The novel regulatory ncRNA, NfiS, optimizes nitrogen fixation via base pairing with the nitrogenase gene *nifK* mRNA in *Pseudomonas stutzeri* A1501

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Unlike most *Pseudomonas*, the root-associated bacterium *Pseudomonas stutzeri* A1501 fixes nitrogen after the horizontal acquisition of a nitrogen-fixing (*nif*) island. A genome-wide search for small noncoding RNAs (ncRNAs) in *P. stutzeri* A1501 identified the novel *P. stutzeri*-specific ncRNA NfiS in the core genome, whose synthesis was significantly induced under nitrogen fixation or sorbitol stress conditions. The expression of NfiS was RNA chaperone Hfq-dependent and activated by the sigma factor RpoN/global nitrogen activator NtrC/*nif*-specific activator NifA regulatory cascade. The *nfiS*-deficient mutant displayed reduced nitrogenase activity, as well as increased sensitivity to multiple stresses, such as osmotic and oxidative stresses. Secondary structure prediction and complementation studies confirmed that a stem-loop structure was essential for NfiS to regulate the nitrogenase gene *nifK* mRNA synthesis and thus nitrogenase activity. Microscale thermophoresis and physiological analysis showed that NfiS directly pairs with *nifK* mRNA and ultimately enhances nitrogenase activity by increasing the translation efficiency and the half-life of *nifK* mRNA. Our data also suggest structural and functional divergence of NfiS evolution in diazotrophic and nondiazotrophic backgrounds. It is proposed that NfiS was recruited by *nifK* mRNA as a novel regulator to integrate the horizontally acquired *nif* island into host global networks.

nitrogen fixation | *Pseudomonas stutzeri* | regulatory ncRNA | NfiS | *nifK* mRNA

A number of noncoding RNAs (ncRNAs) (also called sRNAs) have been identified in the past years in *Escherichia coli* and other bacterial species, including members of the *Pseudomonas* genus (1–4). These RNAs were found to play a role at the posttranscriptional level, being involved in the regulation of diverse physiological processes, such as stress responses, virulence, motility, biofilm formation, quorum sensing, and metabolic control (5–14). In addition to cis-encoded antisense RNAs, many trans-encoded RNAs exert their regulatory roles through base pairing with the complementary mRNA target, which involves the chaperone protein Hfq to mediate the sRNA–mRNA interaction (4, 14, 15). However, the presence of regulatory ncRNAs has not previously been investigated in *Pseudomonas stutzeri*.

The *Pseudomonas* genus constitutes a wide group of species that colonize diverse niches, including plant and animal pathogens as well as nonpathogenic species from water, soil, plant rhizospheres, and marine environments. They play important roles in the nitrogen and carbon cycles (16). However, the biological nitrogen fixation ability, a process by which bacteria reduce molecular N_2 gas to ammonia, is rarely encountered in this genus (16–18). It is now established that nitrogen-fixing strains can be found within the *P. stutzeri* species. Determination of the *P. stutzeri* A1501, DSM4166, and BAL398 genome nucleotide sequences led to the identification of a conserved, 49-kb nitrogen-fixing island acquired by horizontal gene transfer (HGT) in these three strains (17–20). A recent in silico analysis revealed that the island is also conserved in other available genomes of nitrogen-fixing *P. stutzeri* (ref. 21 and references therein). The regulation of *nif* gene transcription in most diazotrophs depends on the sigma factor RpoN/global nitrogen activator NtrC/*nif*-specific activator NifA regulatory cascade (22). *P. stutzeri* A1501, which is isolated from the rice rhizosphere in China, is the best studied strain regarding nitrogen fixation. It possesses a general nitrogen regulatory system in the core genome (NtrBC and related genes) (23–25), but, during its evolution, it acquired a *nif*-specific regulatory system (NifLA) (25) from a diazotrophic ancestor. Thus, the regulatory network controlling nitrogen fixation in A1501 may result from regulatory systems of different evolutionary origins. Colonization of the plant rhizosphere implies that nitrogen-fixing *P. stutzeri* strains adapt to a range of physiological conditions, such as environmental stresses and nutrient availability (18, 23, 26). Despite the identification of a number of genes potentially involved in rhizosphere competence in the A1501 genome, little is known about the mechanisms of adaptation and survival in the rhizosphere (17). A search for ncRNAs in other nitrogen-fixing organisms was reported (5, 6, 14, 27–29), but none have been described so far as being involved in the regulation of *nif* gene expression.

Significance

The biological nitrogen fixation process responsible for the reduction of atmospheric nitrogen to ammonia represents the primary source of nitrogen supporting extant life. We have identified a novel noncoding RNA (ncRNA) in the *Pseudomonas stutzeri* core genome, called NfiS, that is involved in the stress response and controls the expression of genes located in a genomic nitrogen-fixing (*nif*) island. NfiS was found to optimize nitrogen fixation by the direct post-transcriptional regulation of nitrogenase gene *nifK* mRNA. The acquisition of the *nif* island and the recruitment of NfiS by *nifK* mRNA are evolutionary events that seem to contribute to fine-tuned regulation of nitrogenase activity in *P. stutzeri*. This study provides a new regulatory pathway, mediated by an ncRNA for optimal nitrogen fixation, that may operate in other diazotrophs.

