The *Rhizobium etli metZ* Gene Is Essential for Methionine Biosynthesis and Nodulation of *Phaseolus vulgaris*

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A mutant strain (CTNUX23) of *Rhizobium etli* carrying Tn5 unable to grow with sulfate as the sole sulfur source was isolated and characterized. Sequence analysis showed that Tn5 is inserted into a *metZ* (*O*-succinylhomoserine sulphydrylase)-homologous gene. The CTNUX23 mutant strain had a growth dependency for methionine, although cystathionine or homocysteine, but not homoserine or *O*-succinylhomoserine, allowed growth of the mutant. RNase protection assays showed that the *metZ*-like gene had a basal level of expression in methionine- or cysteine-grown cells, which was induced when sulfate or thiosulfate was used. The *metZ* gene was cloned from the parent wild-type strain, CE3, and the resulting plasmid pAR204 relieved, after transformation, the methionine auxotrophy of both strains CTNUX23 of *R. etli* and PAO503(*metZ*) of *Pseudomonas aeruginosa*. Unlike strain CE3 or CTNUX23 (pAR204), strain CTNUX23 showed undetectable levels of *O*-succinylhomoserine sulphydrylase activity. Strain CTNUX23 was unable to produce flavonoid-inducible lipo-chitin oligosaccharides (Nod factors) or to induce nodules or nodulelike structures on the roots of *Phaseolus vulgaris*, unless methionine was added to the growth medium. These data and our previous results support the notion that cysteine or glutathione, but not methionine, is supplied by the root cells to bacteria growing inside the plant.

Additional keywords: nodulation factors.

Gram-negative soil bacteria of the genus *Rhizobium* have the unique ability to form N₂-fixing nodules on the roots of leguminous plants. Nodule formation has been divided into three major stages: preinfection, infection with the concomitant nodule formation, and nodule function. From the point of view of the bacterial partner, this multistage process involves

bacterial multiplication in the rhizosphere, the recognition and infection of the root hairs, bacterial growth inside a network of infection threads, and the release of bacteria from the threads into the cytoplasm of host cells. Within the infected plant cells the bacteria differentiate into nondividing bacteroids that are equivalent to plant cell organelles able to reduce atmospheric dinitrogen (for reviews, see Panagiota et al. 1995; Van Rhijn and Vanderleyden 1995). It has been established that bacteroids depend on their host plant for carbon and energy, but it is largely unclear what sources (such as carbon, sulfur, or nitrogen) the bacteria require or have available to them during growth in the rhizosphere, inside the infection threads, and during differentiation. However, by analyzing the symbiotic behavior of auxotrophic mutants, it has been demonstrated that various metabolic pathways of *Rhizobium* bacteria, although they may not pertain exclusively to symbiosis, are nevertheless important for an efficient symbiotic interaction (Pain 1979; Kerppola and Kahn 1988; Noel et al. 1988).

We are studying the sulfur metabolism of *Rhizobium etli*, with the aim of understanding which sulfur sources are used by this bacterium during its symbiotic interaction with *Phaseolus vulgaris* (common bean) (Taté et al. 1997). It was earlier observed that methionine auxotrophic mutants of *R. leguminosarum* formed effective nitrogen-fixing nodules (Nod⁺/Fix⁺) on the roots of pea (Pain 1979). Furthermore, it has been observed that mutants of *R. meliloti* (Kerppola and Kahn 1988) with defects in methionine biosynthesis formed nodules (Nod⁺) on the roots of *Medicago sativa*, but these nodules were ineffective, i.e., unable to fix nitrogen (Fix⁻). The different symbiotic behavior of the methionine-requiring strains of *Rhizobium* may be due to the step affected by the mutations or it may be due to strain-specific requirements. However, in all cases described, Met⁻ mutants were able to elicit nodule formation, thus indicating that they were able to produce and release active nodulation factors, the Nod factors. It has been shown that purified Nod factors are able to provoke multiple host reactions including deformation of roots hairs, division of root cortical cells, and formation of nodulelike structures (for reviews, see Carlson et al. 1994; Spaink and Lugtenberg 1994; Long 1996).

We report here on the isolation of a mutant strain of *R. etli*, called CTNUX23, that fails to grow with sulfate as the sole sulfur source. Strain CTNUX23 contains a single Tn5 insertion in a gene showing a high degree of identity with the *metZ* gene (*O*-succinylhomoserine sulphydrylase). The MetZ protein

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catalyzes the second step of the biosynthesis of methionine in *Pseudomonas aeruginosa* (Fogolino et al. 1995). When tested on the roots of *P. vulgaris*, strain CTNUX23 was able to induce root hair deformation and curling, but later neither nodule primordium nor infection threads were observed (Nod⁻ phenotype). Furthermore, in the presence of methionine (its nutritional requirement), strain CTNUX23 induced the formation of nodules, but these nodules contained only a few infected cells and were functionally ineffective (Fix⁻). Based on these data, and our previous evidence, the pathway of sulfur organization of *R. etli* and the nature of the sulfur source used by this bacterium during symbiotic interaction will be discussed.

RESULTS

Isolation of Met⁻ strains of *R. etli*.

The wild-type strain of *R. etli*, CE3, grows on a chemically defined medium containing only inorganic sulfur sources (e.g., sulfate, sulfite, or thiosulfate), thus indicating that it is capable of synthesizing sulfur amino acids such as methionine and cysteine. To isolate Met⁻ strains, the suicide plasmid pSUP1011 (Simon et al. 1983) was used to deliver Tn5 to *R. etli* CE3. A control experiment was performed with pSUP101 carrying the genetic markers of pSUP1011, except for Tn5 (Simon et al. 1983). Plasmid DNA was introduced into *R. etli* by electroporation, and kanamycin-resistant bacteria were obtained at high frequency only with pSUP1011.

