy, trans-translation deletion
mutants of Y. pseudotuberculosis are deficient in the delivery of Yop
proteins by a Type III secretion system [24]. The existence of a
common trans-translation dependent check-point mechanism
required for the assembly of these two secretion systems is an
attractive hypothesis that will need further investigation.

**SsrA in H. pylori: a one piece molecule induced during
acid stress**

Using RACE (Rapid Amplification of cDNA Ends) mapping for
SsrA of H. pylori, Dong et al. [45] obtained two bands that were
interpreted as indicative of a two-piece sQmRNA like in C. crescentus
and all the related ρ- proteobacteria [45,46]. In contrast, northern
blotting experiments presented in this study indicated that SsrA is
an abundant one-piece molecule in H. pylori. In cells grown at
neutral pH, SsrA was detected as one band with molecular weight
please provide the rest of the text. It appears to be cut off.
extracts migrated through SDS-PAGE with a peroxidase-coupled anti-peroxidase antibody (Sigma) as in [49]. Intensities were quantified with the Quantity One software (Bio-Rad).

Measurement of transformation efficacy

*H. pylori* N6 strain harboring pILL786, pILL788, pILL791, pH792, pILL793, pILL794, and pH2328 (Table 1 and S1) were grown on blood agar plates, harvested after 24 h and suspended in peptone broth (Difco). Bacterial ODs were adjusted to OD 1.5, then 50–200 µl (approx 5 × 10⁷ cells) of these preparations were spotted in duplicates on non-selective plates and left to grow. Four hours later, one patch was taken for enumeration in order to determine the number of viable bacteria. One µg of plasmid DNA or PCR product was added on the other patch in order to inactivate the chromosomal copies of *ssrA* or *supB* genes, respectively. Twelve hours later these bacteria were plated on selective media, and four days later the total numbers of transformants were counted. Transformation rates represent the number of transformants obtained per viable cell for 1 µg of DNA.

Motility tests and mouse model for colonization

*H. pylori* strain X47-2AL and its isogenic mutants expressing SsrADD and SsrASTOP were grown on plates for 18 h and harvested in 500 µl of peptone broth (approx OD 1.5). To test the motility of the strain, 2 µl of the preparations were inoculated on Brucella Broth (Difco) soft–agar plates, 0.035% Bacto-Agar (Difco), 10% (v/v) decomplemented FCS (Eurobio) by piercing the agarose. The plates were left to grow for 7 days at 37°C. Motility was measured by determining the diameter of the spread around the inoculation spot.

The *in vivo* colonization capacities of *H. pylori* strain X47-2AL and its isogenic mutants expressing SsrADD and SsrASTOP were assessed as in [36].

Sensitivity tests

Overnight liquid cultures of wild type *H. pylori* strain 26695 or of the two isogenic tag- mutants SsrADD and SsrASTOP were used to inoculate BHI medium containing 10 % FCS at an initial OD of 0.15 and left to grow for 6 h. Cultures growing exponentially were used to perform the following tests. Serial dilutions of the bacteria were spotted on plates containing different concentrations of chloramphenicol (Sigma) 2 or 2.5 µg ml⁻¹; Amoxicillin (Clamoxyl, GlaxoSmithKline) 0.2 or 0.6 µg ml⁻¹ and plates were incubated under microaerophilic conditions. The controls consist of culture grown without these antibiotics. Counting of surviving bacteria was performed 5 days later.

To determine the sensitivity of the strains to oxidative stress, 1 ml (approx 10⁷ bact) of cells in exponential phase were placed into 12-wells plates containing BHI supplemented with 0.5 or 0.75 µM paraquat (methyl Viologen, Sigma). Cultures were incubated at 37°C under microaerophilic conditions while shaking at 160 rpm. After 18 h, bacterial counts were performed on blood agar plates.

RNA extraction and Northern blotting

Exponentially growing liquid cultures of *H. pylori* wild type 26695 or isogenic mutants (OD 0.6) were centrifuged at room temperature for 10 min at 3000 g. Pellets were suspended in preheated BHI medium adjusted to pH 2.0, pH 4.5 or pH 7.0 at an OD of 0.2, left for 30 min. RNA was extracted using the phenol-chloroform method [36]. Four µg of total RNA were separated on 4% acrylamide-urea denaturing gels, blotted onto Hybond-N+ membrane (Amersham) with a transblotter (40 min, 10 mV) and U.V. cross-linked. 5S rRNA and a 300-nucleotides-long internal fragment of *ssrA* ³²P-labeled riboprobes were synthesized with the StripAble RNA Probe Synthesis and Removal Kit (Ambion). 5S rRNA probed on the same membranes served for calibration. Hybridization was performed at 65°C for 4 h with UltraHyb (Ambion). Quantitative analyses of blots were performed with Quantity One software (Bio-Rad).

Supporting Information

Table S1

Found at: doi:10.1371/journal.pone.0003810.s001 (0.10 MB DOC)

Table S2

Found at: doi:10.1371/journal.pone.0003810.s002 (0.19 MB DOC)

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Author Contributions

Conceived and designed the experiments: MT HDR. Performed the experiments: MT JMT. Analyzed the data: MT HDR. Wrote the paper: MT HDR.

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