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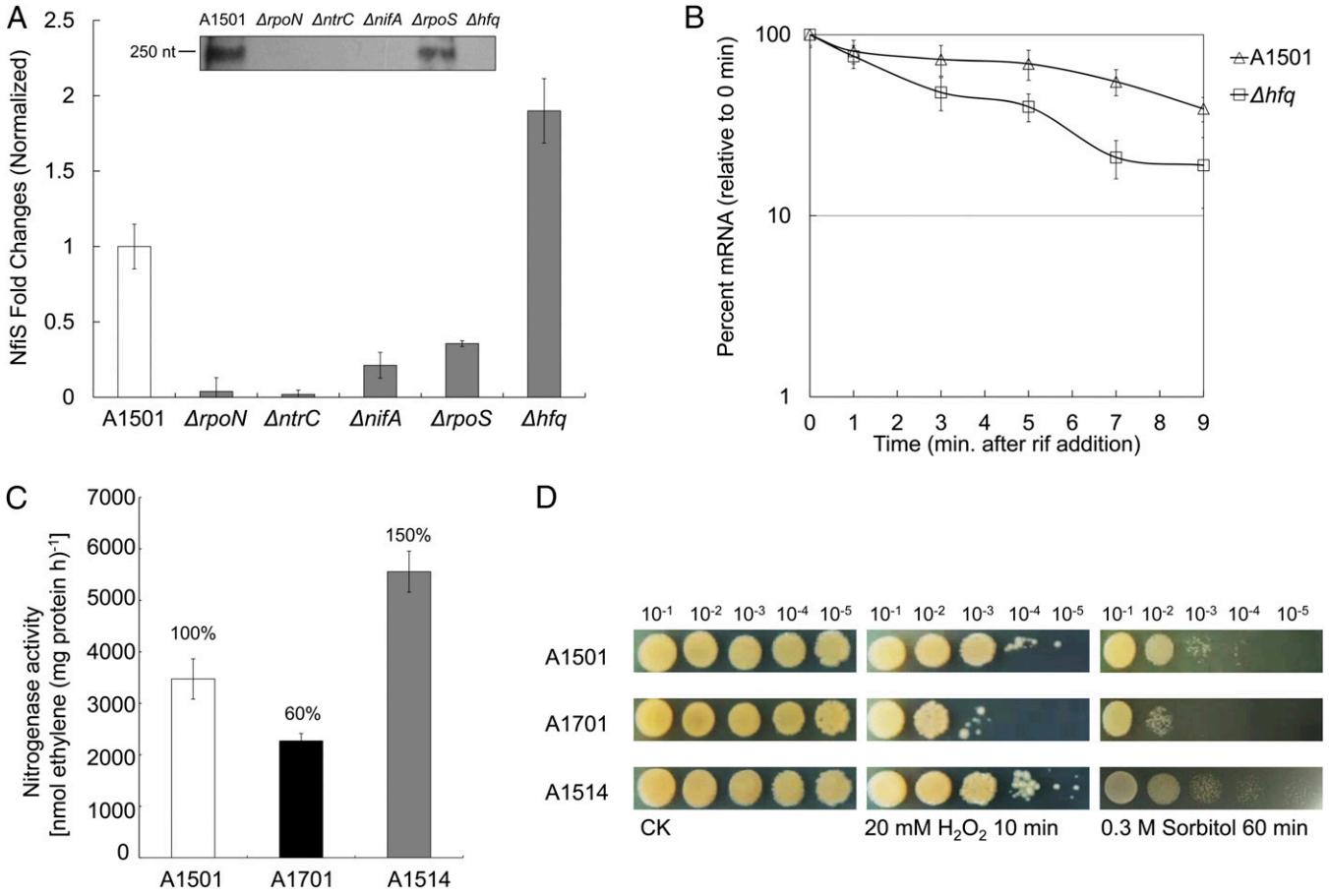

Fig. 1. Transcriptional and functional analysis of NfiS ncRNA in *P. stutzeri* A1501. (*A*) *nfiS* transcription under nitrogen fixation conditions in the WT A1501 and five mutant strains. Total RNA was extracted, and the expression of target genes was measured using qRT-PCR. (*Inset*) RNA Northern blot assay using RNA extracted from the same strains under the same conditions and hybridized with the *nfiS*-specific probe. Measurements were normalized to the WT values, and fold differences are plotted. (*B*) Analysis of the NfiS half-life in the WT A1501 and *hfq* mutant strains. Rifampicin (400 μg/mL) was added at time 0. At the time indicated, an equal volume of frozen media was added to bring the temperature immediately to 4 °C. RNA was extracted, followed by qRT-PCR. (*C*) Nitrogenase activity in the WT A1501, *ΔnfiS* A1701, and NfiS overexpression strain A1514. Error bars represent the SD calculated from three sets of independent experiments. (*D*) Growth upon oxidative or osmotic stresses. Serial 10-fold dilutions of OD-standardized WT A1501, *ΔnfiS* A1701, and *nfiS* overexpression strain A1514 were spotted on LB plates after exposure to 20 mM H_2O_2 or 0.3 M sorbitol. CK, untreated culture control.

A search for ncRNAs in the genome of *P. stutzeri* A1501 was performed (26), and we now report on the identification of a novel RNA, named NfiS, specific for *P. stutzeri* strains, involved in the control of nitrogen fixation and the response to oxidative and osmotic stresses. This ncRNA controlled nitrogenase activity directly by the posttranscriptional regulation of *nifK* mRNA and also indirectly through the induction of the RpoN/NtrC/NifA regulatory cascade via unidentified mechanisms. Further analysis showed that the *nfiS* genes in diazotrophic and nondiazotrophic *P. stutzeri* exhibited remarkable differences in secondary structure and regulatory function and evolved in different ways due to different niches and selection pressures. During evolution of *P. stutzeri*, NfiS was apparently recruited by *nifK* mRNA as a novel activator to optimize nitrogenase activity in response to specific environmental cues. It is hypothesized that, although not identical to NfiS, the involvement of ncRNAs in the regulation of nitrogen fixation is likely to represent a novel regulatory mechanism that is widely distributed among nitrogen-fixing bacteria.

Results

NfiS Is Hfq-Dependent and Activated by the RpoN/NtrC/NifA Cascade. Preliminary experiments revealed that ncRNA01 expression was strongly induced both under conditions of nitrogen fixation and in response to osmotic stress (Fig. S1*A*). This finding led us to investigate the role of ncRNA01 in the regulation of nitrogen fixation. We therefore renamed this ncRNA the "nitrogen fixation condition-inducible small ncRNA" (NfiS). The *nfiS* gene (254 nt) is flanked by a gene encoding a low-molecular-weight penicillin-binding protein and a gene encoding a TonB-dependent receptor (Fig. S1*B*). Furthermore, the gene is conserved in other *P. stutzeri* strains whose genomes are available and in particular showed 100% identity with the corresponding gene in DSM4166 (19), another nitrogen-fixing strain of *P. stutzeri* (Fig. S1*C*). Because we did not identify homologs in any other bacterial species by BLAST search and did not find any match to the RNA families in the Rfam database (rfam.xfam.org), NfiS is likely a novel ncRNA specific to *P. stutzeri*.

The determination of the *nfiS* transcription initiation site (Fig. S1*B*) showed that the transcript starts from an adenine residue, which is the 13th nucleotide downstream from the conserved C residue of the predicted element located at position −12 of the putative RpoN-dependent promoter (GG-N10-GC) (22). No binding site for NtrC or NifA was identified. A band corresponding to the migration of a 250-nt molecule was detected by Northern blot analysis in the WT strain A1501, but it was not detected when the analysis was performed with RNA extracted from deletion mutants for *rpoN, ntrC*, or *nifA* under nitrogen fixation conditions (Fig. 1*A*, *Inset*, lanes 1–4), which are involved in the regulation of nitrogen

fixation (17, 18, 24, 25). The band intensity was decreased when the analysis was performed with RNA extracted from an *rpoS* deletion mutant, known in other systems as the RNA polymerase sigma factor of the general stress response (30) (Fig. 1*A*, *Inset*, lane 5). These results suggested that NfiS transcription is RpoN-dependent and under the control of NtrC, NifA, and RpoS. Quantitative real-time PCR (qRT-PCR) analysis led to similar conclusions (Fig. 1*A*).

