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Isolation of carbon- and nitrogen-deprivation-induced loci of *Sinorhizobium meliloti* 1021 by Tn*5–luxAB* mutagenesis

Anne Milcamps,[1,2] Daniel M. Ragatz,[1,2] PyungOk Lim,[1,2] Kelly A. Berger[2] and Frans J. de Bruijn[1,2,3]

Author for correspondence: Frans J. de Bruijn. Tel: +1 517 353 2229. Fax: +1 517 355 9614. e-mail: debruijn@pilot.msu.edu

NSF Center for Microbial Ecology[1], MSU-DOE Plant Research Laboratory, Rm 306, Plant Biology Building[2] and Department of Microbiology[3], Michigan State University, East Lansing, MI 48824, USA

Soil bacteria, such as *Sinorhizobium meliloti*, are subject to variation in environmental conditions, including carbon- and nitrogen-deprivation. The ability of bacteria to sense changes in their environment and respond accordingly is of vital importance to their survival and persistence in the soil and rhizosphere. A derivative of Tn*5* which creates transcriptional fusions to the promoterless *luxAB* genes was used to mutagenize *S. meliloti* 1021 and 5000 insertion mutants were subsequently screened for gene fusions induced by selected environmental stresses. The isolation of 21 gene fusions induced by nitrogen-deprivation and 12 induced by carbon-deprivation is described. Cloning and partial DNA sequence analysis of the transposon-tagged loci revealed a variety of novel genes, as well as *S. meliloti* genes with significant similarity to known bacterial loci. In addition, nodule occupancy studies were carried out with selected Tn*5–luxAB* insertion mutants to examine the role of the tagged genes in competition.

Keywords: *Rhizobium* starvation, nutrient-deprivation, luciferase activity, Tn*5*–*lux*, competition

INTRODUCTION

Soil bacteria are frequently exposed to nutrient-deprivation conditions. In fact the bulk non-rhizosphere soil is so oligotrophic that it has been called a nutritional desert (Metting, 1985). Consequently, bacterial growth is extremely limited and non-growth, or very slow growth, may be considered the norm (Lynch, 1988). Generations of bacteria in the soil have been estimated to range from less than 1 to 80 per year (Shields *et al*., 1973; Gray, 1976; Metting, 1985; Lynch, 1988; Matin, 1991), whilst generation times in a laboratory setting are measured in hours or days. Understanding how bacteria survive and persist under oligotrophic conditions is important to both increase our fundamental knowledge of microbial ecology and for applications such as the release of genetically engineered micro-organisms (GEMs) into the environment. In addition, the isolation of tightly regulated promoters will aid in designing GEMs for specific purposes, such as bioremediation, where nutrient-deprivation-regulated promoters can be used to induce gene expression.

The manner in which bacteria deal with the problem of nutrient-deprivation varies considerably. Some bacteria, such as *Bacillus* and *Myxococcus* spp., sporulate when confronted with nutrient-deprivation (Losick *et al*., 1986; Kaiser, 1986). Matin *et al*. (1989) have described two fundamental ways in which Gram-negative bacteria confront nutrient-deprivation: (i) by becoming more efficient scavengers of scarce nutrients, and/or (ii) by becoming more resistant to stress in general. Understanding which genes are regulated before, during and after the initiation of deprivation conditions is crucial for the elucidation of the mechanisms by which these processes occur.

Most of our knowledge regarding the molecular basis of deprivation responses in non-differentiating bacteria derives from work with *Escherichia coli*, *Salmonella typhimurium* and *Vibrio* spp. (see Kjelleberg, 1993). In *E. coli*, over 70 polypeptides are induced or enhanced during carbon, phosphorus, or nitrogen deprivation, as determined by two-dimensional PAGE (2D-PAGE)

Abbreviations: 2D-PAGE, two-dimensional PAGE; Km, kanamycin; Sm, streptomycin.

The GenBank/EMBL accession numbers for the sequences determined in this work are given in the text.

(Groat *et al*., 1986). Many of these polypeptides are uniquely induced by deprivation of a single nutrient. However, several are induced by more complex deprivation conditions and a group of 15 proteins constitutes a core set which is induced under all deprivation conditions examined. Similar results have been observed using 2D-PAGE analysis of *S. typhimurium* proteins where six polypeptides were induced by three or more stress conditions (Spector *et al*., 1986), and a *Vibrio* sp. where three proteins were commonly induced during nutrient-deprivation conditions (Nyström *et al*., 1990). Insertional mutagenesis with promoter probe elements, such as Mu*d-lacZ* and Tn*phoA*, has been used in *E. coli* and *S. typhimurium* to isolate and characterize genes activated under nutrient-deprivation conditions (Groat *et al*., 1986; Spector *et al*., 1988; Lange & Hengge-Aronis, 1991; Alexander *et al*., 1993; Alexander & St John, 1994; Weichart *et al*., 1993).

Although much is known about bacterial gene expression patterns from laboratory studies involving primarily enteric or marine bacteria, comparatively little is understood about gene regulation in soil bacteria under environmental conditions. Only recently has the effect of nutrient-deprivation on *Pseudomonas putida*, a common Gram-negative soil bacterium, been described (Givskov *et al*., 1994). A large number of proteins induced by C- and N-deprivation were detected using 2D-PAGE protein analysis, including a group of eight proteins induced by multiple stresses. The synthesis of these proteins appeared to be temporally regulated, as has been observed in *E. coli* and *Vibrio* spp. In soil bacteria such as *Sinorhizobium meliloti*, *P. putida* and *Pseudomonas fluorescens*, mutagenesis with Tn5 derivatives carrying reporter genes has generated collections of strains that carry gene fusions induced in stationary phase and under C- or P-limitation conditions (Kim *et al*., 1995; Uhde *et al*., 1997, Kragelund *et al*., 1997). Yet little is known about how the transposon-tagged loci affect survival and competition in the soil or rhizosphere.

