Research Note

A Purine-Related Metabolite Negatively Regulates *fixNOQP* Expression in *Sinorhizobium meliloti* by Modulation of *fixK* Expression

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5-aminoimidazole-4-carboxamide nucleotide (AICAR) is a negative effector of cytochrome terminal oxidase *cbb3* production in *Rhizobium etli*. In this work, the effect of AICARiboside (AICAr), the precursor of AICAR on the expression of the *Sinorhizobium meliloti* *fixNOQP* operon encoding the symbiotic terminal oxidase *cbb3*, was analyzed. AICAr reduced the microaerobic induction levels of *fixN-lacZ* and *fixT-lacZ* gene fusions 18- and sevenfold respectively, and both genes were activated by the transcriptional activator FixK. A *fixK-lacZ* fusion presented 14-fold-reduced induction levels in microaerobic cell cultures in the presence of AICAr. AICAr also reduced three-fold the microaerobic expression levels of the *nifA-lacZ* fusion, whose expression as well as that of *fixK* is controlled by the two-component system FixL–FixJ. In contrast, AICAr had no effect on the expression levels of a *hema-lacZ* fusion. These data suggest that AICAr prevents *fixNOQP* induction by the inhibition of *fixK* transcription.

Bacteria of the genera *Bradyrhizobium* and *Rhizobium* may establish a specific symbiotic relationship with their legume host plant. Bacteria elicit the formation of new organs and root nodules, where bacteroids reduce atmospheric nitrogen to ammonia and supply the host plant with combined nitrogen. In response to specific flavonoid molecules synthesized by the plant (Shultze et al. 1994), *nod* genes coding for enzymes involved in the formation and excretion of lipooligosaccharide factors (Nod factors) are activated. Nod factors cause root-hair curling and nodule meristem initiation (Shultze et al. 1994). *Rhizobia* spp. are attached to curled-hair roots and, soon afterward, a tubular structure (the infection thread carrying the bacteria) is formed. The infection threads grow into the root cortex then release the dividing bacteria to the nodule meristem. Finally, after bacteria invade nodule cortical cells, bacterial genes involved in nitrogen fixation are induced in response to the microaerobic environment of the

nodule (Batut and Boitard 1994). The characterization of purine auxotrophs from several *Rhizobium* species (Djordjevic et al. 1996; Kim et al. 1988; Newman et al. 1992) revealed that an intermediate in AICAR production is essential in the early stages of symbiosis, specifically for promoting infection-thread development and nodule morphogenesis (Djordjevic et al. 1996; Newman et al. 1995).

In addition to the inability of *Rhizobium etli* purine auxotrophs to infect *Phaseolus* spp. (Newman et al. 1995), they showed increased levels of the symbiotic terminal oxidase in free-living aerobic cultures (Soberón et al. 1997). Analysis of the transcription of the *fixNOQP* operon, encoding the symbiotic terminal oxidase *cbb3* in different *R. etli* mutants of the purine–thiamin biosynthetic pathway suggests that a molecule related closely to the 5-aminoimidazole-4-carboxamide nucleotide (AICAR) is a negative effector of the expression of these genes (Miranda et al. 1997; Soberón et al. 1997). A mutant in the *purH* gene that determines the conversion of AICAR to 5'-phosphoribosyl-5-formamidoimidazole-4-carboxamide (FAICAR) and inosine expressed low levels of *fixNOQP* in contrast to eight independent mutants affected in five previous steps of the purine biosynthetic pathway that expressed threefold-higher levels of *fixNOQP* (Soberón et al. 1997). Neither the molecular mechanism by which AICAR or a related metabolite modulates *fixNOQP* expression nor the physiological significance of this regulation is known.

As an approach to understanding the molecular mechanism by which AICAR negatively modulates *fixNOQP* expression, we studied the effect of AICARiboside (AICAr), the precursor of AICAR (the riboside is transported more easily to the cell than the nucleotide), on *fixNOQP* expression of *Sinorhizobium meliloti* because the molecular mechanism leading to *fixNOQP* expression is well documented in this organism (Batut and Boitard 1994). In *S. meliloti*, the expression of *fixNOQP* is regulated mainly in response to O₂ through the O₂-sensing cascade comprising the *fixL*, *fixJ*, and *fixK* gene products (Batut et al. 1989; David et al. 1988; Gilles-Gonzalez 1994; Lois et al. 1993; Renalier et al. 1987). Besides inducing *fixK* expression, the two-component system FixL–FixJ activates the expression of *nifA*, whose gene product is responsible for the activation of the nitrogenase structural genes *nifHDK*

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(Agron et al. 1993; Batut and Boitard 1994). *fixK* codes for a transcriptional activator that is homologous to Fnr and CRP and activates *fixNOQP* expression (Batut et al. 1989). FixK also induces the expression of *fixT*, whose gene product negatively regulates *fixK* and *nifA* expression by counteracting FixL–FixJ activities (Foussard et al. 1997; Garnerone et al. 1999). The effects of AICAr on the expression of *fixK*, *fixT*, and *nifA* also were studied. Our data suggest that a molecule related to AICAr negatively regulates the expression of *S.*

meliloti fixNOQP by the inhibition of *fixK* transcription. A preliminary report of these results was presented at the 16th North American Symbiotic Nitrogen Fixation Conference in Cancun, México (Soberón et al. 1999).