Most base-pairing small RNAs require the RNA chaperone Hfq to form stable sRNA-target-mRNA hybrids or to maintain their own stability (15). An *hfq* gene was identified in A1501 and found to be involved in the regulation of nitrogen fixation because its inactivation caused a significant decrease in nitrogenase activity (Fig. S2). Although expression of the *nfiS* gene was up-regulated in the *hfq* deletion background as determined by qRT-PCR, Northern blot analysis showed that the *nfiS* transcript was hardly detectable in the absence of Hfq (Fig. 1*A*, *Inset*, lane 6), implying that Hfq affected the stability of the NfiS transcript. This possibility was checked by measuring the half-life of the NfiS transcript, which was 7 min for the WT and 3 min for the *hfq* mutant strain (Fig. 1*B*), indicating that Hfq had a positive effect on the stability of NfiS.

Inactivation and Overexpression of NfiS Affect both Nitrogenase Activity and Stress Resistance. To evaluate the effect of NfiS expression on nitrogenase activity, the *nfiS*-knockout mutant A1701 (*ΔnfiS*) was constructed, as well as strain A1514 corresponding to A1501 harboring a plasmid expressing the WT *nfiS* gene (pLAnfiS-A1501) (*SI Materials and Methods* and Table S1). Both A1701 and A1514 displayed growth properties similar to that of the WT strain. The *nfiS* deletion in A1701 resulted in about 40% reduction in nitrogenase activity whereas activity of the NfiS-overexpressing strain A1514 showed an increase to 150% compared with the WT (Fig. 1*C*). Moreover, it was checked, by Western blot analysis, that the production of the nitrogenase MoFe protein polypeptides (NifK and NifD) was decreased in the *ΔnfiS* mutant strain (Fig. S3). This finding suggested a correlation between the NfiS level and the nitrogenase synthesis and activity. Therefore, NfiS is a positive regulator required for maximal expression of nitrogenase.

Bacterial ncRNAs are involved in responses to generalized stresses (5, 9, 14, 29). In the presence of 20 mM H_2O_2 or 0.3 M sorbitol, the *nfiS* mutant (A1701) was more sensitive than the WT whereas the overexpression of NfiS (A1514) led to enhanced resistance (Fig. 1*D*). Thus, NfiS plays an important role in the response to oxidative or osmotic stress, possibly contributing to optimal nitrogen fixation, which can be significantly affected by environmental stresses.

NfiS Contributes to the Global and Positive Regulation of Nitrogen Fixation and Stress Resistance-Related Genes. The expression level of a set of nitrogen fixation-related genes was compared in the WT, *ΔnfiS* (A1701), and NfiS overexpressing (A1514) strains under nitrogen fixation conditions. Deletion of *nfiS* resulted in decreased expression of the assayed *nif* and *nif*-related genes. In contrast, their expression was remarkably enhanced by overproduction of NfiS. In particular, *nifA*, coding for the transcriptional activator of all *nif* operons (18, 24, 25), showed a threefold increase, and *glnK,* coding for a PII family protein (31, 32), showed a sixfold increase (Fig. S4*A*). In *P. stutzeri,* GlnK is essential for both NifA synthesis and activity, in particular by preventing the inhibitory effect of NifL on NifA activity (26, 32). These data were in agreement with the observation that inactivation and overexpression of NfiS affected nitrogenase activity (Fig. 1*C*), suggesting a role in positive regulation of the *nif* genes. Similarly the expression of the *rpoN, glnK*, and *ntrC* genes was decreased in the *nfiS* mutant strain (A1701) and increased in the NfiS overexpressing strain (A1514), indicating that NfiS could also play a role in the global regulation of nitrogen metabolism. Apparently, this global effect could solely be due to an effect of NfiS on RpoN because all of the genes analyzed here are σ^{54}-dependent. However, the level of NfiS was reduced in mutant strains carrying deletions of *nifA* or *ntrC* (Fig. 1*A*), suggesting a more complex regulatory circuitry.

The expression levels of oxidative or osmotic stress-related genes were compared in the three strains under sorbitol stress. Deletion and overexpression of *nfiS* resulted in down- and up-regulation, respectively, of genes such as *proC* and *katA*, showing that expression of these genes is NfiS-dependent (Fig. S4*B*). The *proC* gene encodes pyrroline carboxylate reductase, a key enzyme in the last step in proline biosynthesis, and *katA* encodes catalase, both of which play important roles in oxidative or osmotic stress responses (33, 34). Based on these studies, NfiS likely controls nitrogenase activity at the transcriptional level through the regulation of both the RpoN/NtrC/NifA regulatory cascade and certain stress response-related genes via unknown mechanisms.

A Stem-Loop Is Essential for NfiS to Regulate Optimal Nitrogen Fixation. Using bioinformatics tools, we found putative pairing between NfiS and the transcript of the A1501 *nifK* gene (Fig. 2*A*)