Mutants showing methionine-dependent growth were identified by being screened simultaneously on plates of sulfate-containing minimal medium (RMM) with or without supplemental methionine (25 μ M). Two strains, CTNUX22 and CTNUX23, able to grow only in the presence of methionine, were recovered from 1,500 Tn5 recipients. Both putative Met⁻ strains were isolated and confirmed by their failure to grow on sulfate-containing RMM medium.

Cloning of the Tn5 flanking region from the CTNUX23 mutant.

Probing of the total genomic DNA of strains CTNUX22 and CTNUX23 with a labeled Tn5 DNA fragment established that, in both cases, the insertion was located on a single 10-kb *Eco*RI fragment. Moreover, when the genomic DNAs were digested with *Bam*HI, two fragments with sizes of 5.5 and 9 kb hybridized (data not shown). Therefore, each strain carried only one Tn5, located in a very closely linked genomic site.

Since both strains showed identical growth phenotypes, only one of them (strain CTNUX23) was chosen for further characterization.

By selecting kanamycin-resistant transformants of *Escherichia coli* DH5 α , the plasmid pAR193, carrying a copy of Tn5 plus the adjacent genomic DNA, was isolated from strain CTNUX23 (Fig. 1A). Appropriate restriction fragments from clone pAR193 were subcloned into pGEM7Zf⁺ (plasmid pAR194 and pAR195) (see Figure 1). The DNA sequence of the region flanking Tn5, including that of the insertion point, was established with DNA of clone pAR194 as template and the Tn5out oligonucleotide as primer. Sequence analysis revealed a putative open reading frame (amino acids [aa] 113 to 235 of the deduced peptide sequence shown in Figure 2A) highly similar to those of numerous prokaryotic and eukaryotic pyridoxal 5'-phosphate (PLP)-dependent enzymes. These proteins belong to a family that includes cystathionine γ -lyase, cystathionine γ -synthase, and *O*-acetylhomoserine sulfhydrylase, which are enzymes involved in the biosynthesis of the sulfur amino acids. Among the PLP-dependent enzymes, the strongest similarity was obtained with the deduced protein sequence of *O*-succinylhomoserine sulfhydrylase, the *metZ* gene product of *P. aeruginosa* (Fogolino et al. 1995). Since a mutation of the *metZ* gene of *P. aeruginosa* leads to methionine auxotrophy, it is likely that the mutant strain of *R. etli*, CTNUX23, contains a *metZ*::Tn5 insertion.

Cloning, sequence, and sequence analysis of the *R. etli metZ*-like gene.

Southern hybridization of various restriction digests of DNA obtained from the wild-type strain of *R. etli*, CE3, probed against a DNA fragment (420-bp *Sal*I/*Pst*I fragment) from pAR198 (Fig. 1B), yielded at least one strongly hybridizing band for each digest (data not shown). Of the hybridizing bands, one (an approximately 2.8-kb *Eco*RI/*Bam*HI fragment) was chosen for the construction of an enrichment library (see Materials and Methods). The vector used was pMP220, a low copy number plasmid that specifies tetracycline resistance (Spaink et al. 1987). Strain CTNUX23 was transformed by electroporation with the ligation mixture and the resulting bacteria were plated on RMM agar containing sulfate as the sole sulfur source. Two colonies were isolated and retested extensively. From one of them plasmid DNA (pAR204) was isolated (see Figure 1) and used to transform strain CTNUX23 again. As a control, strain CTNUX23 was

Fig. 1. A, Strategy to clone the Tn5 DNA flanking region from strain CTNUX23 of *Rhizobium etli*. A partial restriction map of the Tn5 DNA is shown. The Tn5-outward reading primer is indicated by arrowhead. **B,** Physical map of the *R. etli* DNA cloned into pAR204 with partial restriction map. Boxed area represents translated sequence of *metZ* and *apaG* genes. Position of Tn5 insertion in mutant strain CTNUX23 of *R. etli* is indicated. Restriction site positions are indicated as follows: B, *Bam*HI; E, *Eco*RI; Hp, *Hpa*I; P, *Pst*I; S, *Sac*I.

transformed with plasmid pMP220 (vector alone). Since strain CTNEX23(pAR204) could grow with sulfate as the sole sulfur source, whereas strain CTNEX23(pMP220) could not, it was possible to conclude that the DNA fragment carried by pAR204 contains the information necessary to complement the Tn5 mutation. Moreover, since the vector used, plasmid pMP220, is a promoter-probing vector (Spaink et al. 1987), it is likely that the *metZ* gene carried by pAR204 is transcribed from its own promoter.

By Southern hybridization analysis of various restriction digests of pAR204, overlapping DNA fragments were identified, cloned, and sequenced (Fig. 2). The sequence was confirmed with synthetic oligonucleotides. Sequence analysis re-

vealed two major open reading frames (ORFs). The first ORF is of 1,182 bp, with an ATG start codon at position 221 of the nucleotide sequence and a TGA stop codon at position 1403 (Fig. 2). The deduced protein, with a size of 394 aa, shares 50.3% identical residues (59.5% similar over 394-aa overlap) with the product of the *Mycobacterium tuberculosis metZ* gene (GenBank accession no. Z84725), and 43.9% identical residues (53.4% similar over 394-aa overlap) with the product of the *P. aeruginosa MetZ* gene (Swiss-Prot accession no. P55218). Thus, a *metZ*-homologous gene appears to be present in *R. etli*. The length of the *R. etli metZ* gene (1,185 bp, 394 aa) is similar to that of *metZ* of *P. aeruginosa* (1,212 bp, 403 aa) and the coding region was preceded by a putative ri-