Bacterial strains and plasmids used are listed in Table 1. Cells were cultured microaerobically in minimal succinate (MS) medium with succinate (10 mM) and ammonium as carbon and nitrogen sources respectively (GTS without the addition of glucose as carbon source) (Putnoky and Kondorosi 1986).

Table 1. Bacterial strains and plasmids

Designation	Relevant characteristics	Reference
<i>Sinorhizobium meliloti</i>		
GMI5595	$\Delta(nod\ fix)$ JB16	David et al. 1988
GMI5682	$\Delta(nod\ fix)$ JB16 <i>fixK</i> , 2.60::Tn5	Batut et al. 1989
<i>Escherichia coli</i>		
XL1-blue	<i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>hsdR17</i> , <i>supE44</i> , <i>lac</i> (F' <i>proAB</i> , <i>lacIq</i> <i>lacZ</i> Δ M15, Tn10 [Tc^r]), <i>thi</i>	Bullock et al. 1987
Plasmids		
pGMI931	Tc^r , <i>fixN-lacZ</i>	David et al. 1988
pGD106	Tc^r , <i>fixT-lacZ</i>	Foussard et al. 1997
PMF457	Tc^r , <i>fixK-lacZ</i>	Foussard et al. 1997
pCHK57	Tc^r , <i>nifA-lacZ</i>	Ditta et al. 1987
pXLGD4	Tc^r , <i>hemA-lacZ</i>	Leong et al. 1985

Fig. 1. *fixNOQP* and *fixT* are negatively regulated by AICAr. **A**, Expression of *fixN-lacZ* (pGMI931) **B**, Expression of *fixT-lacZ* (pGD106) in GMI5595 and GMI5682 *fixK* mutant cultured microaerobically (shaded boxes) or aerobically (white boxes). **C**, Expression of *hemA-lacZ* (pXLGD4) in GMI5595 cultured microaerobically. β-galactosidase activities are expressed as μmol of onitrophenol produced per min per mg of protein. Error bars represent standard deviations of means of four replicate cultures.

A higher cytochrome content was found in cells cultured in MS medium than in those cultured in minimal glucose succinate medium (GTS; data not shown). The preparation of cell inoculum for microaerobic-inducing cultures was critical to obtain reproducible effects of an AICAr addition on *fix* gene expression. To prepare cell inoculums, bacteria were cultured on MS plates for 4 days, used to inoculate 50-ml MS medium in 2,500-ml Erlenmeyer flasks at an optical density (OD) at 540 of 0.025, and incubated for 36 h. AICAr was added to the cell culture (150 µg per ml, 0.6 mM) after this incubation period, if indicated, and cultures were incubated for an additional 12 h. Several AICAr concentrations were tested, which showed that 0.6 mM was enough to see a reproducible effect of AICAr (data not shown). With the use of these cells as inoculum, microaerobic cultures of *S. meliloti* were prepared by diluting cells to an initial cell density of 0.05 OD₅₄₀ in 50-ml fresh MS, with or without the addition of AICAr. The cultures were then flushed with a stream of 1,200 ml per min of sterile gas mix of N₂ with 2% O₂ for 5 min and cultured for 8 h at 30°C with shaking at 200 rpm. After this time, cells were harvested and β-galactosidase was determined by measuring orthonitrophenol production, as described (Maniatis et al 1982). Activities are expressed as a µmol of onitrophenol produced per min per mg of protein. Protein was measured as described (Markwell et al. 1978), with bovine serum albumin as standard.

AICAr inhibits expression of *fixNOQP* and *fixT* genes in free-living cultures of *S. meliloti*.

To study the effect of the addition of AICAr to *S. meliloti* cell cultures on the expression of the *fixNOQP* and *fixT* genes, strain GMI5595 and its derivative *fixK* GMI5682 carrying a

Fig. 2. Effect of AICAr on *fixK* and *nifA* expression. **A**, Expression of *fixK-lacZ* (pMF457) in GMI5595 and GMI5682 *fixK* mutant and **B**, *nifA-lacZ* (pCHK57) in GMI5595 cultured microaerobically. β-galactosidase activities are expressed as µmol of onitrophenol produced per min per mg of protein.

