Inhibition of the FixL Sensor Kinase by the FixT Protein in Sinorhizobium meliloti*

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Anne-Marie Garnerone, Didier Cabanes‡, Marie Foussard‡, Pierre Boistard, and Jacques Batut§

From the Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes, CNRS-INRA BP27, Castanet-Tolosan Cedex, 31326 France

Nitrogen fixation in symbiotic rhizobia is subject to multiple levels of gene regulation. In Sinorhizobium meliloti, the alfalfa symbiont, the FixLJ two-component regulatory system plays a major role in inducing nitrogen fixation and respiration gene expression in response to the low ambient O2 concentration of the nodule. Here we report on the mode of action of the FixT protein, a recently identified repressor of nitrogen fixation gene expression in S. meliloti. First, we provide evidence that FixT prevents transcription of the intermediate key regulatory genes nifA and fixK by counteracting the activity of the FixLJ two-component system under otherwise inducing microoxic conditions. Second, we demonstrate that FixT acts as an inhibitor of the sensor hemoprotein kinase FixL, preventing the production or the accumulation of its phosphorylated form. FixT is thus a new example of a regulatory protein that blocks signal transduction in two-component systems at the level of the sensor kinase.

In legumes, nitrogen fixation takes place in a specialized organ, the nodule, that rhizobia elicit on the roots of their host plants. Within the nodule, rhizobia acquire the capacity to reduce N2 to ammonia that is assimilated by the plant cells. In the Rhizobium-legume symbiosis, as in all nitrogen fixing systems that have been studied so far, nitrogen fixation is tightly regulated, primarily but not uniquely, at the transcriptional level. In Sinorhizobium meliloti (formerly Rhizobium meliloti), the alfalfa symbiont, both positive and negative control of nitrogen fixation gene expression have been described, although positive control is by far the best understood. Positive control is exerted by a two-component regulatory system, FixLJ, that senses the microoxic conditions that prevail inside the nodule (1, 2). Indeed, the legume nodule is a microoxic environment whose dissolved O2 concentration is in the 10–30 nm range. Under microoxic (or anoxic) conditions, the sensor hemoprotein kinase FixL autophosphorylates from ATP on a conserved histidine residue (His295) (3) and subsequently transfers its phosphate to one of the conserved aspartate residues (Asp54) of the regulator protein FixJ (4). Phosphorylation of FixJ dramatically enhances its affinity for the promoters of two intermediate regulatory genes, nifA and fixK, that control expression of genes involved in the biosynthesis of nitrogenase and of an oxidase complex with high affinity for oxygen, respectively (5–7).

Early observations indicated that expression of nifA or fixK was enhanced in a fixK mutant (8). More recently, it was demonstrated that repression by fixK actually involved activation of a repressor gene, called fixT (9), located downstream of the fixLJ operon (Fig. 1A). We now provide evidence that the FixT protein prevents transcription of nifA and fixK by counteracting the activity of the master FixLJ two-component system under microoxic conditions. We further demonstrate that FixT behaves as an inhibitor of the FixL sensor kinase, preventing synthesis or accumulation of its phosphorylated form. Biological implications of this finding are discussed with respect to the regulation of symbiotic nitrogen fixation as well as in terms of signal processing by two-component regulatory systems in general.

EXPERIMENTAL PROCEDURES

Bacterial Strains—GMI939, -940, and -941 are derivatives of GMI211 (lac Sm′), constructed as described in Ref. 9. Briefly, GMI939 was derived from GMI211 by site-directed inactivation of the –10 promoter region of the fixT1 gene. GMI940 carries an 5-kilobase pair deletion that removes the fixT2 gene as well as the adjacent fixK and fixNOQP genes. GMI941 is a derivative of GMI940, in which the 5-kilobase pair deletion has been transduced into GMI939, using a phleomycin resistance marker associated with the deletion (9).