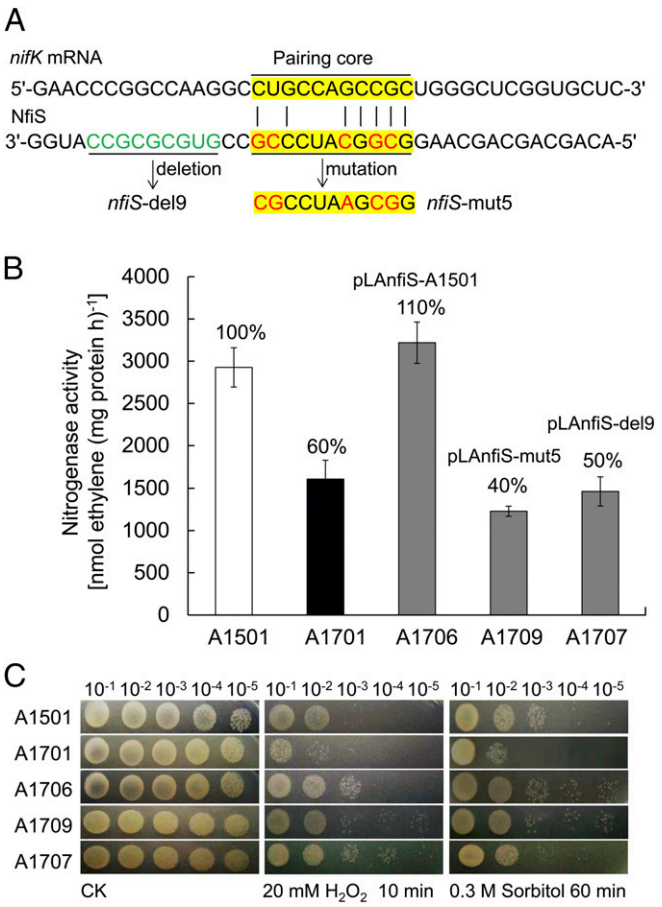

Fig. 2. A stem-loop is essential for NfiS regulation of nitrogenase activity. (*A*) Region of the *nifK* mRNA showing complementarity to the NfiS stem-loop region and relevant genetic constructs. Yellow, 11-nt pairing region with *nifK* mRNA; green, 9-nt region. Deletion of the entire 9-nt region and the 5-nt substitution in 11-nt pairing region are underlined, and mutated nucleotides are highlighted in red. (*B*) Nitrogenase activity in the WT A1501, *ΔnfiS* A1701, and three complemented strains (A1701 containing pLAnfiS-A1501, pLAnfiS-mut5, and pLAnfiS-del9, respectively). Experiments were performed in triplicate with mean values shown. The percentage of nitrogenase activity compared with WT is indicated. (*C*) Serial 10-fold dilutions of OD-standardized cultures were spotted on LB plates after exposure to 20 mM H_2O_2 or 0.3 M sorbitol.

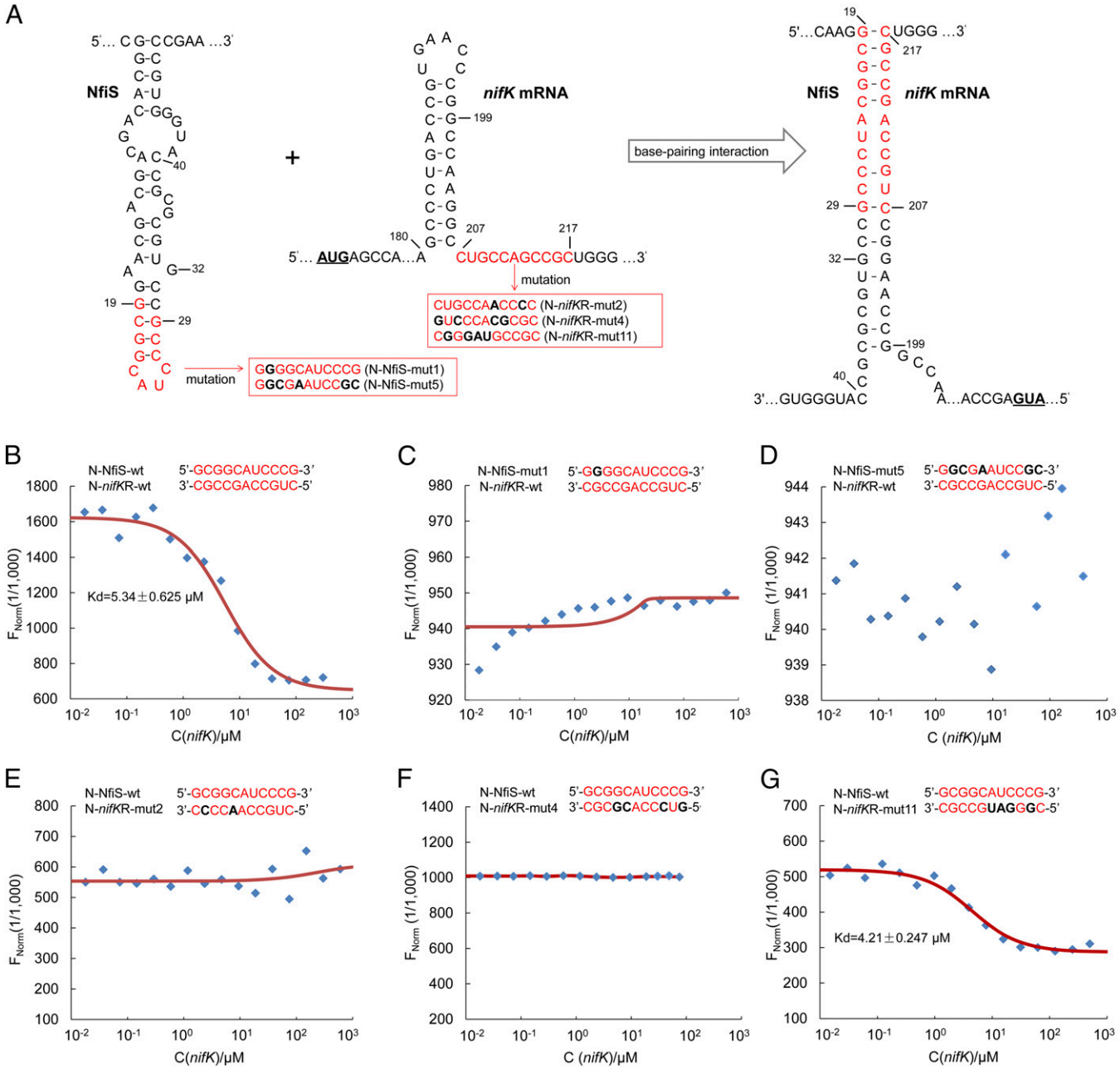

Fig. 3. Binding of NfiS to *nifK* mRNA. (*A*) Schematic representation of the base-pairing complex formation (*Right*) between the NfiS stem loop (*Left*) and the complementary sequence of *nifK* mRNA (*Middle*). Pairing nucleotides are shown in red. Point mutations introduced into synthesized oligonucleotide derivatives in red boxes are shown in black and bold. mut, mutation; N, synthesized oligonucleotide; *nifK*R, *nifK* mRNA; wt, wild type. (*B–G*) Determination of the binding affinity of NfiS to *nifK* mRNA by microscale thermophoresis. The concentration of labeled N-NfiS molecules was constant whereas the concentration of the nonlabeled binding partner N-*nifK*R molecules varied from 10 nM to 300 μM. See Table S2 and *SI Materials and Methods* for details.

encoding the β-subunit of the MoFe protein of the nitrogenase enzymatic complex (24). The predicted secondary structure of NfiS contains a stem-loop (between nucleotides 2 and 49) with an 11-nt consensus sequence (GCGGCAUCCCG) moderately complementary to the 11 nucleotides (CUGCCAGCCGC) at the 5′ end (nucleotides 207–217) of *nifK* mRNA (Fig. S1*D* and Fig. 3*A*). Furthermore, this stem-loop also contains a 9-nt sequence (GUGCGCGCC) present only in diazotrophic *P. stutzeri* strains, although absent in nondiazotrophic strains (Fig. S5*A*).