We have used the reporter gene approach to identify C- and N-deprivation-induced loci by mutagenizing *S. meliloti* 1021 with a Tn5 derivative (Tn5-1063) containing the promoterless luciferase *luxAB* genes (Wolk *et al*., 1991; Meighen & Dunlap, 1993). The use of Tn5 has been shown to be an extremely useful tool for random mutagenesis in many Gram-negative bacterial systems, including *S. meliloti* (de Bruijn & Rossbach, 1994). In addition, luciferase has been a useful reporter in studies of microbial gene expression (Carmi *et al*., 1987; Heitzer *et al*., 1994), as well as a useful marker to track organisms in the soil and rhizosphere (de Weger *et al*., 1991; Beauchamp *et al*., 1993; Boelens *et al*., 1993; Möller *et al*., 1994; Jansson, 1995). Previously, we have used Tn5-1063 successfully in *P. fluorescens* to isolate gene fusions whose bioluminescence is induced under N- and P-deprivation conditions (Kragelund *et al*., 1995).

In this paper, we describe the isolation and characterization of a collection of 33 *S. meliloti* mutants generated by random Tn5-1063 mutagenesis carrying gene fusions induced by N- and/or C-deprivation, and report our preliminary results on the role that some of the Tn5-1063-tagged loci may play in competition. The word 'starvation' has been widely used in the literature, but as this implies a demonstrated physiological response of the bacterium, we have chosen to use the word 'deprivation' in this paper.

METHODS

Bacterial strains and plasmids. *S. meliloti* 1021 (Sm^r) has been described by Meade *et al*. (1982), *E. coli* DH5α by Hanahan (1983) and *E. coli* HB101 by Boyer & Roulland-Dussoix (1969). Plasmid pRK2013 has been described by Ditta *et al*. (1980). Plasmid pRL1063a (Wolk *et al*., 1991) was kindly provided by Dr P. Wolk (Michigan State University, USA).

Media and growth conditions. *S. meliloti* 1021 was grown at 28 °C in TY (Beringer, 1974) or GTS medium (per litre: 0·1 g K_2HPO_4; 1 g NaCl; 3 g Tris; 246 mg $MgSO_4.7H_2O$; 11 mg $CaCl_2$; 0·27 mg $FeCl_3.6H_2O$; 0·242 mg $Na_2MoO_4.2H_2O$; 3 mg H_3BO_3; 2·23 mg $MnSO_4.4H_2O$; 0·287 mg $ZnSO_4.7H_20$; 0·125 mg $CuSO_4.7H_2O$; 0·065 mg $CoCl_2$; 2 mg biotin; 2 g $(NH_4)_2SO_4$; 2 g glucose; 2·7 g sodium succinate) (Kiss *et al*., 1979). The N sources in GTS medium used were either 0·2 % $(NH_4)_2SO_4$, 0·2 % KNO_3 or 0·2 % glutamine. *E. coli* strains were grown at 37 °C in LB medium (Silhavy *et al*., 1984). Antibiotics were used at the following final concentrations: 250 μg streptomycin (Sm) ml^{-1} for *S. meliloti*; 200 μg kanamycin (Km) ml^{-1} for *S. meliloti*; 20 μg Km ml^{-1} for *E. coli*.

Transposon Tn*5*-1063 mutagenesis. *E. coli* strain DH5α, carrying plasmid pRL1063a or pRK2013, and the recipient *S. meliloti* 1021 strain were grown in LB-Km (*E. coli* strain) and TY-Sm (*S. meliloti* strain) respectively, washed twice with TY and concentrated fivefold in TY medium. Equal amounts of donor, helper and recipient cells were spotted on TY plates. After 24 h incubation at 28 °C, the mating mixtures were resuspended in sterile distilled water and plated on selective plates of GTS medium with Sm (250 μg ml^{-1}) and Km (20 μg ml^{-1}). Five thousand Km^r colonies were isolated, single-colony-purified, grown in liquid TY medium and stored in microtitre plates at −70 °C. Conjugation conditions and general protocols for Tn5 insertions were carried out as described by de Bruijn & Rossbach (1994).

Screening of the Tn*5*-1063 insertion strains for N- and C-deprivation-induced gene fusions. The *S. meliloti* strains carrying Tn5-1063 were grown overnight and spotted in groups of 48 onto 2 sets of membrane filters (Nucleopore) using an inoculating manifold and incubated on solid GTS media for 36 or 48 h, before transfer to nutrient-deprivation media.

To create N-deprivation conditions, one set of membranes was transferred to GTS medium lacking N. The other set was transferred to unmodified GTS medium (control set). Both sets were incubated for an additional 6–24 h. Luminescent cell patches were identified by spreading 50 μl n-decanal inside the lid of a glass Petri dish, placing it on top of the plate containing the filter, exposing the cells to n-decanal for 60 s and measuring light emission as described by Wolk *et al*. (1991), using the Hamamatsu Photonic System model C1966-20 (Photonic Microscopy), coupled to a Nikon 35 mm *f* 2·0 lens (camera aperture of 0·8 and variable gain). Strains carrying Tn5-1063-generated gene fusions whose luminescence was weak or absent before transfer, low or absent under non-

starvation conditions (control set) but strong after transfer to N-deprivation media, were selected for further analysis. These strains were re-screened by growing cells in liquid TY medium until an optical density of 1·0 was reached, spotting them on membranes in 2 µl droplets, incubating for 36 h and transferring to N-deprivation medium.