Fig. 2. DNA sequence of *metZ* and *apaG* and the nucleotide 5' flanking region. Translated sequences are shown in single-letter code, based on their presumed start codon. Stop codon is indicated by stop, exact position of Tn5 insertion in strain CTNEX23 and restriction enzyme recognition sites corresponding to those indicated in Figure 1 are indicated. A putative ribosome-binding site (*rbs*) and a putative Rho-independent transcriptional terminator (---> <---) are indicated. The amino acid sequence that fits with the consensus sequence for the binding of PLP (pyridoxal 5'-phosphate) cofactor (Prosite accession no. PD0C00677) is underlined.

bosomal binding site (Fig. 2). One of the best conserved regions with respect to other reported trans-sulfuration enzymes was that corresponding to the consensus pattern for the binding of PLP, which is used as a cofactor by these enzymes (Prosite: accession no. PD0C00677). In one of the groups of PLP-dependent enzymes (including the cystathionine γ -synthase, cystathionine γ -lyase, homocysteine synthase, etc.), nine significant blocks of similarities were previously identified (Cherest et al. 1993). All these blocks were also conserved in the predicted sequence of *R. etli* MetZ-like protein. Thus, the sequence analysis suggests that the gene interrupted by Tn5 in strain CTNUX23 encodes an *O*-succinylhomoserine sulphydrylase.

The second ORF, located in the 3'-flanking region of *metZ* gene (Fig. 2), is of 405 bp with an ATG start codon at position 1617 of the nucleotide sequence and a TGA stop codon at position 2019 (Fig. 2). The predicted protein, with a size of 134 aa, shares 45% identical residues (66% similar over 116-aa overlap) with the product of *Salmonella typhimurium* *apaG/corD* gene (Swiss-Prot accession no. Q56017) and 44% identical residues (65% similar over 116-aa overlap) with the product of *E. coli* *apaG* gene (Swiss-Prot accession no. P05636). It has been previously noted (Swiss-Prot report no. Q56017), that an *apaG* mutant strain of *S. typhimurium* shows a low level of Co^{2+} resistance, and a decreased efflux of Mg^{2+} . As in enteric bacteria, the function of the *apaG*-homologous gene in *R. etli* is currently unknown, but worthy of investigation.

The sulfur sources used by strain CTNUX23.

To test the ability of *R. etli* strains to grow on various sulfur sources, a modified chemically defined RMM medium was used (see Materials and Methods). It was observed that strain CTNUX23 could not grow on sulfate-containing medium,

could grow well in a methionine-containing medium, and could grow but at very low rates with cysteine or glutathione. Therefore, in strain CTNUX23 the pathway of methionine biosynthesis (with sulfate as sulfur source) is not functional and the conversion of cysteine into methionine by a pathway independent of the *metZ* gene product is very inefficient.

The mutant strain, CTNUX23, was further characterized by testing its growth response on RMM liquid medium containing sulfate, but supplemented with the compounds that are known to be intermediates of methionine biosynthesis in various organisms (Fig. 3A). Under all conditions tested, the values measured with the wild-type strain, CE3, were almost the same as those showed for strain CTNUX23-pAR204. Like its parent strain, CE3 and CTNUX23 could grow in the medium containing 25 μM cystathionine, homocysteine, or methionine. On the other hand, unlike strain CE3, the mutant CTNUX23 could not grow in the same medium supplemented with homoserine or *O*-succinylhomoserine. This nutritional requirement corresponds to that of a *metZ* mutant of *P. aeruginosa* (strain PAO503) even though the latter could grow with homocysteine but not with cystathionine (0.5 mM) as the sole sulfur source (Fogolino et al. 1995). However, it has been speculated that the uptake (permeation) of cystathionine may be very inefficient in *P. aeruginosa* (Fogolino et al. 1995).

Strain CTNUX23 showed no *O*-succinylhomoserine sulphydrylase activity.

To obtain more information about the function of *R. etli* MetZ, measurements of *O*-succinylhomoserine sulphydrylase (OSHS) activity were thus performed in different strains of *R. etli* (Fig. 3B). Bacteria grown in TYR rich medium were harvested by low speed (2,500 $\times g$) centrifugation, washed, and resuspended in sulfate-containing minimal medium. Cells

Fig. 3. A, Effects of methionine biosynthetic intermediates on growth of *Rhizobium etli*. Growth was determined by measuring absorbance changes in optical density at 590 nm (OD₅₉₀). Strains CTNUX8 (*cysG*) and CTNUX23 (*metZ*) were grown overnight in rich medium (TYR). Bacterial inocula were prepared by washing the cells twice with sulfate-free RMM medium and adjusting the OD₅₉₀ to 0.05. Bacteria were grown at 30°C with shaking (250 rpm, 6-76 rotary shaker, New Brunswick, New Brunswick, NJ) in sulfate-containing RMM medium without any addition (none) or supplemented with cysteine (Cys), glutathione (Glut), homoserine (Hom), *O*-succinylhomoserine (Osh), cystathionine (Cyst), homocysteine (Hcys), or methionine (Met) at the concentration of 25 μM . Growth was monitored at OD₅₉₀ 40 h after the inoculation. **B,** Values represent units of *O*-succinylhomoserine sulphydrylase activity (homocysteine produced) measured with strain CE3 (wild-type) and CTNUX23 (*metZ*) of *R. etli*.

were maintained for 3 h with shaking at 30°C, harvested by high speed (8,000 × g) centrifugation, and disrupted by sonic oscillation. With the resulting crude extracts used as a source of OSHS, the initial rate of sulfide-dependent homocysteine production was measured spectrophotometrically as previously described (Fogolino et al. 1995). The rates were corrected for production of homocysteine in the absence of added sulfide. Considering the OSHS activity measured with the wild-type strain CE3 (97.5 nmol min⁻¹ mg⁻¹) as 100%, the activity of strain CTNUX23(pAR204) was about 90% (88.2 nmol min⁻¹ mg⁻¹), while the activity of the CTNUX23 mutant was not detectable. Therefore, in *R. etli* the *metZ* gene product is essential for the synthesis of homocysteine with sulfide as sulfur donor.