The role of the NfiS stem-loop structure in nitrogen fixation and stress responses was explored further. Two plasmids carrying *nfiS* mutations with either a substitution of five nucleotides in the putative base-pairing site with *nifK* mRNA (pLAnfiS-mut5) or a complete deletion of the 9-nt sequence downstream of the pairing site (pLAnfiS-del9) were constructed (Fig. 2*A*). Complementation of *ΔnfiS* (A1701) showed that both nitrogenase activity and stress resistance were restored to WT levels by pLAnfiS-1501 (A1706; containing WT *nfiS*), but pLAnfiS-mut5 (A1709) and pLAnfiS-del9 (A1707) failed to restore nitrogenase activity (Fig. 2*B*). These two latter plasmids did restore the stress resistance caused by adding 20 mM H_2O_2 or 0.3 M sorbitol to the medium (Fig. 2*C*). These results suggested that the stem-loop structure of NfiS plays a role in optimal nitrogen fixation but is not necessary for oxidative or osmotic tolerances.

NfiS Directly Base Pairs with *nifK* mRNA to Enhance the Translation Efficiency and Half-Life of the Transcript. Based on the prediction of the NfiS secondary structure and on the complementation data using the WT or mutated *nfiS* genes, it was hypothesized that NfiS is recruited and paired with *nifK* mRNA. As shown in Fig. 3*A*, an 11-nt sequence in the stem-loop structure of NfiS (nucleotides 19–29) pairs with its counterpart at the 5′ end of *nifK* mRNA (nucleotides 207–217) by seven nucleotides. This 63% match is moderate but is sufficient for the NfiS and *nifK* mRNA recognition and binding. To assay this hypothesis, a set of 30-nt ssRNA oligonucleotides containing the WT or mutated sequences were synthesized, including an N-NfiS-wt containing the stem-loop structure of NfiS, an N-*nifK*R-wt containing the complementary region of *nifK* mRNA, and two mutations (N-NfiS-mut1 and N-NfiS-mut5) containing site-specific mutations in the stem-loop structure of NfiS (Table S2). The interaction of the fluorescently labeled N-NfiS-wt or N-NfiS-mut probe molecules with the nonlabeled competitor molecule N-*nifK*R-wt was assayed using microscale thermophoresis (MST), allowing sensitive measurement of molecular interactions in solution (35, 36). This analysis indicated that N-NfiS-wt bound to N-*nifK*R-wt at low micromolar concentrations of the titrant, exhibiting a dissociation constant (K_d) of 5.34 ± 0.63 μM (Fig. 3*B*), suggesting a relatively strong interaction. In contrast, the two mutated derivatives (N-NfiS-mut1 and N-NfiS-mut5) carrying substitutions of one or more nucleotides in the 11-nt stem-loop motif displayed a complete defect in binding to *nifK* mRNA (Fig. 3 *C* and *D*), indicating that conservation of this motif is critical to the interaction between NfiS and *nifK* mRNA.

The effect of mutations in the 11-nt sequence at the 5′ end of *nifK* mRNA (residues 207–217) on base pairing with NfiS was in turn investigated using 30-nt ssRNA oligomers containing mismatch mutations (N-*nifK*R-mut2 or N-*nifK*R-mut4) and one compensatory mutation (N-*nifK*R-mut11). The latter showed a perfect match (100%) with the 11-nt targeting sequence of NfiS (Fig. 3*A* and Table S2). We further measured the binding affinity of these altered *nifK* nucleotides with NfiS (N-NfiS-wt). As shown in Fig. 3 *E* and *F*, N-*nifK*R-mut2 or N-*nifK*R-mut4 exhibited no interaction with N-NfiS-wt. We also measured the binding affinity using N-*nifK*R-mut11 (100% match level). The compensatory mutation resulted in enhanced binding affinity to N-NfiS-wt compared with the WT situation, showing a K_d of 4.21 ± 0.25 μM (Fig. 3*G*). These data indicate that *nifK* mRNA is a direct target of the NfiS ncRNA.

The secondary structure of *nifK* mRNA contains a putative hairpin structure (nucleotides 181–206) immediately adjacent to the ncRNA binding site, presumably forming an inhibitory structure that affects translation efficiency (Fig. 3*A*, *Middle*). In the presence of rifampicin, the half-life of *nifK* mRNA in the WT strain was 20 min, but it decreased to 15 min for the *nfiS* mutant (Fig. 4). This transcript stability was restored to WT level by the complementary plasmid (Fig. 4). This result is in good agreement with the results from the complementation assay of nitrogenase activity as shown in Fig. 2*B*. These results led us to conclude that the regulation of nitrogen fixation by NfiS in A1501 occurs at multiple levels, which, at the posttranscriptional level, is through a direct interaction with target mRNA that increases its translation efficiency and half-life and consequently nitrogenase activity.

Structural and Functional Divergences of NfiS Under Diazotrophic and Nondiazotrophic Backgrounds. Analysis of the nucleotide sequence of the *nfiS* gene revealed that the 9-nt sequence identified as a part of a stem-loop required for regulation of nitrogenase synthesis (Fig. 2*A*) is present only in the nitrogen-fixing strains (e.g., A1501 and DSM4166) (Fig. S5*A*). The putative RpoN-binding site of the *nfiS* promoter region is not well-conserved in *P. stutzeri* ATCC17588, RCH2, and SLG358A3-8, which are not diazotrophic (Fig. 5*A*). These structural differences may reflect the functional divergences of NfiS evolution in diazotrophic and nondiazotrophic backgrounds.

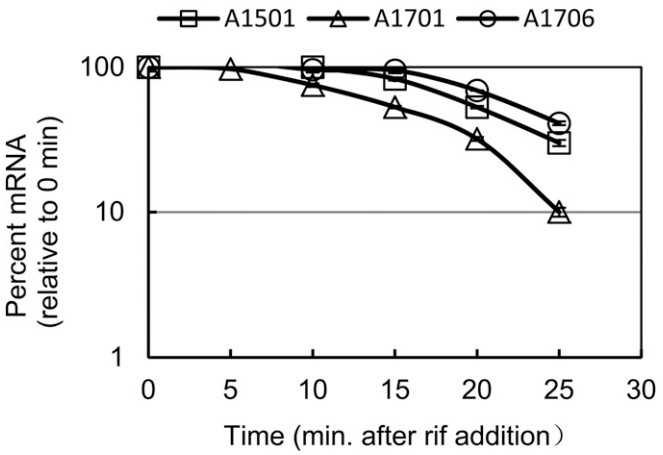

Fig. 4. Effect of *nfiS* mutation on the half-life of *nifK* transcript. Determination of *nifK* mRNA half-life in the WT A1501, *ΔnfiS* A1701, and complemented strain A1706 (A1701 containing complementation plasmid pLAnfiS-A1501) in the same conditions as Fig. 1.