Northern Blot Analysis of fixK Expression—RNAs were extracted from 30-ml cultures (A600 = 0.4) in M9 medium of cells incubated under microoxic conditions (2% oxygen) for 4 h (10). Bacterial cells were collected by centrifugation and incubated for 10 min at 65 °C in 2 ml of prewarmed lysis solution (1.4% SDS, 4 mM EDTA, 75 µg of proteinase K). Proteins were precipitated by adding 1 ml of NaCl 5 M at 4 °C. Nucleic acids were precipitated from the supernatant by the addition of 1 volume of isopropanol and the pellet was resuspended in nuclelease-free water. DNA was eliminated by the addition of 7.5 units of fast protein liquid chromatography-pure RNase-free DNase I (Amerham Pharmacia Biotech). RNAs were further extracted with phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol and then precipitated with ethanol. The RNA pellet was washed with 70% ethanol and resuspended in nuclelease-free water. RNAs were denatured for 10 min at 75 °C before loading on a 2.2 M formaldehyde, 1.5% agarose gel. RNAs were electrotransferred to a Hybond N nylon membrane (American Pharmacia Biotech) and probed with a 32P-labeled BglII–EcoRV fixK internal fragment. Equal loading of the different lanes in Fig. 1 was ensured by hybridization of the membrane with a hemA probe. For the purpose of quantification, a range of appropriate dilutions of the various RNA preparations was transferred to a Nylon membrane, and the hybridization signals were quantified on a phosphor screen apparatus (Fuji).

Reverse Transcriptase-PCR Analysis of nifA Expression—Microaerobic induction of nifA was achieved using the stoppered tube assay procedure (11). RNAs were purified as described above and quantitated by measuring the absorbance at 260 nm. The absence of contaminating DNA in the preparation was ensured by PCR amplification in the

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§ To whom correspondence should be addressed. Tel.: 33 5 61 28 50 54; Fax: 33 5 61 28 50 61; E-mail: jbatut@toulouse.inra.fr.

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absence of reverse transcriptase.

The following oligonucleotides were used to isolate nifA and hemA gene expression: 5′-ATT AGC TTC GCA GAA CA (nifA reverse primer), 5′-CAG CAA GAA CAC CAG AA (nifA sense primer), 5′-GTC GAT CGC GTT CTT (hemA reverse primer), and 5′-TGG ATC GGC TGC ATC A (hemA sense primer) (see Table 1). PCR products were cloned into the pBluescript II SK vector, and inserts were sequenced.

First-strand cDNA synthesis took place in a 17-μl reaction volume, 1 μl of total RNA (100 ng), 7.1 μl of diethyl pyrocarbonate-treated H2O, and 1 μl (50 ng) of the nifA or hemA reverse primer were heated at 70 °C for 10 min and quickly chilled on ice. After a brief centrifugation, 3.4 μl of 5× buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl2) 2 μl of 1.5 mM dithiothreitol, and 1 μl of a 25 mM dNTP solution were added and incubated at 42 °C for 2 min. After the addition of 1 μl (200 units) of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) and thorough mixing by pipetting up and down, the reaction tube was incubated for 50 min at 42 °C. Reverse transcriptase was inactivated by heating at 95 °C for 5 min, since we have observed that heating at 70 °C did not fully inactivate the enzyme.

For PCR amplification, 2.5 μl of 10× PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 1 μl of 50 mM MgCl2, 1 μl of dNTP mix (25 μM), 1 μl of reverse primer (50 ng), 1 μl of sense primer (50 ng), 0.5 μl (2.5 units) of Taq DNA polymerase (Life Technologies, Inc.) were added to the first strand synthesis reaction mix. After gentle mixing, the reaction mix was overlaid with one drop of mineral oil, heated at 94 °C for 3 min, and submitted to 25 cycles of the following sequence (94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min) and one cycle at 72 °C for 10 min. Reverse transcriptase-PCR products were electrophoresed on a 2% agarose gel, blotted onto a nylon membrane (Biodyne A Transfer membrane; Pall), and probed with a 32P-labeled probe internal to nifA or hemA genes.

**Purification of FixT**—FixT was essentially purified by affinity chromatography of a fusion protein between FixT and MalE, the maltose-binding protein (Biolabs). DNA corresponding to the putative nifA gene from S. meliloti was cloned into the expression vector pMAL-c2. The resulting plasmid (pMF116) was transformed into E. coli 1537 (from amino acid position 266 to the end of the protein), positions 50517-50601 were cloned into a pMal2 vector to obtain a fusion protein between FixLC and MalE. DNA fragment corresponding to FixLC was purified with NcoI and BamHI restriction enzymes. The resulting plasmid (pNAMB661) was transformed into the TB1 strain of E. coli, and the fusion protein was purified. Fractions containing phosphorylated (radioactive) FixL22 were individually screened by TLC (polyethyleneimine-cellulose, Schleicher & Schuell) for the presence of contaminating ATP, ADP, and P, using 0.75 m potassium dihydrogenophosphate (pH 3.5) as a buffer. Pure fractions were pooled, concentrated at 4 °C, and used immediately.