For C-deprivation conditions, a modified screening procedure was used since the generation of reduced flavin mononucleotide, a cofactor for luciferase, is closely tied to the C status of the cells and therefore insufficient during C-deprivation conditions. Membranes, containing colonies, were transferred to both GTS and GTS without C sources, and incubated for 6–24 h. Prior to the addition of the aldehyde substrate and the visualization of luminescent colonies, the membranes were transferred to unmodified GTS medium (with 0·2% glucose and 0·2% succinate) and incubated for 30 min. Strains exhibiting enhanced luminescence after starvation were retained. These strains were tested and grown in TY medium (OD 2), spotted on membranes and incubated for 48 h prior to transfer to C-deprivation medium.

Luminescence of the cell patches was measured quantitatively with the Hamamatsu photonic camera (program Total Intensity, window size corresponding to 2025 pixels) and recorded as photons per spot min^{-1}.

DNA isolation and manipulation. Plasmid DNA was prepared by the alkaline lysis method as described by Kragelund *et al.* (1995). Total DNA was isolated from *S. meliloti* strains according to de Bruijn *et al.* (1989). Restriction enzyme digestions, ligations and Southern blotting experiments were carried out as described by Sambrook *et al.* (1989). Labelling of DNA probes and DNA hybridizations were performed using a non-radioactive DNA labelling and detection kit (Boehringer Mannheim) according to the manufacturer's specifications.

DNA sequence analysis. Sequencing of double-stranded plasmid DNA was performed with the dideoxy method of Sanger *et al.* (1977) using Sequenase kits (US Biochemicals). To determine the *S. meliloti* DNA sequence on both sides of the transposon insertion, two Tn5-1063-derived oligonucleotides were designed and synthesized by the Macromolecular Synthesis Facility at Michigan State University, USA, and used as sequencing primers. One primer (corresponding to positions 110–86 of the Tn5-1063 DNA sequence: 5′ TACTAGATTCAATGCTATCAATGAG 3′) was designed to determine the upstream sequence from the Tn5 target site in the antisense direction. The other primer (corresponding to positions 7758–7781 of the Tn5-1063 DNA sequence: 5′ AGGAGGTCACATGGAATATCAGAT 3′) was designed for determining the downstream sequence from the Tn5-target site in the sense direction. These primers were modified from previously described sequencing primers for Tn5-1063 and other Tn5 derivatives (Black *et al.*, 1993; Fernandez-Pinas *et al.*, 1994). DNA sequences (400–500 nt) were analysed using the program Sequencher (Gene Codes Corporation). Similarities were studied with the program BLAST (Altschul *et al.*, 1990). The codon preference profiles were determined by the program CodonUse 3.1 (Codon window size 33, logarithmic range 3), kindly provided by Conrad Halling (University of Chicago, IL, USA). The GenBank/EMBL accession numbers of the sequences are as follows: AF069400 (N1); AF069401 (N3); AF069402 (N5); AF069403 (N8); AF069404 (N9); AF069405 (N12); AF069406 (N15); AF069407 (N25); AF069408 (N30); AF069409 (N110); AF069410 (N113); AF069411 (N127); AF069412 (N150); AF069413 (N161); AF069414 (N183); AF069415 (C1), AF069416 (C101); AF069417 (C18); AF069418 (C19), AF069419 (C27); AF069420 (C37); AF069421 (C47).

Nodulation and N_2-fixation assays. *S. meliloti* strains carrying Tn5-1063 insertions were screened for their symbiotic phenotype by inoculation on alfalfa (*Medicago sativa*) seedling roots. Alfalfa seeds were sterilized by soaking for 3 min in 95% (v/v) ethanol, followed by 3 min in 0·1% $HgCl_2$ and rinsed thoroughly with sterile distilled water. The seeds were placed on sterile Whatman filter paper, in test tubes containing 20 ml of sterile N-free B+D liquid medium (Broughton & Dilworth, 1971). Saturated cultures of strains carrying Tn5-1063 were diluted with sterile H_2O (1:5) and 1 ml aliquots were added to glass test tubes containing one-week-old alfalfa seedlings. Inoculated plants were grown for 6–7 weeks in a growth chamber (16 h light, 28 °C) and examined for the presence or absence of nodules (Nod phenotype).

Nitrogenase activity in nodules was measured in the glass test tubes containing the nodulated plants by capping each tube with a stopper, injecting acetylene (1/10 vol.), withdrawing a 1 ml sample from the tube after 30 min, followed by measurement of acetylene reduction to ethylene by GC analysis.

Nodule competition experiments. For each mutant strain tested, eight seedlings were inoculated with a 1:1 ratio of mutant strain versus reference strain. After 5 weeks, all nodules were harvested from the plants and individually analysed. The nodules were surface-sterilized with 95% ethanol and 3% (v/v) hydrogen peroxide, crushed in 300 µl sterile water and spotted onto plates with either Sm (250 µg ml^{-1}) or Km (200 µg ml^{-1}) to determine the ratio of reference strain versus mutant bacteria.

RESULTS

Tn*5*-1063 mutagenesis of *S. meliloti* 1021

Plasmid pRL1063a, carrying the promoterless *luxAB* transposon Tn5-1063 (Fig. 1a), was used to generate a collection of 5000 *S. meliloti* 1021 insertion mutants. Insertion of Tn5-1063 into a *S. meliloti* gene can result in the creation of a transcriptional fusion, whereby expression of the *luxAB* reporter genes is controlled by the resident *S. meliloti* promoter (Fig. 1b). Due to the presence of an *E. coli* origin of replication in Tn5-1063, the interrupted gene can be easily excised from the genome, ligated to form a self-replicating plasmid and recovered by electroporation or calcium-chloride-mediated transformation (Fig. 1c).