The qRT-PCR measurement of NfiS expression in strain ATCC17588 showed a dramatic increase after sorbitol shock but not under nitrogen limitation conditions, in contrast to the observations with A1051 (Fig. 5*B*). These data suggest the existence of different mechanisms controlling the regulation of NfiS expression in diazotrophic and nondiazotrophic *P. stutzeri* strains. When a plasmid carrying the *nfiS* gene of the ATCC17588 strain (pLAnfiS-ATCC) was introduced into the *nfiS* mutant, restoration of oxidative or osmotic stress-resistant phenotypes was observed whereas there was no complementation for nitrogen fixation ability (Fig. 5 *C* and *D*). This result is in agreement with the requirement of the 9-nt region for optimal nitrogenase activity (Fig. 2*B*), but not for oxidative or osmotic-stress resistances (Fig. 2*C*).

Discussion

This work reports the characterization of NfiS, an ncRNA specific to *P. stutzeri*, which is dramatically induced under nitrogen fixation conditions. The pattern of *nfiS* expression in mutant strains for *rpoN, ntrC*, or *nifA* impaired in the nitrogen fixation process is in agreement with a role for NfiS in nitrogen fixation. In addition, the mutant strain displayed reduced stress responses, and putative target genes of the *kat* and *pro* operons were identified. Structural and functional divergences of NfiS in diazotrophic and nondiazotrophic *P. stutzeri* are schematically shown in Fig. S5*B*, reflecting the evolution of NfiS in different ways due to different niches and selection pressures, which is why it is proposed that the primary function of NfiS in *P. stutzeri* is related to the stress response, and it also implies that NfiS likely contains another nucleotide sequence pairing with the stress resistance target genes, not yet identified.

In the nitrogen-fixing strain *P. stutzeri* A1501, NfiS, which is located in the core genome, controls the expression of genes located in the horizontally acquired *nif* island. This finding raises interesting questions concerning the evolution of nitrogen fixation ability within *P. stutzeri* strains. There is growing evidence that bacteria use gene acquisition to adapt to ecological niches. For example, among nitrogen-fixing systems, the acquisition of symbiotic islands was reported for rhizobia (37); analysis of *Azospirillum* genomes revealed that a large proportion of the genes were acquired to adapt from marine to terrestrial environments (38) and that the evolution of nitrogenase from anoxic

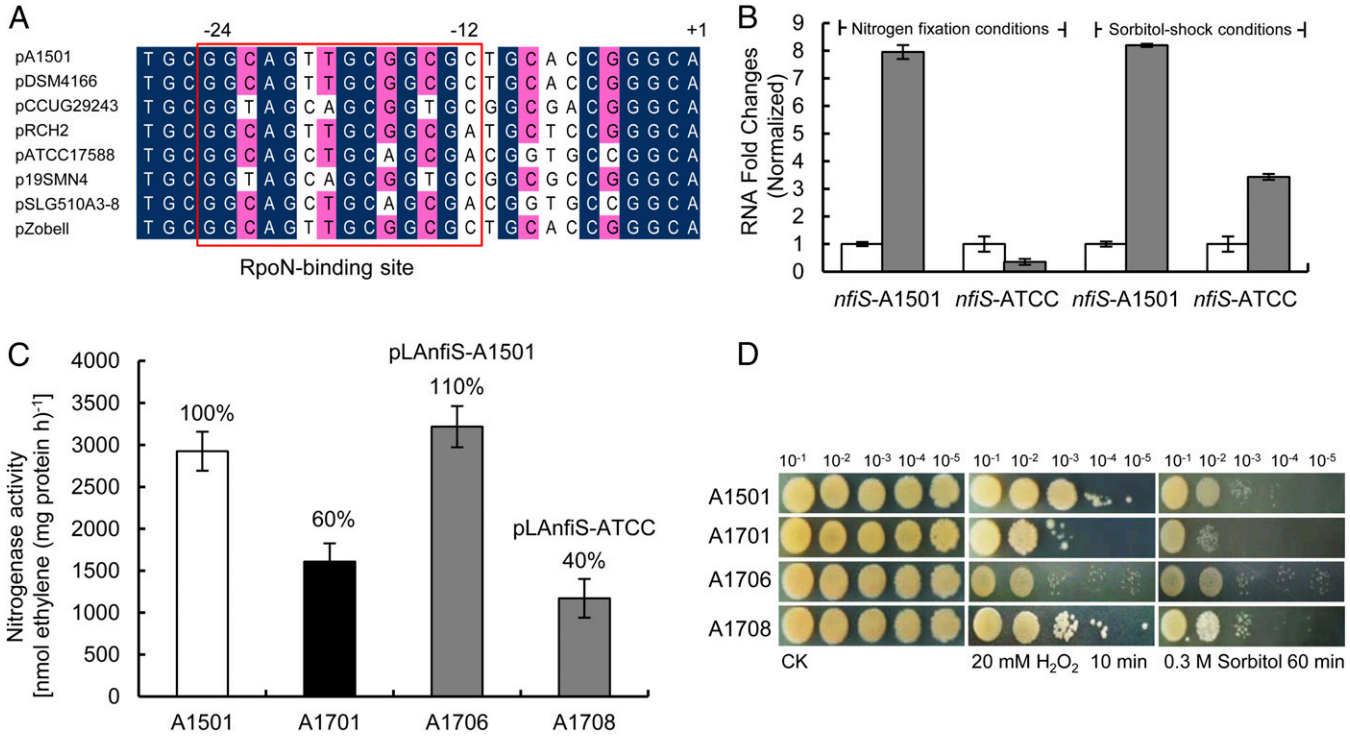

Fig. 5. Structural and functional comparison between the diazotrophic and nondiazotrophic *P. stutzeri* NfiS. (*A*) Alignment of the *P. stutzeri* A1501 *nfiS* promoter region with homologous sequences in *P. stutzeri* DSM4166 (diazotrophic) and CCUG29243, ATCC17588, 19SMN4, SLG510A3-8, and Zobell. The sequence alignment was performed using CLUSTAL X. Nucleotides conserved at 100% and at >70% are highlighted in black and red, respectively. The nucleotide sequences, which may correspond to the RpoN binding site located at position −12 to −24 from the transcription start, are boxed. Transcriptional start sites are shown. (*B*) Induction of the *nfiS* gene in diazotrophic *P. stutzeri* strain A1501 or nondiazotrophic strain ATCC17588 grown under nitrogen fixation (gray bars) or sorbitol stress conditions (gray bars) vs. control (open bars), as measured by qRT-PCR. Error bars as in Fig. 1. (*C*) Nitrogenase activity of the WT A1501, *ΔnfiS* A1701, and two complemented strains (A1701 containing pLAnfiS-A1501 and pLAnfiS-ATCC, respectively). (*D*) Response to sorbitol and H_2O_2 stresses in the same strains as in *C* and the same conditions as in Fig. 2.

to aerobic environments was made possible due to gene acquisition (39).