**Phosphotransfer Assays—** FixT was added after phosphorylation of FixJ by acetylphosphate for 1 h at 25 °C (5). The transcription assay was then performed in the presence of FixT as described above. Alternatively, the FixJ-P preparation was run over a Centricon 3000 microconcentrator (Amicon) in order to eliminate acetylphosphate before adding FixT.

**RESULTS**

**Phosphorylation assays were essentially as described (5) except that [γ-32P]ATP (0.4 mM) was used as labeling source (final specific activity ~5000 dpm/pmol). All reactions were at 28 °C.** Phosphorylation reactions were loaded on SDS-10% polyacrylamide gels. When indicated, reactions were performed under anaerobic conditions. Dried gels were stained with Coomassie Brilliant Blue R-250, and bands were identified by microdensitometry. In all experiments, bands were excised from the gel, and radioactivity was determined by liquid scintillation counting of excised band gels.

**FixT Activity**

**FixT** lowers fixK and nifA transcript levels in vivo—fixT is a duplicated gene on the pSym-a megaplasmid of S. meliloti. One copy of fixT, which we shall call fixT1, lies between the regulatory fixJ and fixK genes (Fig. 1A) and is transcribed from a fixK-dependent promoter (9). The second copy of fixT,
fixT2, is located ~260 kilobase pairs from fixT1 in a reiterated region that includes a second copy of fixK as well as of the fixNOQP operon (14). nifA instead is unlinked to fixT genes.

Northern blot analysis of RNAs extracted from fixT S. meliloti mutant strains and congeneric controls incubated under microoxic conditions showed that the levels of fixK transcript were enhanced in fixT mutants (Fig. 1B). Selective inactivation of fixT1 (9) led to a 10-fold increase in the level of fixK RNA as compared with the wild-type GM1211 strain (lane 4). Similarly, a strain deleted for fixT2 as well as for the adjacent fixK and fixN genes displayed an increase in the level of fixK RNA (lane 2) as compared with GM1211. For a reason that we ignore, this effect was, however, smaller than the one observed with the fixT1 mutant. The combination of the two sets of mutations in the same strain (GM1941, lane 3) resulted in an ~50–60-fold increase in the level of fixK transcript as compared with the wild-type control strain.

We examined the effect of fixT on nifA expression by reverse transcriptase-PCR, since we could not obtain consistent Northern blot hybridization patterns with a nifA probe. Wild-type (GM1211) and fixT (GM1941) S. meliloti cells were incubated under nearly anoxic conditions in order to maximize nifA expression (11). Comparison of the nifA-specific amplification products from extracted RNAs showed higher levels of nifA transcript in the double fixT mutant as compared with the wild-type strain (Fig. 1C, lanes 1 and 2). Expression of the oxygen-insensitive S. meliloti hemA gene (8) was unaffected by mutations in fixT (Fig. 1C, lanes 3 and 4).

These data suggested that fixT affected the synthesis or the stability of fixK and nifA transcripts. We took advantage of the availability of an in vitro transcription assay for these genes to discriminate between these possibilities. Purified FixT Protein Blocks fixK Transcription in Vitro—Previous genetic evidence indicated that fixT encoded a repressor protein (9). The FixT protein, encoded by fixT1, was overproduced in Escherichia coli as a fusion protein with MalE, the maltose-binding protein, and purified by affinity chromatography over an amylose column (see “Experimental Procedures”). In the experiments described below, either the MalE-FixT protein or the cleaved and purified FixT protein has been used and has led to similar results.