The insertional specificity of the Tn5-1063 transposon in *S. meliloti* 1021 was examined by screening the collection of 5000 insertion mutants for auxotrophs and their nature. Sixty-two auxotrophic mutants belonging to 13 different phenotypic groups were identified, suggesting a well dispersed pattern of Tn5-1063 insertions (P. O. Lim, S. Reiser, D. M. Ragatz, & F. J. de Bruijn, unpublished data). Southern blot analysis of genomic DNA of 69 Tn5-1063-tagged strains, probed with pRL1063a DNA, revealed single hybridizing bands of different sizes for 61 of 69 isolates tested, which also suggests a random and simple insertion pattern for Tn5-1063 in *S. meliloti* 1021.

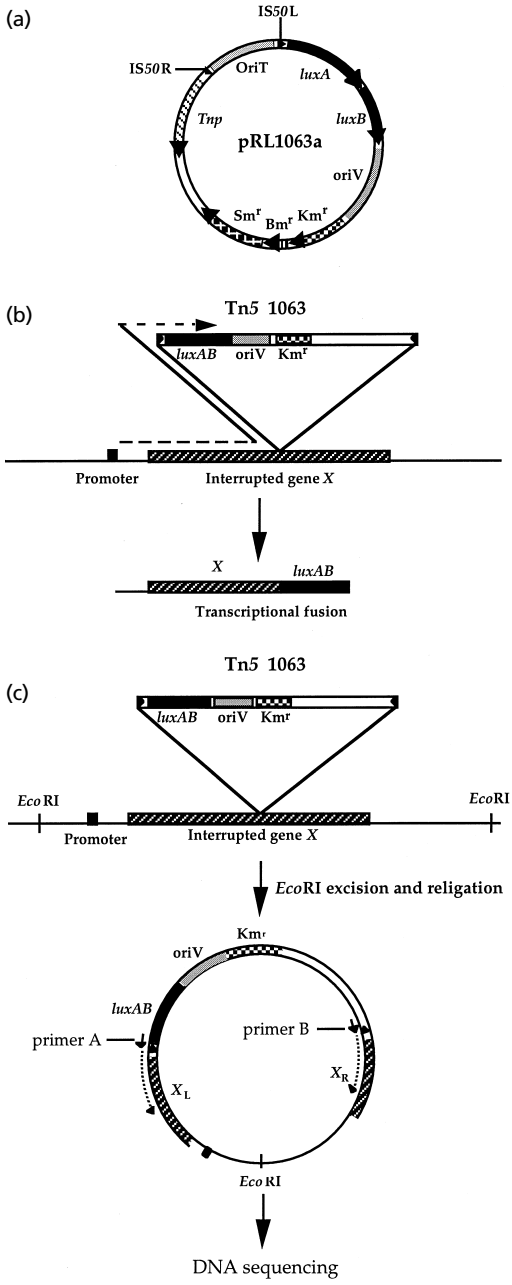

Fig. 1. Strategy for working with Tn*5*-1063. (a) Plasmid pRL1063a carrying Tn*5*-1063 (Wolk *et al.*, 1991); (b) insertion of Tn*5*-1063 in a target gene and generation of a transcriptional fusion; (c) cloning and sequencing of the flanking regions of the Tn*5*-1063 insertion. The position of the DNA sequencing primers (A, B) and the direction of the sequencing reactions are indicated.

Isolation and sequence analysis of *S. meliloti* strains carrying Tn*5*-1063 gene fusions induced by N-deprivation

The collection of 5000 random Tn5-1063 insertion mutants was screened for *luxAB* expression induced by N-deprivation, as described in the Methods section. Twenty-one mutant strains whose bioluminescence was consistently activated or enhanced under N-deprivation conditions were isolated (Fig. 2).

All of the strains examined grew well on ammonium sulfate or glutamine; however, five failed to grow on nitrate (Table 1). In addition, these strains were examined for induction and/or repression of the gene fusions they harboured in the presence of nitrate, ammonium sulfate or glutamine. Ten of the gene fusions were found to be activated in the presence of nitrate, but none were induced in the presence of glutamine or ammonium sulfate as sole N source (Table 1; Fig. 2). However, two of the gene fusions (N112 and N127) were also expressed at a low level on unmodified GTS medium, suggesting a partially constitutive expression pattern. Most strains carrying the gene fusions showed luciferase activity after 6 h of deprivation and an increased level of activity after 24 h of deprivation. Strains N3, N21, N25, N110 and N111 showed a reduced level of luciferase activity after prolonged deprivation.

Luciferase activity was quantified as described in the Methods section and the result of this analysis is shown in Fig. 3. Some strains displayed a high level of luciferase activity (N5, N8, N9, N30). These strains were found to carry the transposon insertion in loci which encode enzymes for the same assimilatory function (see below).

Southern hybridization analysis of the strains carrying N-deprivation-induced *luxAB* fusions revealed that in 19 out of 21 cases single hybridizing bands were present, suggesting a simple Tn*5*-1063 insertion event (Table 1). The two strains that displayed multiple hybridizing bands were not analysed further. The genomic location of selected N-deprivation-induced genes was previously determined by Honeycutt *et al.* (1993) using pulsed-field gel electrophoresis and DNA hybridization. The N-deprivation-induced genes tagged in strains N3, N4, N25 and N123 were found to be located on the chromosome (Table 1). The gene tagged in strain N113 was found to be located on the symbiotic plasmid pSym-a and those tagged in N5 and N149 on the other symbiotic plasmid (pSym-b). Two additional N-deprivation-induced genes, tagged in strains N112 and N110, have been found to be located on pSym-b (Table 1; R. J. Honeycutt, personal communication).

