Research Note

The *purMN* Genes of *Rhizobium leguminosarum* and a Superficial Link with Siderophore Production

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We isolated a mutant of *R. leguminosarum* initially on the basis of reduced production of the siderophore vicibactin on chrome azurol sulfonate (CAS)/agar indicator plates. The mutation was in the *purMN* operon and the mutant was shown to be an adenine auxotroph and defective for nodulation of peas. The siderophore defect appears to be trivial, being due to diminished growth of the auxotroph on agar-based minimal medium, which contains unknown contaminant(s) that allow it grow poorly. Transcriptional fusions showed that *purMN* was transcribed at relatively high levels in media containing purines. Expression was enhanced, approximately twofold, if purines were omitted.

Additional keywords: N₂ fixation, purine auxotrophy.

Bacteria known as the “rhizobia” induce N₂-fixing nodules on legumes. Purine auxotrophs of these bacteria are known to be symbiotically defective. In most legumes, they induce small, nonfixing nodules (Pankhurst and Schwinghamer 1974; Pain 1979; Djordjevic et al. 1996; Newman et al. 1995). It appears not to be the purine requirement per se that causes the symbiotic defect, but rather the lack of a precursor, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) (Newman et al. 1995).

Rhizobium leguminosarum makes vicibactin, a trihydroxamate cyclic siderophore, comprising three repeating units based on D-oronithine and butyric acid (Dilworth et al. 1998). In a screen of mutants altered in vicibactin production (Stevens et al. 1999), we isolated one vicibactin nonproducer that, as described here, was a purine auxotroph. Here, we describe why the link between the adenine requirement and vicibactin synthesis is probably trivial and describe features of the mutated genes, *purM* and *purN*.

R. leguminosarum bv. *viciae* 8401pRL1JI (see Table 1 for this and other strains used) was mutagenized with Tn5 by introducing into it by conjugation the “suicide” plasmid pSUP2021 (Simon et al. 1986), which contains the transposon in a plasmid that is mobilizable to *Rhizobium* spp. but which

does not replicate in that host. Kanamycin-resistant transconjugants (specified by Tn5) arose at a frequency of approximately 10⁻⁶. Survivors were picked to the siderophore indicator chrome azurol sulfonate (CAS) plates (Schwyn and Neilands 1987). Of 2,500 colonies tested, one, termed J225, had a very small halo, compared with wild type.

To try to quantify the vicibactin made by this mutant, strain J225 was inoculated into Y minimal medium (Beringer 1974) but it did not grow, indicating that it was auxotrophic. This was unexpected since CAS tests are done on Y minimal medium agar and there was no obvious defect in the growth of the bacterial stabs. However, when J225 was streaked on minimal medium, solidified with agarose rather than the less pure agar, there was no growth. Thus, agar has a contaminant that lets J225 grow.

By streaking J225 on minimal media supplemented with different nutrients, it was found to be a purine auxotroph, growth being restored by adenine (30 µg ml⁻¹). Growth of J225 on minimal agarose media was also restored by 30 µg ml⁻¹ AICAR, a late intermediate in purine biosynthesis (Zalkin and Nygaard 1996). Djordjevic et al. (1996) also found that *pur* mutants of *Rhizobium* sp. strain NGR234 grew on agar-based media but not on agarose.

To examine the CAS phenotype on agarose media, mutant J225 was streaked on agarose plates plus CAS and various concentrations of adenine (0.1, 1.0, 10, and 30 µg ml⁻¹). Surprisingly, there was no growth on any of these media. A wild-type control (8401pRL1JI) was also streaked on these same media. Again, there was no growth. Thus, it appears that CAS, in agarose-based media, is inhibitory to growth of *R. leguminosarum*, but on agar-based substrate it has no such obvious, deleterious effects.

J225 was plated on agar-based medium + CAS supplemented with the various concentrations of adenine or of AICAR. Normal-sized halos arose with both compounds at 10 and 1.0 µg ml⁻¹. With either compound at 0.1 µg ml⁻¹ or on unsupplemented agar-CAS plates, there was no staining although growth was essentially the same as that of the wild-type controls. Thus, it seems that when the J225 is stressed for lack of purine, siderophore synthesis and/or export is inhibited even though growth seems normal.

We also had available several other *ade* mutants of *R. leguminosarum* bv. *viciae*, isolated in the period 1973 to 1978.

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Nucleotide and/or amino acid sequence data are to be found at EMBL data base as accession number AJ243305.