A transcription assay for fixK was previously set up (5) that relies on the ability of phosphorylated FixJ protein to drive fixK transcription in vitro, at the genuine promoter, in the presence of E. coli σ70-RNA polymerase holoenzyme. The assay is designed in such a way that transcription strictly depends upon phosphorylation of FixJ. It consists of three steps (see “Experimental Procedures” for details). 1) Pure FixD protein is phosphorylated by incubation with pure FixL protein and ATP for 20 min. The FixL protein that is used, FixL122, is a truncated, soluble, oxygen-responsive form of the full-length FixL protein (5, 12). 2) Closed complexes are allowed to form for 10 min by the addition of E. coli RNA polymerase-σ70 holoenzyme and a cloned fixK DNA transcription template to phosphorylated FixJ 3) Isomerization of closed complexes and elongation of transcription is started upon the addition of the three missing nucleotides. The first two steps are performed under anoxic conditions, since they are readily reversible in air.

The addition of highly purified MalE-FixT protein at the onset of FixJ phosphorylation (step 1 above) resulted in a dramatic decrease in the level of fixK mRNA synthesized (Fig. 2A, lane 3). The effect of MalE-FixT on fixK transcription was specific, since all other transcripts, which originate from transcription of the vector template DNA genes by E. coli holoenzyme, were unaffected. Thus, FixT specifically blocked initiation of transcription of fixK or elongation of the nascent fixK RNA. The inhibitory effect of FixT was also observed when FixT was added 10 min after FixJ phosphorylation was started (Fig. 2A, lane 4; see “Discussion”). Interestingly, no inhibition of fixK transcription was observed when FixJ was phosphorylated from acetylphosphate (Fig. 2B), a low molecular weight phosphodonor (5, 15), instead of being phosphorylated from FixL-phosphate. Specifically, FixT had no effect on FixJ-driven transcription if FixT was added to FixJ at the end (Fig. 2B) or at the beginning of FixJ incubation with acetylphosphate (data not shown). Furthermore, FixT did not inhibit the transcriptional activity of a FixJ-phosphate preparation from which acetylphosphate had been eliminated by molecular sieving (data not shown). This demonstrated first that FixT did not directly dephosphorylate FixJ-phosphate (since the transcription assay relies on phosphorylated FixJ) and, second, that FixT did not inhibit FixJ-
phosphate transcriptional activity per se. Rather, these data suggested an implication of FixL in inhibition by FixT.

FixT Inhibits FixL-Phosphate Synthesis—When purified FixL is incubated in the presence of γ-labeled [32P]ATP, the amount of (auto)phosphorylated FixL protein rises linearly over at least the first 15 min of the reaction (16) (Fig. 3). The amount of (auto)phosphorylated FixL protein rises linearly over at least the first 15 min of the reaction (16) (Fig. 4).

When purified FixT protein was preincubated with FixL prior to the addition of γ-labeled ATP (400 μM), no FixL-phosphate accumulation could be detected after 10 min (Fig. 3A) or after 60 min of incubation (data not shown). In a second set of experiments (Fig. 3B), FixL was incubated with γ-labeled ATP for 5 min prior to the addition of FixT, thus allowing a detectable amount of FixL-phosphate to be synthesized. Upon the addition of FixT, the level of phosphorylated FixL stabilized but did not decrease, thus excluding a prominent phosphatase activity associated with FixT. This was confirmed by purifying FixL-phosphate from ATP (and ADP) nucleotides in order to prevent further FixL phosphorylation. It was then observed that the addition of FixT (Fig. 3C) or FixT plus ATP (data not shown) did not enhance the rate of FixL-P dephosphorylation. This is evidence that FixT is not a phosphatase.

We also ruled out the possibility of an ATPase activity associated with FixT, since no Pi production or depletion of the ATP pool could be detected on TLC plates after extended incubation of FixT with γ-labeled ATP, even in the presence of FixL (data not shown). We were also unable to detect any (reasonably stable) phosphorylation of FixT after it was incubated with either radiolabeled ATP or FixL-phosphate and separated by SDS-PAGE under neutral pH conditions (data not shown). Thus, the model we presently favor is that FixT either blocks autophosphorylation of FixL from ATP (FixL + ATP → FixL-P + ADP) or enhances the rate of the reverse reaction (i.e., FixL-P + ADP → FixL + ATP).

Both explanations are consistent with the further observation that adding FixT to FixL 60 min after the addition of ATP (i.e., when the phosphorylation reaction FixL + ATP = FixL-P + ADP had reached equilibrium) resulted in the disappearance of most of FixL-phosphate within 15 min (Fig. 4).