To characterize the N-deprivation-induced genes, the Tn5-1063-tagged loci of strains carrying a single transposon insertion were cloned from the *S. meliloti* genome, as shown in Fig. 1. The DNA sequence of the *S. meliloti* DNA fragments flanking the Tn5-1063 inverted repeats was determined using unique primers (Fig. 1c) corresponding to the left and right ends of the Tn5-1063, respectively (see Methods). As expected, a 9 bp duplication at the insertion site of Tn5-1063 was observed in most cases, and removed before the fused DNA sequences were analysed to identify significant ORFs, using a codon-usage program as described in Methods. In all cases a highly significant ORF was identified (data not shown).

The amino acid sequences deduced from the primary

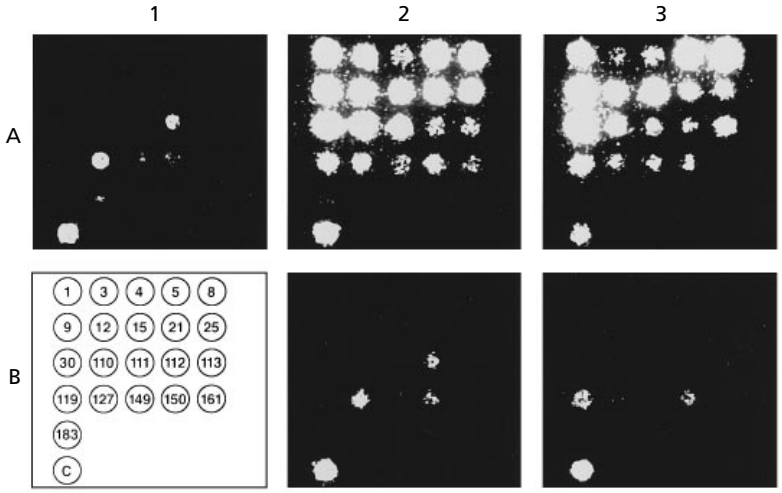

Fig. 2. Luciferase activity determination of fusions induced by N-deprivation. Analysis of strains with N-deprivation-induced *lux* gene fusions, using the Hamamatsu photonic camera. Row A, analysis of 21 N-deprivation-induced fusions 0, 6 and 24 h (columns 1, 2 and 3, respectively) after transfer to N-free medium; row B, luciferase activity of the fusions on non-starvation medium (unmodified GTS). The strain designations are the same in all panels and are outlined in row B, column 1. Strain C is a Tn*5*-1063-tagged derivative of strain 1021 displaying a constitutively expressed luciferase activity.

Table 1. Phenotypes and genome location of the *S. meliloti* N-deprivation-induced tagged loci

Strain	*lux* fusion induction by*:			Growth on†:		Symbiotic phenotype‡:		Hybridizing fragments§	Genome location‖
	−N	$+NO_3^-$	+Gln	$+NO_3^-$	+Gln	Nod	Fix		
Rm N1	+	+	−	+	+	+	+	1	ND
Rm N3	+	−	−	+	+	+	+	1	Chr
Rm N4	+	−	−	+	+	+	+	1	Chr
Rm N5	+	+	−	−	+	+	+	1	pSym-b
Rm N8	+	+	−	−	+	+	+	1	ND
Rm N9	+	+	−	−	+	+	+	1	ND
Rm N12	+	−	−	+	+	+	+	1	pSym-b¶
Rm N15	+	+	−	−	+	+	+	1	ND
Rm N21	+	+	−	+	+	+	+	2	ND
Rm N25	+	+	−	+	+	+	+	1	Chr
Rm N30	+	+	−	−	+	+	+	1	ND
Rm N110	+	+	−	+	+	+	+	1	pSym-b¶
Rm N111	+	−	−	+	+	+	+	1	ND
Rm N112	+	−	−	+	+	+	−	1	pSym-b
Rm N113	+	−	−	+	+	+	+	1	pSym-a
Rm N119	+	+	−	+	+	+	+	2	ND
Rm N127	+	−	−	+	+	+	+	1	ND
Rm N149	+	−	−	+	+	+	−	1	pSym-b
Rm N150	+	−	−	+	+	+	+	1	ND
Rm N161	+	−	−	+	+	+	+	1	ND
Rm N183	+	−	−	+	+	+	+	1	Chr

*Induction of Tn*5*-1063 fusions by growth of mutants on GTS without a N source (−N), with 0·2% nitrate ($+NO_3^-$) and with 0·2% glutamine (+Gln).

†Growth of mutants on GTS with 0·2% nitrate ($+NO_3^-$) or 0·2% glutamine (+Gln) as sole N source.

‡Symbiotic phenotype of alfalfa roots inoculated with mutant strains: presence of nodules on roots (Nod) and N_2-fixation capacity as determined by acetylene reduction (Fix).

§Number of fragments of genomic DNA cut with *Eco*RI which hybridize to a probe of whole pRL1063a plasmid DNA.

‖Genomic location of Tn*5*-1063 insertions. Chr, chromosome; ND, not determined.

¶R. J. Honeycutt, personal communication.