Two such alleles (*ade27* and *ade28*) had been mapped to one of two chromosomal locations (Beringer et al. 1978); others had not been mapped. These auxotrophs were also streaked on the media described above. The results are summarized as follows:

(i) All Ade⁻ auxotrophs grew on agar-based but not agarose-based media lacking adenine. Most of these mutants were originally “non-leaky,” indicating that the contaminant was not in the agar when they were first isolated.

(ii) None of the mutants stained on agar/CAS plates lacking adenine or AICAR.

(iii) Some strains (56, 159, 192, and 1076) were restored to normal growth on agarose plus AICAR. Others (1027, 1075, and 1060) were not, indicating that they were blocked later in purine biosynthesis. However, both classes were defective in siderophore production on CAS plates.

It was thus apparent that *R. leguminosarum ade* auxotrophs, when grown on low concentrations of adenine, were defective in siderophore synthesis. To see if deprivation of other nutrients had the same effect, three double auxotrophs were depleted of their “second” requirement. Strains 1075 (Ade⁻ Liv⁻) 56 (Ade⁻ Cys⁻), and 1027 (Ade⁻ Phe⁻) were streaked on CAS agar supplemented with adenine at 30 µg ml⁻¹ plus different concentrations of leucine + isoleucine + valine (strain 1075), cysteine (strain 56), or phenylalanine (strain 1027). For all three, growth appeared normal at concentrations as low as 1.0 µg ml⁻¹. However, at 1.0 µg ml⁻¹ there was no visible staining on CAS for any of them while at 10 and 30 µg ml⁻¹ CAS staining was indistinguishable from that on the wild type. Thus, as with the adenine requirement, the ability to make vicibactin is lost when bacteria are stressed for different nutrients, even if growth seems normal.

We conclude that there is no *direct* link between adenine auxotrophy and vicibactin synthesis; rather, the original observation with strain J225 was due to general effects of nutrient starvation on siderophore production.

To identify the gene(s) mutated in strain J225, plasmids constituting a gene bank of *R. leguminosarum* 8401pRL1JI

DNA cloned in the wide-host-range vector pLAFR1 (deLuca et al. 1998) were introduced en masse into J225 with pRK2013 as a helper plasmid (Figurski and Helinski 1979). Several transconjugants that were restored to normal growth on agarose-based minimal medium were obtained. These also made normal-sized halos on agar/CAS plates. Plasmids were isolated from three Ade⁺ transconjugants, transformed into *Escherichia coli* 803, and reintroduced into J225; the transconjugants were prototrophic and wild type on CAS plates.

EcoRI digestion of these plasmids (see Sambrook et al. 1989) showed that they shared several fragments. One plasmid, pBIO700, was studied further (see Table 1 for this and other plasmids used here). Various *EcoRI* restriction fragments were cloned from pBIO700 into the wide-host-range vector pRK415 and the resulting plasmids mobilized into mutant J225. One fragment, 4.1 kb in size, in a plasmid named pBIO704, corrected the Ade⁻ and CAS⁻ phenotypes. This same fragment was also present in the three cosmids that corrected J225. This fragment was cloned into pUC18 to form plasmid pBIO705, which was used to probe *EcoRI*-digested genomic DNA (Downie et al. 1983) from J225 and from the wild-type parent. A single 4.1-kb fragment from the wild type hybridized, but in J225 the band was larger, by 5.6 kb, corresponding in size to Tn5. Thus, the adenine requirement was caused by a Tn5 insertion in this *EcoRI* fragment.

DNA sequencing was done on both DNA strands of the 4.1-kb *EcoRI* fragment by the di-deoxy chain termination method with an A. L. F. sequencer (Pharmacia, Uppsala, Sweden). In some cases, sequencing was done commercially by M. W. G. (Eberberg, Germany). This revealed five open reading frames, two of whose deduced products were relevant to the phenotype of J225. (The other three open reading frames were transcribed divergently from *purMN*. They had homologues in other bacteria but these appeared to have no relation to purine biosynthesis and were not studied further; see, however, comments on them in EMBL accession number AJ243305). These were homologues of *purM* and *purN* that, respectively,