FixT Promotes FixL-Phosphate Dephosphorylation in the Presence of FixJ—When FixL and FixJ are incubated together in the presence of γ-labeled ATP under anoxic conditions, the phosphate first enters FixL and then readily transfers to FixJ as shown above (Fig. 2). This is evidence that FixT is not a phosphatase.

Evidence for a Stoichiometric Interaction between FixT and FixL—When FixL and FixJ are incubated together in the presence of γ-labeled ATP under anoxic conditions, the phosphate first enters FixL and then readily transfers to FixJ so that only FixJ-phosphate accumulates (Fig. 5, lanes 1–3). The level of phosphorylated FixJ-phosphate levels off after a 30–40 min incubation in our assay conditions because of the phosphatase activity associated with FixL (3, 17). When FixT was added after a 60-min incubation, the level of phosphorylated FixJ decreased dramatically within 15 min (FixT). FixT thus promoted dephosphorylation of FixJ-phosphate. As pointed out above (Fig. 2B), this was not due to a direct dephosphorylation of FixJ-phosphate by FixT. Rather, FixJ-phosphate disappearance was a consequence of the block in FixL-phosphate synthesis that prevented replenishing of the FixJ-phosphate pool (see “Discussion”). In addition, we observed that FixT did not affect phosphotransfer between FixL-phosphate and FixJ as monitored with purified (sieved), nucleotide-free, FixL-phosphate (data not shown).

Fig. 4. FixT action results in a depletion of the FixL-phosphate pool. FixL122 (6 μM) was incubated with [γ-33P]ATP (0.4 mM) at 28 °C under aerobic conditions. After a 65-min incubation (lane 1), the mix was split into two tubes to which either buffer (-; lanes 3, 5, and 7) or MalE-FixT protein (+; 9 μM) was added (lanes 4, 6, and 8). Samples were withdrawn at 70 min (lanes 3 and 4), 75 min (lanes 5 and 6), and 80 min (lanes 7 and 8) for SDS-PAGE analysis. Lane 2, positive control. In this tube, MalE-FixT protein was present throughout the phosphorylation reaction of FixL122.
FixL122 (6 μM monomer), FixJ (6 μM), and [γ-32P]ATP (0.4 mM) was incubated at 28 °C under anoxic conditions. After a 65-min incubation (arrow), the mix was split into two reaction tubes to which either buffer (open bar) or purified MalE-FixT protein (9 μM) was added (black bar). Incubation was prolonged, and samples were withdrawn at 70, 75, and 80 min for the purpose of SDS-PAGE analysis.

FixT promotes FixJ-phosphate dephosphorylation in the presence of FixL. A mix of FixL122 (6 μM monomer), FixJ (6 μM), and [γ-32P]ATP (0.4 mM) was incubated at 28 °C under anoxic conditions. After a 65-min incubation (arrow), the mix was split into two reaction tubes to which either buffer (open bar) or purified MalE-FixT protein (9 μM) was added (black bar). Incubation was prolonged, and samples were withdrawn at 70, 75, and 80 min for the purpose of SDS-PAGE analysis.

FixT targets the carboxyl-terminal kinase domain of FixL—FixL is a modular protein (12, 19, 20) made up of three domains: 1) a N-terminal membrane-anchoring domain, which is not essential for activity or for oxygen sensing in vitro (this domain is missing in the soluble FixL122 protein used in this work), 2) a central oxygen-sensing heme-containing domain whose structure has been solved recently (20); and 3) a C-terminal kinase domain homologous to the kinase domain of bacterial sensor kinases. This isolated domain, previously called FixLC, is capable of constitutive autophosphorylation independently of oxygen, although with a lower efficiency than the full-length protein (19). We examined whether this isolated kinase domain was prone to inhibition by FixT.

A FixLC protein was purified that consisted of the last 240 amino acids fused to MalE (see “Experimental Procedures”). FixT significantly inhibited phosphorylation of FixLC, although inhibition was not complete (Fig. 7A, lane 4), even at a molar ratio of 6 between FixT and FixLC (data not shown). We conclude that the C-terminal kinase domain of FixL is the primary target for FixT, although we do not exclude the possibility that the central oxygen-sensing domain of FixL may contribute to the interaction with FixT.