However, it has long been established that acquisition of new metabolic properties could decrease host fitness. Small regulatory RNAs together with the chaperone Hfq were reported to play critical roles in the general regulatory pathways operating in the host (4, 14, 40, 41). For example, Skippington and Ragan revealed that more than 80% of genes targeted by Hfq-associated core sRNAs were horizontally acquired within the *E. coli–Shigella* clade (4). A large number of phenotypic changes were reported in *hfq* mutant strains of *Sinorhizobium meliloti*, which were particularly impaired in nodulation of the host plant (42, 43). A recent analysis of Hfq RNA targets in *S. meliloti* revealed extensive control of symbiotic regulons and of operons involved in the stress response (14). It is proposed that the Hfq chaperone plays a role in the regulation of nitrogenase activity (Fig. S2) and the stability of NfiS (Fig. 1*B*), which likely results in better integration of laterally acquired *nif* genes into existing regulatory networks.

Analysis of the secondary structure of NfiS led to the hypothesis that an 11-nt sequence was critical in the control of nitrogen fixation, particularly in the putative pairing with the mRNA of *nifK* (Fig. S1*D* and Fig. 2*A*). Mutational analysis and microscale thermophoresis experiments support the hypothesis that NfiS was recruited for nitrogen fixation and the conclusion that NfiS plays a role in the stabilization of the *nifK* mRNA resulting in increased nitrogenase activity (Figs. 2*B*, 3, and 4). What is commonly referred to as the "nitrogenase" (Mo-Nitrogenase) is an enzymatic complex, composed of two proteins: the MoFe protein, a heterotetramer encoded by the *nifD* and *nifK* genes, and the Fe protein, a dimer encoded by *nifH* (44–46). The nitrogenase structural genes (*nifHDK*) are organized in one operon in most *nif* systems (22). The *nifK* gene is located in the distal position, thereby exhibiting relative lower transcriptional and translational activities than proximal genes. In a former work (23), it was shown that, under nitrogen fixation conditions, the relative factors of expression for *nifH*, *nifD*, and *nifK* were of 94-, 54-, and 38- fold, respectively, suggesting that NifK synthesis is a rate-limiting step. Similar decreases in *nifHDK* transcript abundance were observed in *Azotobacter vinelandii*, which seem to be affected by RNA secondary structures between three *nif* structural genes (47). Therefore, the involvement of NfiS in the translation efficiency of the *nifK* gene, as shown in Fig. 3*A*, is critical for the synthesis of nitrogenase polypeptides in a correct ratio for optimal nitrogenase activity.

The involvement of NfiS in the posttranslational regulation of *nifK* raised the question as to whether similar mechanisms could exist in other diazotrophs. Because the complementary sequence of *nifK* mRNA was identified in the corresponding region of 31 *nifK* genes from 42 representative diazotrophs (Table S3 and Fig. S5*C*), it is proposed that similar mechanisms, involving as yet unidentified ncRNA(s), might operate in other diazotrophs. It is also tempting to speculate that other ncRNA targets could be different from *nifK* mRNA in some systems because NfiS is *P. stutzeri*-specific. Former reports suggested that an Hfq homolog, NrfA, was required for *nifA* expression in *Azorhizobium caulinodans* and *Rhodobacter capsulatus* although the involvement of an RNA molecule in this regulation was not shown (48–50). The *nif* islands in *P. stutzeri* isolated from diverse niches and geographical origin revealed relatively high degrees of conservation (18–21), also implying that this ncRNA-based regulatory role is not limited to A1501 but can be extended to other

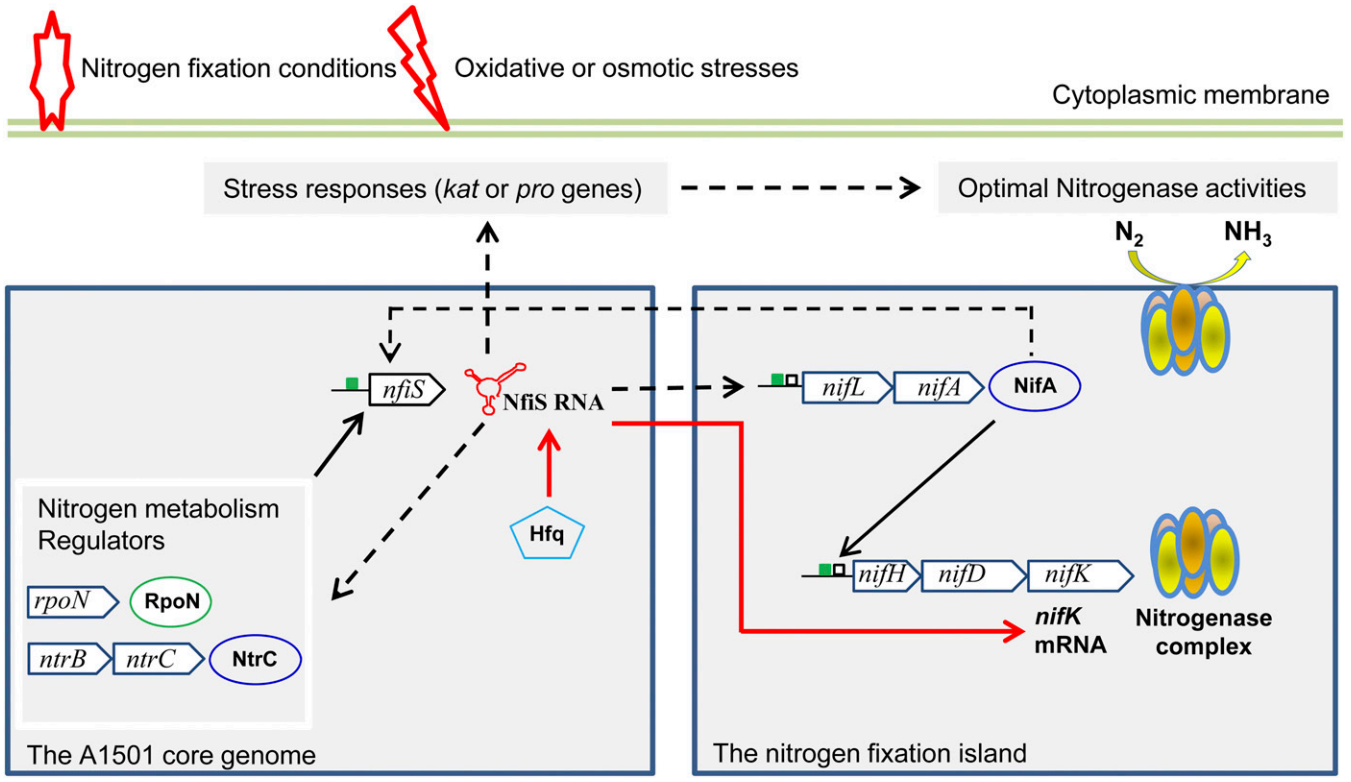

Fig. 6. Proposed model of NfiS-mediated regulation of optimal nitrogenase activity. The figure shows the interactions between NfiS in the core genome and the horizontally acquired *nif* island. The green box represents the putative RpoN-dependent promoters, and the black box represents the upstream activator sequences. Solid black and red arrows indicate transcriptional regulation and posttranscriptional regulation, respectively, and dashed lines represent unknown mechanisms. For more details, see the *Discussion*.

nitrogen-fixing isolates, including the unique *Pseudomonas azotifigens* strain DSM17556 that is phylogenetically close to *P. stutzeri* (18, 21, 51).