Table 1. Strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	Source
Bacteria		
<i>Escherichia coli</i>		
7803	Used for transformation	Wood 1966
<i>Rhizobium leguminosarum</i>		
8401pRL1JI	Wild-type <i>R. leguminosarum</i> bv. <i>viciae</i> , str ^r	Downie et al. 1983
J225	8401pRL1JI; <i>adeI</i> ::Tn5	This work
J250	8401pRL1JI <i>purM1</i> ::Tnlac	This work
J251	8401pRL1JI <i>purN1</i> ::Tnlac	This work
56	<i>cys1 ade8</i>	This work
159	<i>ade17 leu8</i>	This work
192	<i>ade21 str</i>	This work
1027	<i>phe1 ade27 rif</i>	Beringer et al. 1978
1060	<i>phe1 ade28 rif</i>	Beringer et al. 1978
1075	<i>liv1 ade29 rif</i>	This work
1076	<i>liv1 ade30 str</i>	This work
Plasmids		
pPH1JI	P1-group plasmid, used for marker exchange; gent ^r	Downie et al. 1983
pRK415	Wide host-range cloning vector; tet ^r	Keen et al. 1988
pRK2013	Helper plasmid for conjugation	Figurski and Helinski 1979
pBIO700	pLAFR1-based cosmid containing ca. 25 kb <i>R. leguminosarum</i> DNA, including <i>purMN</i>	This work
pBIO704	4.1-kb <i>EcoRI</i> fragment from pBIO700, cloned in pRK415; contains <i>purMN</i>	This work
pBIO705	4.1-kb <i>EcoRI</i> fragment, containing <i>purMN</i> , cloned in pUC18; used for sequencing	This work
pBIO706	Derivative of pBIO700 containing <i>purM1</i> ::Tnlac	This work
pBIO707	Derivative of pBIO700 containing <i>purN1</i> ::Tnlac	This work

catalyze 5'-phosphoribosylaminoimidazole synthetase and 5-phosphoribosylglycinamide transformalase N (Smith and Daum 1986, 1987). In *E. coli*, *purM* and *N* are also in a two-gene operon (Zalkin and Nygaard 1996). Djordjevic et al. (1996) conducted limited sequencing around the site of a transposon insertion in an Ade⁻ auxotroph in *Rhizobium* sp. strain NGR234 and found homology to *purM* and *purN*, but the entire sequence of those genes was not determined nor is it available in data bases.

Both reactions specified by *purM* and *N* precede the formation of AICAR, consistent with growth of J225 being restored by this compound. The similarity of *R. leguminosarum* PurM and PurN was greatest to those of *E. coli* (51.3 and 35.8% identity, respectively). In *R. leguminosarum*, *purM* and *N* are separated by 124 bp; the intervening DNA had no secondary structure nor obvious promoters, suggesting that, as in other bacteria, they are in the same operon (see below). Preceding the *E. coli* genes that are regulated by *purR* is a conserved motif (Zalkin and Nygaard 1996). No such sequence was seen upstream of *R. leguminosarum purM*.

Beringer et al. (1978) found that *ade* alleles of *R. leguminosarum* were located in three unlinked parts of the chromosome. Plasmid pBIO700 was mobilized into strains containing alleles at two of these loci; these were strains 1027 (*ade27*) and 1060 (*ade28*). Neither mutant was restored to adenine prototrophy. Unfortunately, the strain containing the *ade71* allele, which is in the third *ade* locus on the *R. leguminosarum* chromosome, was no longer available. Therefore, it was not possible to know if *ade71* is mutated in *purMN* or if *purMN* are at another, unidentified locus, not identified.

To study the expression of the *purMN* genes identified here, plasmid pBIO700 was mutagenized with Tnlac (Simon et al. 1989) as described by Downie et al. (1983). Mutant derivatives were mobilized into J225 to identify any that no longer corrected its Ade⁻ phenotype. Two such plasmids (pBIO706 and pBIO707) were obtained. Restriction mapping showed that, in both, Tnlac was in the 4.1-kb *EcoRI* fragment identified above. The junction of Tnlac in pBIO706 and pBIO707 was done by M. W. G.; an oligonucleotide corresponding to the 5' end of *lacZ* was used as a primer in cycle sequencing reactions in which the templates were the Tnlac mutant derivatives of pBIO700. By sequencing the junctions of Tnlac with *Rhizobium* spp. DNA, in pBIO706, *lacZ* was located in *purM*, with *lacZ* and *purM* in the same orientation. In pBIO707, Tnlac was in *purN*, but with *lacZ* in the "wrong" orientation. These two mutations were each introduced by marker exchange (see Downie et al. 1983) into the genome of *R. leguminosarum* bv. *viciae* 8401pRL1JI to form strains J250 (*purM1*) and J251 (*purN1*). As expected, both were Ade⁻ and had small halos on agar/CAS plates.

Plasmids pBIO706 and pBIO707 were each transferred into strains J250 and J251. None of the transconjugants was corrected for their adenine requirement, confirming by this complementation test that *purM* and *N* are in the same transcriptional unit.