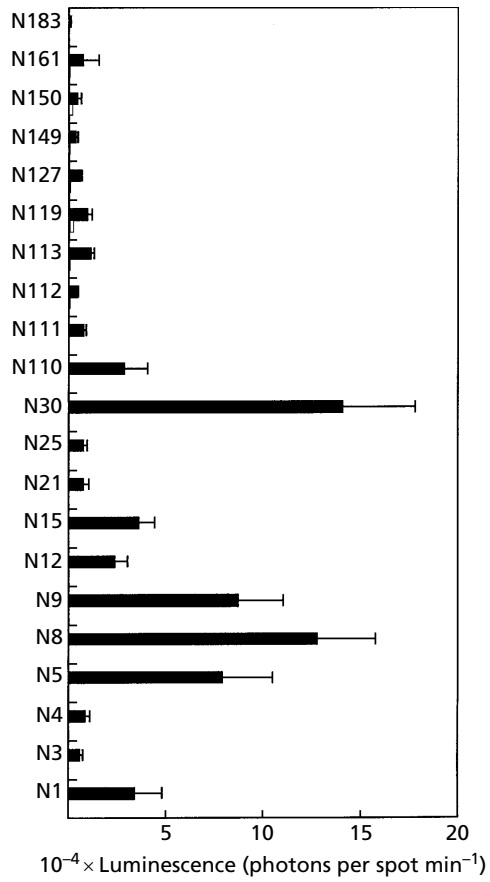

Fig. 3. Quantification of luciferase activity of fusions induced by N-deprivation. Luminescence was measured after 24 h of N-deprivation. The data are a representative sample of three repeated experiments, with measurements of three individual spots for each strain. Error bars indicate the standard error within a single experiment ($n = 3$). ■, Deprivation conditions; □, non-deprivation conditions.

DNA sequence of the N-deprivation-induced Tn5-1063-tagged loci were compared to sequences in the non-redundant protein database at GenBank and the sequence similarity significance values are shown in Table 2. The Tn5-1063-tagged loci of strains N30 and N15 were found to share significant similarity with the assimilatory nitrate reductase genes of several bacteria, including *Klebsiella pneumoniae* (*nasA*; Lin *et al*., 1993), *Oscillatoria chalybea* (*narB*; Unthan *et al*., 1996), *Synechococcus* sp. (*narB*; Omata *et al*., 1993), as well as the *E. coli* formate dehydrogenase genes *fdnG* (Berg *et al*., 1991) and *fdoG* (Plunkett *et al*., 1993). Since both the N30 and N15 mutant strains were found to be unable to grow on nitrate as sole N source (Table 1), it was likely that the gene encoding assimilatory nitrate reductase of *S. meliloti* had been tagged in these strains. In addition, these gene fusions were induced in the presence of nitrate (Table 1), which has also been observed for the *K. pneumoniae nasA* gene (Goldman *et al*., 1994). The alignment of the deduced amino acid sequence of the Tn5-1063-tagged locus of strain N30 with nitrate reductases and formate dehydrogenases is shown in Fig. 4. A conserved four cysteine consensus [4Fe-4S] cluster-binding motif, marked by triangles in the amino-terminal region, has been postulated to contribute to iron–sulfur binding (Berg *et al*., 1991; Breton *et al*., 1994). Although the tagged loci in strains N30 and N15 show similarity with the same gene, the location of the transposon within the gene is different.

The Tn5-1063 insertions in strains N5 and N9 were also found to be located in the same ORF, which shares significant similarity with the *Bacillus subtilis* assimilatory nitrite reductase gene *nasD* (Ogawa *et al*., 1995), the *K. pneumoniae* assimilatory nitrite reductase gene *nasB* (Lin *et al*., 1993) and the *E. coli* NADH-dependent nitrite reductase gene *nirB* (Peakman *et al*., 1990; Fig. 4). The tagged locus in strain N8 was found to share similarity with the *B. subtilis nasE* and is preceded by an ORF sharing similarity with the *B. subtilis nasD* gene. In *B. subtilis*, NasD and NasE constitute the subunits of nitrite reductase. Strains N5, N9 and N8 failed to grow on minimal medium with nitrate as the sole N source (Table 1), confirming that the assimilatory nitrite reduction pathway has been inactivated in all three strains. As in the case of strains N30 and N15, the gene fusions in strain N5, N9 and N8 were also found to be induced in the presence of nitrate (Table 1).

The Tn5-1063-tagged loci of strains N112 and N149 were found to correspond to the *S. meliloti exoY* and *exoF* genes, respectively (Müller *et al*., 1993). The *exo* genes of *S. meliloti* are organized as contiguous genes in a large operon (Glucksmann *et al*., 1993). *S. meliloti exoY* and *exoF* mutants are described as being unable to produce exopolysaccharides and unable to induce functional nodules ($Exo^-Nod^+ Fix^-$ phenotype; Long *et al*., 1988; Gray *et al*., 1990; Leigh & Walker, 1994). As expected, strains N112 and N149 did not display the mucoid phenotype associated with the other Tn5-1063-carrying strains under N-deprivation conditions. In addition, when strains N112 and N149 were inoculated onto alfalfa roots (Table 1), nodule-like structures were formed which lacked N_2-fixation activity (Fix^-), as previously observed for Exo^- mutant strains (Leigh *et al*., 1985). All other strains displayed a $Nod^+ Fix^+$ phenotype (Table 1).