The *metZ* genes of *R. etli* and *P. aeruginosa* are functionally equivalent.

To confirm that the cloned sequence encodes a protein functionally homologous to the MetZ protein, its ability to suppress the methionine auxotrophy caused in *P. aeruginosa* by a *metZ* mutation was tested. For this purpose strain PAO503(*met-9011*) of *P. aeruginosa* (Fogolino et al. 1995) was transformed by electroporation with either plasmid pAR204 (carrying the *metZ* gene of *R. etli*) or pMP220 (the vector alone). The resulting strains were tested for their ability to grow on sulfate-containing M9 minimal plates at 37°C. Colonies of strain PAO503(pAR204) were evident 24 h after plating, while colonies of strain PAO503(pMP220) were never observed. On the other hand, both strains PAO503(pMP220) and PAO503(pAR204) could grow when plated on TY agar or on M9 agar supplemented with methionine. Therefore, it is possible to conclude that the *metZ* genes of *R. etli* and *P. aeruginosa* are functionally equivalent.

The level of *metZ* mRNA depends on the sulfur source.

RNAse protection analysis (Fig. 4) was carried out to determine the level of the *metZ* mRNA when *R. etli* was grown in various conditions. RNA purified from the wild-type CE3 strain grown on sulfate, thiosulfate, cysteine, or methionine as the sole sulfur source was hybridized to an excess of ³²P-labeled antisense RNA (riboprobe) generated from clone pAR198 (see Figure 1B) and digested with RNase A and T₁. Quantitative analysis of the protected bands indicated that the intracellular level of *metZ* mRNA was higher (at least threefold) when strain CE3 was grown on sulfate, relative to cysteine or methionine. The level of *metZ* mRNA was even higher (at least fivefold) in bacteria grown with thiosulfate. Hybridizing the same preparations of RNA against total genomic DNA of *R. etli* yielded comparable intensities for all RNA samples (Fig. 4, bottom), indicating that the observed differences in the *metZ* transcript levels were not due to variations in the amount of RNA used. These data show that the transcription of *metZ* gene, or the mRNA stability, is regulated by the sulfur source.

The CTNUX23 mutant has a Nod⁻ phenotype.

To test the capacity of strain CTNUX23 (Met⁻) of *R. etli* to nodulate *P. vulgaris*, roots of germinated seeds were inoculated with strain CTNUX23 or with the wild-type strain, CE3, used as a control. Three weeks after inoculation, nodules were observed on roots of plants inoculated with strain CE3. In contrast, uninoculated controls and plants inoculated with

strain CTNUX23 showed no nodules. Therefore, on the common bean, strain CTNUX23 of *R. etli* has a Nod⁻ phenotype, i.e., unable to induce nodules.

To determine the step(s) at which the symbiotic interaction was arrested, the first stages of nodule induction were studied (Fig. 5). The process of root hair deformation was evident 1 to 2 days post inoculation (dpi) on the primary roots inoculated with the mutant strain, CTNUX23 (Fig. 5A). The same root-hair reactions were observed on the root inoculated with the strain CE3, used as a control (data not shown). The mutant was also able to provoke (2 to 3 dpi) root hair curling (Fig. 5B, C). Later on (3 to 4 dpi), the induction of mitotic activity of cell from the root outer cortex and the formation of infection threads were observed only with the complemented mutant, strain CTNUX23(pAR204) (Fig. 5D) or with strain CE3 (Fig. 5E). In contrast, in the case of strain CTNUX23, the root cells closely related to the curled root hairs showed neither mitotic activity nor infection threads. In some cases a very weak mitotic activity was observed near the curled root (Fig. 5C). Furthermore, the number of nodules invaded by strain CTNUX23 transformed with plasmid pAR204, but not with the vector pMP220 alone, was nearly the same as the number of nodules invaded by the wild-type strain. These experiments showed that inactivation of *metZ* gene led to a decreased ability of the mutant CTNUX23 to induce nodule primordium on the roots of *P. vulgaris*, and that the DNA fragment carried by pAR204 was able to rescue the loss of nodulation ability of strain CTNUX23.

Since purified Nod factors are able to induce mitotic activity of root cells, whereas the bacterial division should be the force to drive the growth of infection threads (for a review,

Fig. 4. Effects of various sulfur sources on transcription of the *metZ* gene. The riboprobe was hybridized to RNA (30 µg) extracted from the wild-type strain, CE3, of *Rhizobium etli* grown on chemically defined medium (RMM) containing sulfate (lane 1), thiosulfate (lane 2), cysteine (lane 3), or methionine (lane 4) as the sole sulfur source. The riboprobe alone (lane P) and the riboprobe annealed in the absence of RNA (lane Pt, showing no protected fragment) are also shown. The molecular weight markers (M) are indicated on left. A slot blot experiment was performed with aliquots of the same RNA preparation and the labeled *R. etli* genomic DNA as a probe.

see Panagiota et al. 1995), it is reasonable to speculate that the symbiotic phenotype of the *metZ* mutant strain of *R. etli* is due to its incapacity to release enough (or active) Nod factors and/or its inability to grow inside the plant.

Production of lipo-chitin oligosaccharides (Nod factors) by strain CTNUX23 of *R. etli*.