In *E. coli*, transcription of *pur* genes is repressed by purine in the growth medium, this being mediated by the repressor *purR* (Zalkin and Nygaard 1996). We examined expression of the chromosomal *purM1::lac* fusion and, as control, we measured expression of the *purN1::lac* fusion in which *lac* was in the opposite orientation. Since we wished to determine the

effects of exogenous purine on *purMN* expression, the purine auxotrophy of J250 and J251 was corrected by introducing wild-type plasmid pBIO700. Derivatives of strains J250 and J251, each with pBIO700, were grown in Y minimal medium with or without adenine or AICAR, each at 30 µg ml⁻¹. For J250/pBIO700, β-galactosidase activities were reduced, approximately twofold, by the purines in the medium (Table 2). However, even in their presence, there was significant expression of the *purM::lacZ* fusion, unlike in *E. coli*, where *purR*-mediated repression in the presence of purines in the medium reduces expression of *pur* structural genes to very low levels. As expected, β-galactosidase activity with the wrongly oriented *purN1::lac* fusion was at background levels.

The mutants J250 and J251 were used to inoculate peas and the effects on nodulation were examined as described by Downie et al. (1985). Derivatives of these two mutants carrying the cloned *purMN* genes in pBIO704 were also used as inoculants. As found for purine auxotrophs in several rhizobial strains, including *R. leguminosarum* bv. *viciae*, the *purM* and *purN* mutants induced low numbers (approximately 10 to 15 per plant) of small, white nodules that failed to fix N₂. Although the mutants induced root hair curling, no infection threads were seen. As expected, transconjugants with pBIO704, which were corrected for their adenine requirement, nodulated and fixed N₂ normally.

Inoculated seedlings were also grown on agar containing adenine or AICAR (both at 30 µg ml⁻¹). As has been found by others, the numbers of nodules increased and they were slightly larger than the unsupplemented media, but the nodules still did not fix N₂ at detectable levels.

This study confirms that purine auxotrophs of *Rhizobium* spp. are defective in symbiotic N₂ fixation. We showed that *purM* and *purN*, which specify early steps in purine biosynthesis, are needed for nodulation of peas by *R. leguminosarum* bv. *viciae*. Unusual, for genes involved in a biosynthetic pathway, is that *purMN* of *R. leguminosarum* are expressed at relatively high levels even when the metabolic product of the pathway is present. However, expression of *purMN* was significantly greater in bacteria grown in the absence of purine than in its presence. This suggests that these genes are under some regulation; it remains to be seen if the *purMN* of *R. leguminosarum* are controlled by a *purR*-like gene, as in *E. coli*.

We identified the *purMN* mutants initially on the basis of their defect in siderophore synthesis. We now believe that this "pleiotropic" effect is essentially trivial since it was mimicked by reducing the levels of nutrient supply to other completely different types of auxotrophs (Liv⁻, Phe⁻, or Cys⁻). Thus, vicibactin production by *Rhizobium* spp. is very sensitive to nu-

Table 2. Effect of adenine and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) on β-galactosidase activities with the *pur::lac* fusions^a

Strain	Purine added		
	None	Adenine	AICAR
8401pRL1JI/pBIO700	61 ± 17	42 ± 18	50 ± 17
J250/pBIO700 (<i>purM</i> fusion)	1,560 ± 82	744 ± 47	677 ± 61
J251/pBIO700 (<i>purN</i> fusion)	46 ± 12	41 ± 8	56 ± 10

^a Cells were grown to late log phase in Y media plus the supplements shown and were assayed, in triplicate for β-galactosidase, assayed as described by Rossen et al. (1985).

tritional stress. Therefore, care should be taken before ascribing loss of siderophore production in undefined mutants directly to a defect in the siderophore biosynthetic pathway. It remains to be seen if other mutations that reduce fitness of *Rhizobium* spp. also affect vicibactin production. For example, Yeoman et al. (1997) found that *cyc* (*ccm*) mutants that are defective in *c*-type cytochrome maturation are also affected in vicibactin production. At least in the case of *R. leguminosarum*, however, *ccm* mutants grow at normal rates in media in which vicibactin production is virtually absent (K. H. Yeoman, unpublished observations).

Other mutants, identified only on the basis of their CAS phenotype, are Fix⁻ on legumes (e.g., see Barton et al. 1996). Again, it remains to be seen if it is the siderophore defect per se that accounts for the symbiotic defect of these uncharacterized mutants.

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