DNA sequence analysis of the loci tagged in strains N3, N12, N25, N110, N127 and N183 revealed significant similarities to genes involved in amino acid transport and metabolism. Strain N127 carried a tagged locus with significant similarity to the *arcC* gene of several bacteria, including *Rhizobium etli*, *Pseudomonas aeruginosa*, *Haemophilus influenzae* and a *Synechocystis* sp. (D'Hooghe *et al*., 1997; Baur *et al*., 1989; Fleischmann *et al*., 1995; Kaneko *et al*., 1996). The *arcC* gene encodes carbamate kinase, an enzyme in the major arginine degradation pathway. The tagged locus of strain N3 shared similarity with the agmatine ureohydrolase gene *speB* of *Synechocystis* spp. and *Streptomyces clavuligerus* (Kaneko *et al*., 1996; Aidoo *et al*., 1994). Agmatine ureohydrolase is also involved in arginine

Table 2. Sequence similarities for the *S. meliloti* N- and C-deprivation-induced tagged loci

Strain	Gene similarity*	Function of similar gene	Significance of similarity†
Rm N1	*fmdF*	Putative transport protein	$4{\cdot}0 \times 10^{-16}$
Rm N3	*speB*	Agmatine ureohydrolase	$1{\cdot}1 \times 10^{-18}$
Rm N4	ND		
Rm N5	*nasD*	Nitrite assimilatory reductase subunit	$2{\cdot}7 \times 10^{-75}$
Rm N8	*nasE*	Nitrite assimilatory reductase subunit	$6{\cdot}2 \times 10^{-11}$
Rm N9	*nasD*	Nitrite assimilatory reductase subunit	$5{\cdot}0 \times 10^{-14}$
Rm N12	*braF*	Branched-chain amino acid transporter	$4{\cdot}4 \times 10^{-23}$
Rm N15	*nasA*	Nitrate assimilatory reductase	$1{\cdot}8 \times 10^{-23}$
Rm N21	ND		
Rm N25	MTH194	Glutamate synthase	$4{\cdot}8 \times 10^{-10}$
Rm N30	*nasA*	Nitrate assimilatory reductase	$1{\cdot}5 \times 10^{-26}$
Rm N110	*livH*	Branched-chain amino acid transporter	$1{\cdot}8 \times 10^{-43}$
Rm N111	ND		
Rm N112	*exoY*	Exopolysaccharide synthesis	$2{\cdot}1 \times 10^{-61}$
Rm N113	*subE*	Protease precursor	$1{\cdot}9 \times 10^{-10}$
Rm N119	ND		
Rm N127	*arcC*	Carbamate kinase	$4{\cdot}2 \times 10^{-44}$
Rm N149	*exoF*	Exopolysaccharide synthesis	$2{\cdot}3 \times 10^{-73}$
Rm N150	NS		
Rm N161	NS		
Rm N183	*dapA*	Dihydropicolinate synthase	$2{\cdot}3 \times 10^{-15}$
Rm C1	NS		
Rm C4	ND		
Rm C18	*thcD*	Ferredoxin reductase	$1{\cdot}7 \times 10^{-18}$
Rm C19	NS		
Rm C22	ND		
Rm C27	*rbsA*	Ribose transport ATP-binding protein	$1{\cdot}8 \times 10^{-19}$
Rm C35	ND		
Rm C37	ORF-f375	Unknown	$2{\cdot}2 \times 10^{-12}$
Rm C47	*xoxF*	Putative dehydrogenase	$8{\cdot}4 \times 10^{-80}$
Rm C55	ND		
Rm C67	ND		
Rm C101	NS		

* GenBank sequences showing similarity with the Tn*5*-1063 fusions. ND, Not determined; NS, no significant similarity.

† Significance of similarity at protein level, indicated by *P*-value (probability that such a match would occur merely by chance as given by the BLAST algorithm).

degradation, although via a pathway distinct from the carbamate kinase pathway. The locus tagged in strain N183 shared similarity with the *dapA* gene of *H. influenzae*, encoding dihydrodipicolinate synthase, an enzyme involved in the biosynthesis of diaminopimelate and lysine (Fleischmann *et al.*, 1995). Strain N25 contained a tagged locus which has similarity with a *Methanobacterium thermoautotrophicum* gene encoding glutamate synthase (Smith *et al.*, 1997). The loci tagged in strains N12 and N110 were found to share similarity with the *livH* and *braG* genes encoding high-affinity branched-chain amino acid transport ATP-binding proteins of several bacteria, including *Synechocystis* sp. and *P. aeruginosa* (Kaneko *et al.*, 1996; Hoshino & Kose, 1990). A similar locus (y4th) has also recently been discovered on the symbiotic pNGR234a plasmid of *Rhizobium* sp. NGR234 (Freiberg *et al.*, 1997).

In the case of strain N113, the deduced amino acid sequence of the tagged locus, 3′ downstream of the transposon insertion, revealed a weak similarity with the *B. subtilis subE* gene, encoding an extracellular protease precursor (Sloam *et al.*, 1988). The tagged locus in strain N1 was found to encode a protein similar to a putative transport protein of *Methylophilus methylotrophus* (Mills *et al.*, 1998).

Even though significant ORFs were identified in each of the remaining N-deprivation-induced loci, no significant similarity of these loci to sequences in the non-redundant protein database at GenBank was found, suggesting that the tagged genes are ‘novel’.