translational *lacZ* gene fusions to the *fixN* (pGMI931) (David et al. 1988) or *fixT* (pGD106) (Foussard et al. 1997) promoters, respectively, were used. The levels of expression of *fixNOQP* and *fixT* were 14- and fivefold higher in microaerobic cultures compared with aerobic cultures (Fig. 1), as previously reported (Batut et al. 1989; Foussard et al. 1997). In accordance with previous reports (Batut et al. 1989; Foussard et al. 1997), the induction of *fixN-lacZ* and *fixT-lacZ* depended upon FixK because in GMI5682 (*fixK*), the expression of either of these genes was not induced (Fig. 1A). When GMI5595 cultures were grown in the presence of AICAr, the induction levels of *fixN-lacZ* and *fixT-lacZ* were lower. In microaerobic conditions with AICAr, the expression levels of *fixN-lacZ* were 18-fold lower than without AICAr (Fig. 1A). Similarly, the *fixT-lacZ* exhibited sevenfold lower induction levels in the presence of this compound (Fig. 1B). The effect of AICAr on the expression of *hemA-lacZ* gene fusion (pXLGD4) (Leong et al. 1985) was studied because *hemA* transcription is constitutive (Leong et al. 1985). *hemA-lacZ* expression levels in cells cultured in the presence of AICAr were very similar to the induction levels found in cells cultured without AICAr (Fig. 1C). *fixN-lacZ* expression was not affected by other metabolites related to AICAR production such as histidine, inosine, adenosine or guanosine (data not shown).

Effect of AICAr on *fixK* expression.

In order to study whether reduction in the levels of expression of the *fixNOQP* and *fixT* genes by AICAr was the result of inhibition of the transcription of *fixK*, we analyzed *fixK* expression in the same culture conditions in strains GMI5595 and GMI5682 carrying a translational *fixK-lacZ* gene fusion (pMF457) (Foussard et al. 1997). Figure 2A shows that the expression of *fixK-lacZ* fusion in microaerobic cultures was higher in the GMI5682 background than in the GMI5595 strain, which is in agreement with the proposed role of FixK in the negative regulation of its own expression (Batut et al. 1989; Foussard et al. 1997). In microaerobic cultures of GMI5595 and GMI5682, the addition of AICAr lowered the induction levels of *fixK-lacZ* 14- and tenfold, respectively (Fig. 2A). Thus, the negative effect of AICAr on *fixNOQP* and *fixT* expression was exerted at the transcription of *fixK*. We currently cannot rule out, however, that AICAr also controls, to some extent, the transcriptional activity of FixK because in aerobic conditions there was no effect on *fixK* expression, in contrast to a fourfold inhibition of *fixN-lacZ* or *fixT-lacZ* expression levels by AICAr (data not shown), however, this remains to be determined.

Effect of AICAr on *nifA* expression.

In order to study whether AICAr had any effect on the activities of the response regulators FixL–FixJ controlling *fixK* expression (Batut et al. 1989), the effect of AICAr on *nifA* expression in strain GMI5595 carrying a translational *nifA-lacZ* gene fusion (pCHK57) (Ditta et al. 1987) was studied. Figure 2B shows that in cells cultured microaerobically with AICAr, the expression levels of *nifA-lacZ* were nearly threefold lower than in cell cultures without AICAr.

The inhibition of FixL–FixJ activities by AICAr could explain the low transcription levels of *fixK* and *nifA* in the presence of this compound. We cannot explain, however, the

different ratios of gene expression inhibition on *fixK* (tenfold) and *nifA* (threefold) by AICAR. Therefore, it still remains to be analyzed whether AICAR modulates FixL–FixJ activities because the data presented here does not exclude the possibility of AICAR acting independently of FixL–FixJ.

Inoculum preparation was crucial in finding a reproducible effect of AICAR on *fix* gene expression. AICAR had no effect if it was added only to the inducing microaerobic cultures and had a slightly larger effect on *fix* gene expression when it was not added to the inducing microaerobic cultures. Moreover, it was present only in the inoculum preparation cultures (data not shown). These results suggest that AICAR could be metabolized to a related molecule, which could be the real effector, yet remains to be determined.

Characterization of *R. etli* purine auxotrophs showed that the purine biosynthetic pathway of the bacteria is required for infection-thread development and nodule morphogenesis (Newman et al. 1992; Newman et al. 1995; Noel et al. 1988). The metabolic intermediate of the purine biosynthetic pathway AICAR was suggested to be the effector (Newman et al. 1995). Our data suggest that AICAR or a related molecule modulates infection-thread development in the plant and may be involved in the differentiation of the bacteria as it modulates the production of the symbiotic terminal oxidase. During the infection process, the *cbb₃* terminal oxidase production might be down regulated by a metabolite related to AICAR. In accordance with this hypothesis, *fixNOQP* genes were not expressed inside infection threads of pea nodules induced by *Rhizobium leguminosarum* bv. *viciae* when *fixN* expression was monitored with *fixN–gusA* gene fusion (A. Y. Borisov, *personal communication*). Reduction of the synthesis of this terminal oxidase could favor the utilization of another respiratory chain, which might be more efficient for bacterial growth during this process.

In conclusion, our data suggest that a molecule related to AICAR might be a novel symbiotic signal modulating the expression of important nitrogen fixation genes in the bacteria as well as infection-thread development in the plant. Thus, this signal might be important for modulating gene expression in both symbionts during the infection process.

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