FixT is not active on the heterologous NtrB, DegS, and KinA sensor kinases—Since the C-terminal kinase domain of FixL is conserved between the sensor proteins of the two-component family, it was of interest to test whether other targets could be identified for FixT. Three purified kinases were kindly provided by Dr. T. Mzadek: the NtrB protein from Salmonella typhimurium and the DegS and KinA sensor proteins from Bacillus subtilis. Two different phosphorylation conditions were tested. In a first set of experiments, KinA, NtrB, and DegS were phosphorylated in the presence of 2.5 μM [γ-32P]ATP, as described before (13). No effect of FixT on the phosphorylation of the heterologous kinases was observed (Fig. 7B). In a second set of experiments, we assayed phosphorylation of NtrB, DegS, and KinA under the conditions used for FixL (i.e. 0.4 mM ATP) and, again, found no effect of FixT (data not shown). Similar data were obtained with higher concentrations of FixT (27 μM) (data not shown).

**DISCUSSION**

Mode of action of FixT—fixT was originally identified by genetic studies that demonstrated its ability to repress expression of the regulatory genes fixK and nifA in S. meliloti (9). However, the mode of action of fixT was completely unknown. Using in vitro methods, we have now demonstrated that FixT counteracts the activity of the FixLJ two-component system that mediates nifA and fixK expression in response to oxygen in S. meliloti. Specifically, we provide evidence that FixT acts by inhibiting primarily FixL-phosphate synthesis.

How FixT achieves its effect is not completely clear yet. When added prior to ATP, FixT completely prevented incorporation of phosphate into FixL (Fig. 3A). This is consistent with FixT blocking autophosphorylation of the FixL protein from ATP. For example, FixT may prevent access of ATP to the nucleotide binding pocket of FixL or slow down histidyl-phosphate formation. By contrast, FixT had no detectable ATPase activity (data not shown) and thus certainly does not act by depleting the pool of ATP. Alternatively, FixT may favor rapid dephosphorylation of newly synthesized FixL-phosphate. We have presented evidence that FixT is not a phosphatase for FixL-P (Fig. 3C) and does not phosphorylate itself from FixL-P (data not shown). By contrast, we do not exclude the possibility that FixT may enhance the rate of dephosphorylation of FixL-phosphate in the presence of ADP (FixL-P + ADP → FixL + ATP),

![FixT Activity](image-url)

**Fig. 7. FixT activity on FixLC and other kinases.** A, FixL122 (lanes 1 and 2) and FixLC (lanes 3 and 4) at 6 μM were phosphorylated from [γ-32P]ATP (0.4 mM) for 15 min in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of cleaved FixT protein (9 μM). Open triangles mark the position of the FixL122 and FixLC proteins. B, FixT activity was tested against 3 μM KinA (lanes 1 and 2), NtrB (lanes 3 and 4), and DegS (lanes 5 and 6). Lanes 1, 3, and 5, without FixT. Lanes 2, 4, and 6, with FixT (9 μM). See “Experimental Procedures” for details.
**FixT Activity**

i.e. may favor the reverse reaction of autophosphorylation. FixT does not resemble any known protein, nor does it have any prominent motif that could give indications regarding its mode of action. However, we presently have three clues: 1) FixT acts directly on FixL; 2) FixT targets the C-terminal domain of FixL; 3) the interaction between the two proteins is likely to be stoichiometric. Our working model is that FixT makes a protein-protein interaction with the carboxyl-terminal kinase domain of FixL that may either inhibit autophosphorylation of FixL or favor the reverse dephosphorylation reaction.

No other effect of FixT was observed besides its effect on FixL phosphorylation. First, FixT did not detectably impair phosphotransfer between FixL-phosphate and FixJ (data not shown). Second, FixT had no effect on the stability of FixL-phosphate (in the absence of FixL), as assayed by transcriptional activity (Fig. 2B). Note that direct assessment of FixL-phosphate dephosphorylation by FixT was not appropriate because of the very low specific activity of acetyl-phosphate that can be achieved (5). Instead, transcription is an indirect but reliable assay for FixL-phosphate dephosphorylation. Thus, the primary, if not unique, role of FixT is to inhibit FixL phosphorylation.