The growth medium of the *metZ* mutant strain CTNUX23 of *R. etli* was investigated for the presence of flavonoid-

inducible lipo-chitin oligosaccharides by radiolabeling the cells with D-[1-¹⁴C]-glucosamine (Fig. 6). The radiolabeled compounds were extracted and analyzed by thin-layer chromatography (TLC), essentially as previously described (Spaink et al. 1992). In the presence of the inducer, naringenin, the wild-type strain, CE3, used as a control, produced two main radiolabeled spots (Fig. 6, lane 2). As previously observed (Cardenas et al. 1995), metabolites that show similar chromatographic behavior were also detected, although at

Fig. 5. Root hair phenotypes of *Phaseolus vulgaris* induced after inoculation with *Rhizobium etli* (A–C) CTNUX23, (D) CTNUX23-pAR204, or (E) CE3. Germinated seedlings were inoculated and, at different times after inoculation, the whole root system was detached, fixed, cleared, and stained as described under Materials and Methods. Mitotic activity (star) of the root cortex cells located in the vicinity of the infection threads (arrowhead) is indicated. Bars indicate a length of 100 μ m.

lower levels, in the uninduced culture (Fig. 6, lane 1). Moreover, the addition of methionine (25 μ M) to the growth medium of strain CE3 showed no effect on the production of flavonoid-inducible compounds. In contrast, strain CTNUX23 was unable to produce radiolabeled compound either in the presence or in the absence of naringenin (Fig. 6, lanes 5 and 6), unless methionine (its nutritional requirement) was added to the growth medium (Fig. 6, lanes 7 and 8).

Supplementation experiments.

To test the effect of the addition of methionine on the nodulation ability of the *metZ* mutant strain, CTNUX23, nodulation experiments were performed with a sulfate-containing plant food minimal medium (Jensen 1942), but supplemented with methionine (25 μ M) (the nutritional requirement of the auxotrophic mutant). The methionine-containing medium was added to the inoculated plants only for 7 days. This period of time was that required to visualize (with the naked eye) primordium formation on the roots of germinated seeds inoculated with the wild-type strain, CE3. After that, the plants were maintained on sulfate-containing Jensen medium. Under these conditions, the mutant strain CTNUX23 was able to induce the formation of nodules. However, at 15 dpi, these nodules were white or slightly pink, and showed a very low (almost undetectable) acetylene reduction activity (Fix⁻). Later on (21 dpi), some nodules showed a very low acetylene reduction activity (up to 2%), compared with effective nodules (Fix⁺) elicited by strain CE3 under the same conditions. The histological organization of 21-day-old nodules induced, in the presence of methionine, was observed (Fig. 7). Nodules elicited by either strain (CE3 and CTNUX23) appeared organized as differentiated structures with a distinct endodermis that separated the outer cortex

Fig. 6. Thin-layer chromatographic analysis of radiolabeled compounds produced by *Rhizobium etli*. Bacteria were grown overnight at 30°C (from an initial OD₅₉₀ value of 0.05) in RMM medium containing D-[1-¹⁴C]-glucosamine (1 μ Ci, 50 mCi mmol⁻¹). Nitrate and mannitol were used as the nitrogen and carbon source, respectively. The culture medium (1 ml) was extracted with water-saturated *n*-butanol (0.5 ml). The *n*-butanol was removed by evaporation and the dry pellet was resuspended in 20 μ l of *n*-butanol. Samples (3 μ l) were loaded on a reverse-phase C₁₈-coated silica ODS (100% octadecyl silanization) plate (Sigma, Milan, Italy) with acetonitrile/water (1:1, vol/vol) as a solvent. The plate was dried and the radiolabeled compounds were visualized with a PhosphorImager gel scanner equipped with Image Quant software (both from Molecular Dynamics, Sunnyvale, CA), after 24 h of exposure. Sample areas are as follows: lanes 1–4, strain CE3(wild-type); lanes 5–8, strain CTNUX23(*metZ*). Bacteria were incubated in the presence of 2 μ M naringenin (lanes 2, 4, 6, and 8) or in the absence of the inducer (lanes 1, 3, 5, and 7). Cultures were also supplemented with 25 μ M methionine (lanes 3, 4, 7, and 8).

from the main body of the nodule and with the vascular bundles distributed at their normal position, i.e., at the periphery of the central tissue. However, in contrast to effective nodules formed by strain CE3 (Fig. 7A), the nodules elicited by strain CTNUX23 (Fig. 7B) showed a very low number of invaded cells (or a lower ratio of invaded to uninvaded cells). Thus, nodules induced by the *metZ* mutant in the presence of methionine were not tumorlike structures and their histological organization appeared different from that of pseudonodules, elicited on the roots of *P. vulgaris* by purine auxotrophs of *R. etli* (VandenBosch et al. 1985; Noel et al. 1988).

To test for the presence of Met⁻ bacteria in nodules formed by strain CTNUX23, bacteria were reisolated and tested for the auxotrophic marker. The nodules were harvested, surface sterilized by sodium hypochlorite treatment, and crushed one at a time. Cells were then diluted and plated on TYR medium with addition of either nalidixic acid or nalidixic acid plus kanamycin to determine the number of CFU present. The same numbers of colonies were observed in both types of plates. Moreover, aliquots of cells were also streaked on plates of defined sulfate-containing RMM medium (with or without supplemental methionine). After 3 days of incubation at 30°C, colonies were observed only on methionine-containing plates, thus indicating that nodules were formed by auxotrophic bacteria and not by prototrophic revertants. However, as judged by the number of CFU obtained, the nodules invaded by strain CTNUX23 released fewer bacteria than nodules invaded by the wild-type strain, CE3. Therefore, in the presence of methionine, its nutritional requirement, strain CTNUX23 had a nodulating phenotype (Nod⁺).