The schematic presented in Fig. 6 tentatively illustrates the role of NfiS in the regulation of nitrogenase activity according to our current knowledge. At the *nif*-specific level, there are at least two potential mechanisms that may contribute to the NfiS-mediated increase of nitrogenase activity. First, the positive effect of NfiS on the transcription of *nif* genes is likely mediated via RpoN and NifA, but how NfiS positively affects *nifA* transcription is not understood at this stage. It is tempting to speculate that this regulation could result from an effect of NfiS on *rpoN*, *ntrC*, and *glnK*, known to be involved in the transcription of *nifA* (24, 25, 32). Second, NfiS is involved in the posttranscriptional regulation of *nifK*, increasing the translation efficiency and the half-life of the mRNA. The Hfq protein is likely involved in the stabilization of the NfiS–*nifK* mRNA complex. Another level of complexity could arise from cross-talk between NfiS and NifA. Data reported in this work suggest that NfiS and NifA positively affect each other. Expression of *nfiS* was strongly decreased in a *nifA* mutant (Fig. 1*A*) whereas expression of *nifA* was decreased in the *nfiS*-deleted background (Fig. S4*A*). In other systems, such as *Bradyrhizobium japonicum,* targets of NifA include not only the *nif* and *fix* genes but also genes that are indirectly related to nitrogen fixation, such as denitrification genes (52, 53). Thus, laterally acquired NifA could affect other core genome-encoded genes via NfiS, establishing a genetic link between nitrogen fixation and other cellular metabolism.

In conclusion, NfiS is, to our knowledge, the first identified Hfq-dependent ncRNA activated by the RpoN/NtrC/NifA cascade in response to environmental signals and is, to our knowledge, the first described case of a bacterial small RNA involved in the regulation of optimal nitrogen fixation via a direct base-paring interaction. At the level of global regulation, NfiS upregulates various regulators, such as RpoN, NtrC, GlnK, and RpoS, controlling the stress response, nitrogen metabolism, and possibly other functions not yet identified.

Materials and Methods

Bacterial Strains, Plasmids, Oligonucleotides, Media, and Growth Conditions. Strains and plasmids are listed in Table S1. Details of plasmid and mutant strain construction are in *SI Materials and Methods* and Fig. S6. *P. stutzeri* A1501 WT (54) and mutant derivatives were grown in LB or in minimal lactate medium (medium K) at 30 °C (24). Antibiotics were used at the following concentrations: 100 μg/mL ampicillin (Amp), 50 μg/mL kanamycin (Km), 10 μg/mL tetracycline (Tc), and 34 μg/mL spectinomycin (Spc).

Nitrogenase Activity Assays. Nitrogenase activity was determined with bacterial suspensions incubated at an OD_{600} of 0.1, in N-free minimal medium [argon atmosphere, containing 0.5% oxygen and 10% (vol/vol) acetylene] at 30 °C, according to the derepression protocol (24). See *SI Materials and Methods* for more details. The specific activity of nitrogenase was expressed as nmol ethylene per hour per milligram of protein. Protein concentrations were determined using a standard protein assay (Bio-Rad) with BSA as the standard. Each experiment was repeated at least three times.

Abiotic Stress-Resistance Assays. *P. stutzeri* WT and mutant derivatives were grown in LB medium at 30 °C to an OD_{600} of 0.6 and then transferred into fresh LB medium in the presence or absence of 0.3 M sorbitol or 20 mM H_2O_2. At the time indicated, 10 times serial dilutions were made, and 10 μL of each dilution was spotted onto solid LB plates.

Northern Blot Analyses for NfiS Expression. Total RNA was isolated from *P. stutzeri* WT and mutant derivatives grown under nitrogen fixation or non-nitrogen-fixation conditions. RNA samples (10 μg) were separated on 1.5% agarose gels in 1× Mops buffer by electrophoresis and electroblotted onto positively charged nylon membranes (Amersham). Membranes were UV

cross-linked and hybridized for 20 h at 65 °C with the suitable digoxigenin-labeled probe. A 30-nt single-strand DNA probe for NfiS was synthesized, and the 5′ end of the synthesized product was labeled with digoxigenin (Sangon Company). Membranes were washed twice in 0.1% SDS/2× SSC buffer and then twice in 0.1% SDS/0.1× SSC buffer before exposure to film (Kodak). Band intensity was analyzed using ImageJ software.

Quantitative Real-Time PCR. Primer pairs used for qRT-PCR are listed in Table S4. Amplifications were conducted on an ABI PRISM 7500 real time PCR system (Applied Biosystems). See *SI Materials and Methods* for more details. Data were analyzed using the ABI PRISM 7500 Sequence Detection System Software (Applied Biosystems).

The 5′ Rapid Amplification of cDNA Ends to Determine *nfiS* Transcriptional Start Site. The 5′ rapid amplification of cDNA ends (RACE) system (Invitrogen) was used according to the manufacturer's instructions (*SI Materials and Methods*). Products were visualized on standard 2% agarose gels stained with ethidium bromide. PCR-generated bands were extracted from the gel, ligated into the pMD18-T vector (Takara), and sequenced.

Western Blot Analysis for NifDK Expression. Western blotting was performed on protein extracts of bacterial cells incubated for 5 h under nitrogen fixation conditions. Proteins separated by SDS/PAGE were transferred to a PVDF membrane (Amersham) by electroblotting. NifD and NifK polypeptides were revealed with antisera raised against the MoFe-protein. See extra details in *SI Materials and Methods*.

Microscale Thermophoresis Measurements. MST experiments were performed according to ref. 55. The dissociation constants (K_ds) were calculated as described (56). Data analyses were performed using Nanotemper Analysis software v.1.2.101 (NanoTemper Technologies). See extra details in *SI Materials and Methods*.

Measurement of NfiS and *nifK* mRNA Half-Lives. Two-milliliter samples were collected at different times (0, 5, 10, 15, 20, and 25 min) right after the addition of rifampicin (400 μg/mL), and total RNA was prepared to estimate mRNA levels by qRT-PCR. The mRNA half-lives were estimated using nonlinear regression analysis with one phase dissociation (*SI Materials and Methods*).

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