The reported effect of FixT on FixL-phosphate synthesis fully accounts for both the FixT-induced FixJ-phosphate dephosphorylation in the presence of FixL (Fig. 5) and the inhibition of fixK transcription (Fig. 2A). Even under low oxygen conditions, phospho-FixJ is continuously and rapidly hydrolyzed because of the phosphatase activity associated with unphosphorylated FixL. In the presence of FixT, the FixL-phosphate pool drops with the consequence that phospho-FixJ cannot be rephosphorylated and thus disappears rapidly. FixL-phosphate dephosphorylation in turn results in a dramatic (more than 100-fold) decrease of its capacity to bind and activate the nifA and fixK promoters (7). The comparison of the kinetics of disappearance of FixJ-phosphate and of inhibition of fixK transcription is consistent with this analysis (compare, for example, Fig. 5, lane 8, and Fig. 2A, lane 4).

**Modulators of Two-component Systems**—Two-component systems are of paramount importance in bacteria, where they probably constitute the most widespread device for signal transduction. *E. coli* and *B. subtilis* each use up to 30 different two-component systems to cope with environmental changes (21, 22). Some two-component systems look very simple; in the FixLJ system, for example, a single protein, FixL, is able to sense its cognate signal (molecular oxygen) and to transduce it to the regulator protein, FixJ, by phosphotransfer. Moreover, both FixL and FixJ proteins have a modular structure (12, 19, 23). As a result, a combination of four domains suffices for oxygen signal transduction. Many two-component systems are more complex, involving more proteins than a simple sensor and regulator pair. Many of these proteins are phosphatases. For example, in *B. subtilis*, RAP phosphatases ensure dephosphorylation of the SpoOF-phosphate regulator protein (24). The SixA protein from *E. coli* is a phosphohistidine phosphatase that promotes dephosphorylation of the sensor ArcB protein (25). The PII protein in enteric ntr circuits is not a phosphatase per se but stimulates the phosphatase activity associated with the NtrB sensor kinase (26).

A more relevant comparison for FixT is KipI, for which the name antikinase was coined in bacteria. kipI was originally identified during the course of the *B. subtilis* genome sequencing project (27). Subsequently, KipI was shown to block sporulation of *B. subtilis* when overproduced in the absence of another protein, KipA, with which KipI normally forms a complex (28). KipI and FixT resemble each other by their inhibitory effect on the (auto)phosphorylation of their cognate sensor kinases, KinA and FixL, respectively. However, FixT and KipI display no amino acid sequence similarity to each other, and, thus, whether they are related in structure or mode of action is not known.

**Biological Role of FixT in *S. meliloti***—The primary role of FixLJ in *S. meliloti* is to control, via nifA and fixK, nif and fix gene expression in response to oxygen availability. The present data reinforce the pivotal role of the FixLJ system in *S. meliloti*, as an integration site for both positive regulation by oxygen and negative regulation by FixT. Typically, molecular devices such as the KipI, FixT, or the above mentioned phosphatases may allow two-component systems to integrate multiple environmental regulatory signals as demonstrated for the RAP phosphatases of *B. subtilis* (24, 28).

Since FixT interacts with FixL, one obvious possibility would be that FixT contributes to oxygen control. Available data indicate that FixL is sufficient for oxygen regulation of nifA and fixK expression both ex planta and in vitro and that fixT does not grossly affect oxygen regulation of the system (9). Furthermore, the central oxygen-sensing domain of FixL does not seem to be the primary target for FixT, and no difference in FixT activity was observed in vitro under anoxic versus oxic conditions (data not shown). Still, FixT may contribute to a fine tuning of the FixLJ response to oxygen, ensuring, for instance, that the FixL protein, which has a very low affinity for oxygen (29), does not become prematurely activated in response to a moderate or transient oxygen deprivation. Alternatively, FixT may prevent activation of the FixLJ system by low oxygen under conditions that would be otherwise antithetical to symbiotic nitrogen fixation such as, possibly, low energy charge, low carbon, or high nitrogen.