DISCUSSION

A Tn5 insertion mutant, strain CTNUX23 of *R. etli*, was isolated and characterized. The mutation in this strain was concluded to be due to a single Tn5::*metZ* insertion based on the following evidence: (i) when DNA prepared from this strain was digested with *EcoRI* (which does not cut Tn5), electrophoresed, and hybridized with a nick translated probe containing Tn5, it was found that only one 10-kb DNA fragment hybridized; (ii) sequence analysis of the Tn5 flanking region (and of the corresponding DNA region cloned from the wild-type strain) showed a significant degree of identity with the sequence of the *metZ* (*O*-succinylhomoserine sulfhydrylase enzyme) gene of bacteria; and (iii) a plasmid (pAR204) carrying the corresponding DNA region from the wild-type strain was shown to rescue prototrophic growth.

As shown in Figure 3, the mutant strain, CTNUX23, could not grow with sulfate as inorganic sulfur source, whereas it could grow in a medium containing methionine, thus indicating that, under the growth conditions tested, the biosynthesis of methionine of *R. etli* depends on *metZ*. It has also been observed that strain CTNUX23 could grow on minimal medium supplemented with cystathionine or homocysteine but not with homoserine or *O*-succinylhomoserine (Fig. 3A), suggesting that in *R. etli* the *metZ* gene product catalyzed the conversion of *O*-succinylhomoserine into cystathionine or homocysteine. In enteric bacteria cystathionine is synthesized from *O*-succinylhomoserine and cysteine (as sulfur donor) in a reaction catalyzed by the cystathionine γ -synthase (the *metB* gene product), while the cystathionine β -lyase (*metC* gene

product) converts cystathionine into homocysteine. However, when a sequence comparison was performed, it was suggested that in strain CTNUX23 the gene interrupted by Tn5 most probably encodes an *O*-succinylhomoserine sulfhydrylase (the *metZ* gene product of *P. aeruginosa*), rather than a cystathionine γ -synthase. As predicted, it was determined by measurements of enzymatic activity that, unlike strain CE3 or CTNUX23-pAR204, the mutant had undetectable levels of OSHS activity (Fig. 3B).

It has previously been shown that a *MET25* (*O*-acetylhomoserine sulfhydrylase activity) mutant strain of yeast could grow with cysteine as the sole sulfur source, due to the presence of a secondary trans-sulfuration pathway (cysteine to methionine) in this organism (Kerjan et al. 1986). The same pathway appears to be present in *P. aeruginosa* (Foglino et al. 1995). However, since cysteine was found to be inefficient (slow growth) as sulfur source, it has been suggested that the direct sulfhydrylation pathway (that is mediated by *metZ* and with sulfide as sulfur donor) is the major route for obtaining methionine in *P. aeruginosa* (see Figure 8). The *metZ* mutant here analyzed (strain CTNUX23) grows very poorly with cysteine (or glutathione), thus suggesting that, in *R. etli*, a putative reverse trans-sulfuration pathway (cysteine to methionine) has very low expression and/or is inactive under the conditions tested.

A *cysG* (siroheme synthetase) mutant strain of *R. etli*, strain CTNUX8, has previously been isolated and characterized (Taté et al. 1997). This mutant could not grow with sulfate as sole sulfur source due to its inability to activate the sulfite reductase complex, but could grow with cysteine, glutathione, or methionine (Taté et al. 1997). To obtain more information about sulfur metabolism in *R. etli*, strain CTNUX8 was tested for its ability to grow in the presence of methionine biosynthetic intermediates (Fig. 3A). Since strain CTNUX8 (*cysG*) was able to grow in a sulfate-containing medium when supplemented with homocysteine, cystathionine, or methionine (Fig. 3A), it is possible to conclude that *R. etli* is able to convert cystathionine, homocysteine, or methionine into cysteine.

To obtain methionine with sulfate as the sole sulfur source, enteric bacteria synthesize cysteine and then, with the cysteine produced as sulfur donor, synthesize methionine (Kredich 1986). Moreover, enteric bacteria cannot use methionine as the sole sulfur source, because there is no efficient pathway from methionine to cysteine. Therefore, it appears likely that in *R. etli* the biosynthesis of sulfur amino acids follows a global pathway resembling that present in *S. cerevisiae* (Cherest and Surdin-Kerjan 1992) and *P. aeruginosa* (Foglino et al. 1995), i.e., a direct sulfhydrylation pathway to synthesize methionine and a pathway to convert methionine into cysteine (Fig. 8).

The Nod factors (lipo-chitin oligosaccharides released by the bacterium) act as morphogenetics signals able to elicit host-specific plant responses such as the induction of cortical cell division (Spaink and Lugtenberg 1994; Long 1996). It has been established that purified Nod factors of *R. etli* can elicit the formation of nodule primordia that develop to the stage at which vascular bundles are formed (Cardenas et al. 1995). In contrast, it was observed that the CTNUX23 strain of *R. etli* induced root hair deformation and curling but failed to induce either nodules (Nod⁺), nodulelike structures, or nodule primordium on the root of *P. vulgaris* (Fig. 5). Since, under free-

living conditions, a *metZ*-dependent methionine biosynthesis is essential to allow the growth of *R. etli* with sulfate (or sulfite) as the sole sulfur source, we propose that in root exudates of germinated seedlings the methionine concentration is too low, if present, to allow the bacterial growth and/or the bacterial production of Nod factors. In fact, a TLC analysis showed that strain CTNUX23 was able to produce flavonoid-inducible compounds only when the growth medium was supplemented with methionine (Fig. 6).