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**REFERENCES**

1. David, M., Daveran, M. L., Batut, J., Dedieu, A., Domergue, O., Ghai, J., Hertig, C., Boistard, P., and Kahn, D. (1988) *Cell* 54, 671–683.
2. Soupène, É., Foussard, É., Beaudouin, P., Truchet, G., and Batut, J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 3759–3763.
3. Monson, E. K., Ditta, G. S., and Helinski, D. R. (1995) *J. Biol. Chem.* 270, 5253–5250.
4. Reyrat, J. M., David, M., Batut, J., and Boistard, P. (1994) *J. Bacteriol.* 176, 1969–1976.
5. Reyrat, J. M., David, M., Blonski, C., Boistard, P., and Batut, J. (1993) *J. Bacteriol.* 175, 6867–6872.
6. Fischer, H. M. (1994) *Microbiol. Rev.* 58, 352–386.
7. Galinier, A., Garnerone, A. M., Reyrat, J. M., Kahn, D., Batut, J., and Boistard, P. (1994) *J. Biol. Chem.* 269, 23784–23789.
8. Batut, J., Daveran-Mingot, M. L., David, M., Jacobs, J., Garnerone, A. M., and Kahn, D. (1989) *EMBO J.* 8, 1279–1286.
9. Foussard, É., Garnerone, A. M., Ni, F., Soupène, É., Boistard, P., and Batut, J. (1997) *mol. Microbiol.* 25, 27–37.
10. de Philip, P., Batut, J., and Boistard, P. (1990) *J. Bacteriol.* 172, 4255–4262.
11. Ditta, G., Virts, E., Palomares, A., and Kim, C. H. (1987) *J. Bacteriol.* 169, 3217–3223.
12. de Philip, P., Soupène, É., Batut, J., and Boistard, P. (1992) *mol. Gen. Genet.* 235, 49–54.
13. Dahl, M. K., Msadek, T., Kunst, F., and Rapport, G. (1992) *J. Biol. Chem.* 267, 14509–14514.
14. Renailier, M. H., Batut, J., Ghai, J., Terauchi, B., Ghérandi, M., David, M., Garnerone, A. M., Vasse, J., Truchet, G., Huguet, T., and Boistard, P. (1987) *J. Bacteriol.* 169, 2231–2238.
15. Lukat, G. S., Mcleary, W. R., Stock, A. M., and Stock, J. B. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 718–722.
16. Gilles-Gonzalez, M. A., and Gonzalez, G. (1993) *J. Biol. Chem.* 268, 16293–16297.
17. Lois, A. F., Weinstein, M., Ditta, G. S., and Helinski, D. R. (1993) *J. Biol. Chem.* 268, 4370–4375.
18. Gilles-Gonzalez, M. A., Ditta, G. S., and Helinski, D. R. (1991) *Nature* 350, 170–172.
19. Monson, E. K., Weinstein, M., Ditta, G. S., and Helinski, D. R. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 4280–4284.
20. Gong, W., Han, B., Manay, S. S., Gonzalez, G., Gilles-Gonzalez, M. A., and Chan, M. K. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 15177–15182.
21. Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessieres, P., Bolotin, A., Borchert, S., Borris, R., Bourrier, L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Brouillet, S., Bruschi, C. V., Caldwell, B., Capuano, V., Carter, N. M., Choi, S. K., Codani, J. J., Connerton, I. F., and Danchin, A. (1997) *Nature* **390**, 249–256
22. Mizuno, T. (1997) *DNA Res.* **4**, 161–168
23. Kahn, D., and Ditta, G. (1991) *Mol. Microbiol.* **5**, 987–997
24. Perego, M., Hanstein, C., Welsh, K. M., Djavakhishvili, T., Glaser, P., and Hoch, J. A. (1994) *Cell* **79**, 1047–1055
25. Ogino, T., Matsubara, M., Kato, N., Nakamura, Y., and Mizuno, T. (1998) *Mol. Microbiol.* **27**, 573–585
26. Keener, J., and Kustu, S. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 4976–4980
27. Akagawa, E., Kurita, K., Sugawara, T., Nakamura, K., Kasahara, Y., Ogasawara, N., and Yamane, K. (1995) *Microbiology* **141**, 3241–3245
28. Wang, L., Grau, R., Perego, M., and Hoch, J. A. (1997) *Genes Dev.* **11**, 2569–2579
29. Gilles-Gonzalez, M. A., Gonzalez, G., Perutz, M. F., Kiger, L., Marden, M. C., and Poyart, C. (1984) *Biochemistry* **33**, 8067–8073