To our knowledge, *metZ* is the first gene of *R. etli*, not apparently correlated with the nodulation (*nod*) genes, that is essential to induce the formation of a nodule primordium on the roots of *P. vulgaris*. Most of the *nod* gene products are enzymes involved in the synthesis of Nod factors and the lack of correlation between *metZ* and *nod* genes is strongly suggested by the following observations: (i) the *metZ* transcription is regulated by the sulfur source used (Fig. 4), whereas the *nod* genes are not expressed in free-living bacteria, with the exception of *nodD*, which is expressed constitutively; and (ii) the capacity of strain CTNUX23(*metZ*) to produce Nod factors (Fig. 6) and to induce nodule formation (Fig. 7) could be restored by the addition of methionine to the medium, thus indicating that a shortage of methionine, and not the absence of MetZ protein, is responsible for the defective nodulation phenotype.

Strain CTNUX23 of *R. etli* was able to induce in the presence of methionine (supplemented during the early steps of symbiotic interaction) only ineffective (Fix⁻) nodules (Fig. 7), indicating that an efficient pathway of methionine biosynthesis is also required at intermediate stages of the symbiotic interaction and that the root cells of *P. vulgaris* are unable to supply the bacterium with enough methionine. Moreover, since strain CTNUX8(Cys⁻) of *R. etli* (which is unable to assimilate sulfate) was able to induce the formation of effective nodule (Nod⁺/Fix⁺) on the roots of common beans, it has been previously hypothesized that an organic source of sulfur such as cysteine, methionine, or glutathione is available for bacterial growth inside the plant (Taté et al. 1997). The experiments presented here allowed us to exclude methionine as a putative sulfur source and, considering that strain CTNUX23 could grow very poorly with cysteine or glutathione as sulfur sources (Fig. 3A), it is reasonable to speculate that the low number of invaded cells observed in nodules elicited by CTNUX23 is simply a consequence of the uncoupling between the timing of nodule development and the rate of bacterial division inside the nodule.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

R. etli (formerly *R. leguminosarum* bv. *phaseoli*) strains were grown at 30°C in TYR medium containing (g liter⁻¹): tryptone (5), yeast extract (3), and CaCl₂·2H₂O (0.88). RMM was used as a chemically defined medium (Hooykaas et al. 1977). *E. coli* strains were grown at 37°C in TY medium containing (g liter⁻¹): tryptone (10), yeast extract (5), and NaCl (10). *P. aeruginosa* strains were grown as described previously (Foglino et al. 1995). M9 medium (in which MgSO₄ was replaced by MgCl₂) was used as sulfate-free medium (Sambrook et al. 1989). Antibiotics, unless otherwise indicated, were added to the medium as needed at

the following concentrations ($\mu\text{g ml}^{-1}$): tetracycline (5), nalidixic acid (20), kanamycin (50), rifampicin (100), and ampicillin (100). All media were solidified with 1.5% agar (Difco, Detroit, MI).

Tn5 mutagenesis and growth phenotype.

R. etli was grown at 30°C with shaking (250 rpm; 6–76 rotary shaker, New Brunswick, New Brunswick, NJ) in TYR medium, and competent cells for electroporation were prepared as previously described (Taté et al. 1997). DNA of plasmid pSUP1011 (Simon et al. 1983) was purified with a Qiagen column (Promega, Madison, WI). Electroporation was performed as previously described (Taté et al. 1997) with a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA). Electroporated cells were resuspended in 1 ml of a rich medium containing (g liter⁻¹): tryptone (20), yeast extract (3), KCl (0.19), CaCl₂ (2.1), MgSO₄ (2.5), and mannitol (10). The cells were incubated with shaking (as described above) for 4 h

at 30°C and then plated on TYR agar containing kanamycin (50 $\mu\text{g ml}^{-1}$). Colonies appearing after incubation for 3 days at 30°C were streaked on plates of rich (TYR) and minimal (RMM) media. Those with growth defects on minimal medium were purified at least twice on nonselective medium and retested extensively.

An RMM medium was prepared with the following modifications: MgSO₄ was replaced with an equimolar amount of MgCl₂, while traces of other sulfate salts (MnSO₄, ZnSO₄, and CuSO₄) were replaced with traces of alternative salts (MnCl₂, ZnCl₂, and CuCl₂). Ammonium chloride (10 mM) and glucose (1%) were used, respectively, as nitrogen and carbon sources. This basal medium was defined by pre-fixing a kind of sulfur compound added at a concentration, unless otherwise indicated, of 25 μM . The sulfur sources used (L-cysteine, L-methionine, sodium sulfate, sodium sulfite, sodium thiosulfate, and glutathione) were sterilized by filtration and added to the sterile basal medium.

Fig. 7. Sections of a 21-day-old determinate-type of nodules elicited by *Rhizobium etli* (A) CE3 or (B) CTNUX23 on *Phaseolus vulgaris*. Nodules of strain CTNUX23 were induced with the Jensen medium, but supplemented with methionine (25 μM). Nodules were harvested, fixed, and stained with methylene blue. Hand-cut sections (made with a razor blade) were obtained and were then viewed by bright-field microscopy. The central tissue (CT), inner cortex (IC), vascular bundles (VB), nodule endodermis (NE), and outer cortex (OC) are indicated. Bars indicate a length of 100 μm .

Genomic hybridization and cloning of the Tn5 flanking region.

Single digestions of *R. etli* CTNUX23 genomic DNA were performed with either *Bam*HI or *Eco*RI. For each digestion, 5 µg of genomic DNA was used, and reaction mixtures were incubated for 4 h at 37°C. DNA-modifying enzymes (Promega) were used as specified by the manufacturer. Southern hybridization against a 5.5-kb *Hpa*I fragment obtained from pSUP1011 and containing the Tn5 DNA was performed under conditions of high stringency (Sambrook et al. 1989).

To clone the Tn5 flanking region, single digestion of genomic DNA (5 µg) from strain CTNUX23 was performed with *Eco*RI, separated by preparative 0.7% agarose gel electrophoresis. The bands of the expected size (about 10 kb) were cut out of the gel and ligated with linearized (*Eco*RI digested) and dephosphorylated pGEM7Zf⁺ (Promega). After transformation into *E. coli* DH5α (Woodcock et al. 1989), two transformants resistant to kanamycin (30 µg ml⁻¹) were isolated and one of them, carrying pAR193 (Fig. 1), was chosen for further analysis.

Construction of an enrichment genomic library.

According to Southern hybridization experiments (hybridized against a ³²P-labeled *Sal*I/*Pst*I [430-bp] DNA fragment from pAR198 used as a probe), the genomic DNA fragments (approximately 2.8-kb *Eco*RI/*Bam*HI) were chosen for the construction of an enrichment library. Genomic DNA (5 µg) of *R. etli* CE3 was digested with *Eco*RI and *Bam*HI and separated by preparative 0.7% agarose gel electrophoresis. The bands of the expected size were cut out of the gel and ligated with linearized (*Eco*RI/*Bam*HI digested) and dephosphorylated pMP220 (Spaink et al. 1987). The ligation mixture was transformed by electroporation into strain CTNUX23 of *R. etli*. These cells were plated on RMM agar medium containing kanamycin (30 µg ml⁻¹), tetracycline (5 µg ml⁻¹), and sulfate as sole sulfur source. Two colonies, able to grow in the absence of methionine, were isolated. From both the plasmid, called pAR204, was isolated and one of them was chosen for further characterization (Fig. 1B).

DNA sequencing.

DNA sequences were determined with a Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham, Milan, Italy). The radioactive nucleotide used for sequencing was either [α-³⁵S]dATP or [α-³²P]dATP (Amersham). Appropriate restriction fragments from clones pAR193 and pAR204 (Fig. 1) were subcloned in either pGEM7Zf⁺ or pGEM4Z and sequenced with the T7 and SP6 promoter primers. A standard Tn5 outward-reading primer, Tn5out (5'-GAAAGGTTCCGT TCAGGACGCTAC-3'), and appropriate synthetic oligonucleotide primers were also used.

RNase protection assays.

RNase protection assays were performed as described elsewhere (Patriarca et al. 1993) with an in vitro transcription system (Promega). Antisense RNA (riboprobe) was synthesized with [α-³²P]UTP (Amersham). Plasmid pAR198 was constructed by ligation of a 430-bp *Sal*I/*Pst*I DNA fragment from pAR196 to *Sal*I/*Pst*I-digested pGEM3Z (Fig. 1). To produce the antisense riboprobe, pAR198 was linearized with *Bam*HI, eluted, and used as the template for SP6 RNA polymerase-mediated transcription. RNA was extracted from *R. etli* cultures as previously described (Patriarca et al. 1993). Protected fragments were run on a denaturing 6% polyacrylamide gel together with a size marker and were quantitatively analyzed with a PhosphorImager gel scanner (Molecular Dynamics, Sunnyvale, CA).

Nodulation test, acetylene reduction activity, and staining of whole fixed roots.

Seeds were surface sterilized and germinated as previously described (Taté et al. 1997). Roots of germinated seeds were inoculated with *R. etli* cells that had been resuspended in the inoculation buffer (50 mM phosphate buffer [pH 7.0]) at the required density (10⁴ to 10⁶ ml⁻¹). Plants were grown in a growth chamber maintained at 65 to 75% relative humidity, 23°C in light, 19°C in dark, with a 16-h photoperiod, with the nitrogen-free Jensen medium (Jensen 1942) containing sulfate as the sole sulfur source. Nitrogenase activity was determined by measuring acetylene reduction activity of nodulated roots, as previously described (Taté et al. 1997).

To test the nodulation ability of *Rhizobium etli* in the presence of methionine, cells of the tested strain, CTNUX23, and the reference strain, CE3, were applied (spotted) to the root of 5-day-old *P. vulgaris* seedlings. Before inoculation, bacteria were grown in TYR medium, centrifuged at low speed, and resuspended in sterilized water. Plants were grown on Jensen medium with or without supplemental methionine (25 µM). Three weeks post-inoculation, nodules (5 nodules per plant, 6 plants), were surface sterilized (5% sodium hypochlorite treatment for 1 min, 70% ethanol for 1 min, and two rinses with sterile water) and crushed in 100 µl of RMM medium with multiple-well (a single nodule for each well) plates (Corning, Corning, NY). Cell suspensions were then streaked (with a toothpick) on plates of TYR agar supplemented either with nalidixic acid (20 µg ml⁻¹) or with nalidixic plus kanamycin (50 µg ml⁻¹). Aliquots of cells were also streaked on defined sulfate medium with or without supplemental methionine. A nodule was considered to be invaded by strain CTNUX23 when only methionine-auxotrophic, kanamycin-resistant bacteria were isolated from that nodule.

Fig. 8. Proposed pathway for methionine biosynthesis in *Rhizobium etli*. Strains CTNUX8 (*cysG*) and CTNUX23 (*metZ*) of *R. etli* are indicated.

Whole plants were collected at different times after inoculation and fixed as previously described (Taté et al. 1994). Fixed roots were cleared with sodium hypochlorite as previously described (Truchet et al. 1989). After clearing, specimens were stained with methylene blue (0.01% in distilled water) and then observed by bright-field or dark-field microscopy. Staining or washing of the material was achieved by gently allowing the solution to flow between the slide and the coverslip.

Nucleotide sequence accession number.

Nucleotide and/or amino acids sequence data are submitted to the EMBL, GenBank, and DDBJ sequence data bases as accession number AJ012295.

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