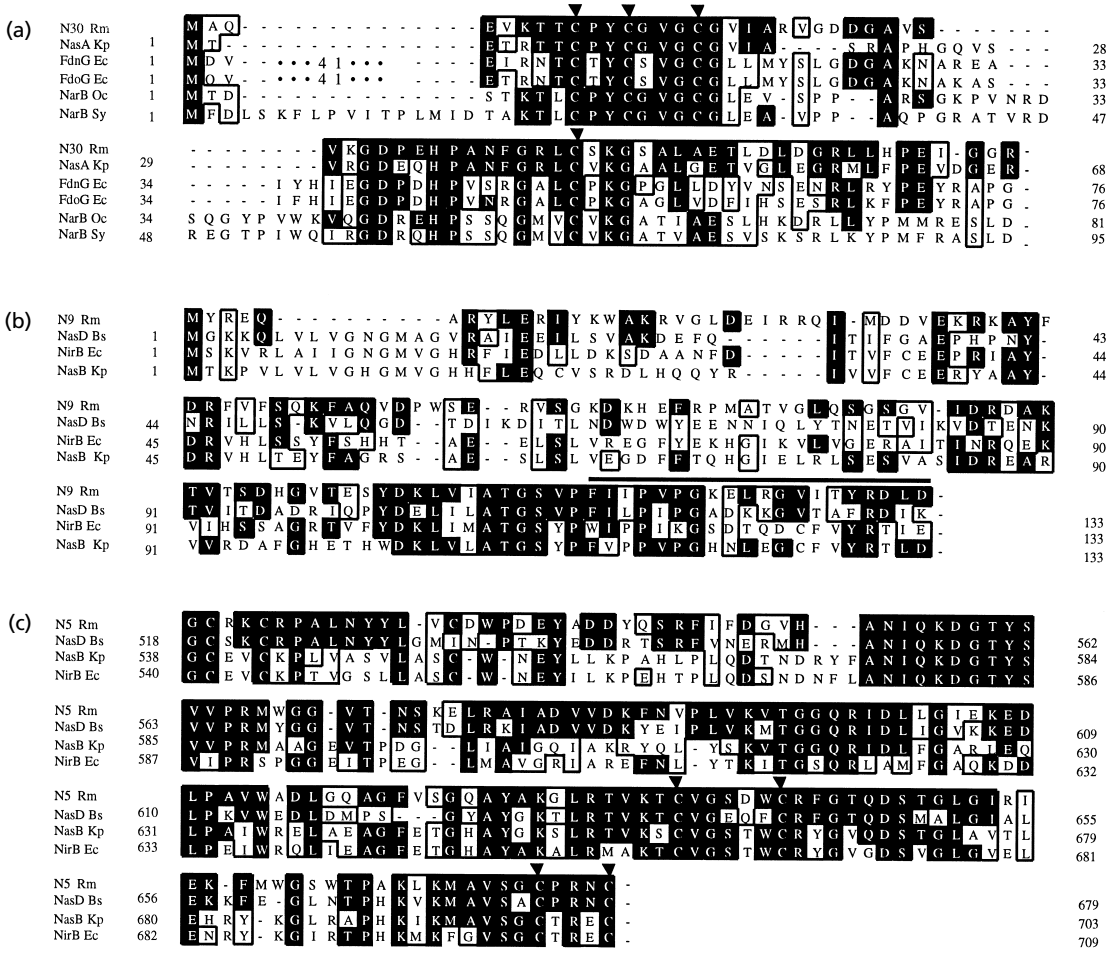

Fig. 4. Similarity of the amino acid sequence deduced from selected Tn*5*-1063-tagged ORFs induced by N-deprivation. (a) Amino acid sequence similarity of the putative protein product of the Tn*5*-1063-tagged ORF of strain N30 (N30 Rm) to nitrate reductases of *K. pneumoniae* (NasA Kp), *Oscillatoria chalybea* (NarB Oc), *Synechococcus* sp. (NarB Sy) and to formate dehydrogenases of *E. coli* (FdnG Ec, FdoG Ec). Triangles mark a conserved four cysteine consensus [4Fe-4S] cluster-binding motif. (b) Similarity of the putative protein products from the Tn*5*-1063-tagged ORF of strain N9 (N9 Rm) to nitrite reductases from *B. subtilis* (NasD Bs), *E. coli* (NirB Ec) and *K. pneumoniae* (NasB Kp). The solid bar indicates the start of an NAD-binding domain. (c) Similarity of the putative protein products from the Tn*5*-1063-tagged ORF of strain N5 (N5 Rm) to nitrite reductases from *B. subtilis* (NasD Bs), *K. pneumoniae* (NasB Kp) and *E. coli* (NirB Ec). The Tn*5*-1063 of strains N9 and N5 is located within the same ORF. The conserved cysteines of the [4Fe–4S]/sirohaem domain found in nitrite reductases and sulfite reductases are indicated with triangles. Identical amino acids are shown in white on black and conserved amino acid residues are boxed (based on the PAM250 matrix). Dashes indicate gaps introduced to maximize alignment. Amino acid residue coordinates are given on each line.

Isolation and sequence analysis of *S. meliloti* strains carrying Tn*5*-1063 gene fusions induced by C-deprivation

A modified screening procedure, including a 30 min incubation on C-source-containing plates after the induction by C-deprivation, was used to isolate C-deprivation-induced genes. This was necessary because the bioluminescence of *lux* fusions was undetectable when rhizobial cells harbouring Tn5-1063 gene fusions were incubated in the absence of a C source during the induction phase (see Methods). This is due to the fact that luciferase activity requires reduced flavin mononucleotide (Meighen, 1991), the generation of which is closely tied to C-metabolism. Using the modified screening procedure, 12 gene fusions whose bioluminescence was consistently induced or enhanced by C-deprivation were isolated. Quantitative measurements of luciferase activity directed by the C-deprivation-induced gene fusions are shown in Fig. 5(a). The luciferase activity of these C-deprivation-induced strains was found to be notably less than the activity of the strains carrying N-deprivation-induced gene fusions. Most strains showed a low level of luciferase activity under non-deprivation conditions and an enhanced level of activity under C-deprivation conditions. However, cells under non-deprivation conditions keep growing whilst growth ceases under C-deprivation conditions. Therefore, the luciferase activity of the C-deprived strains, which appeared to be only slightly enhanced in

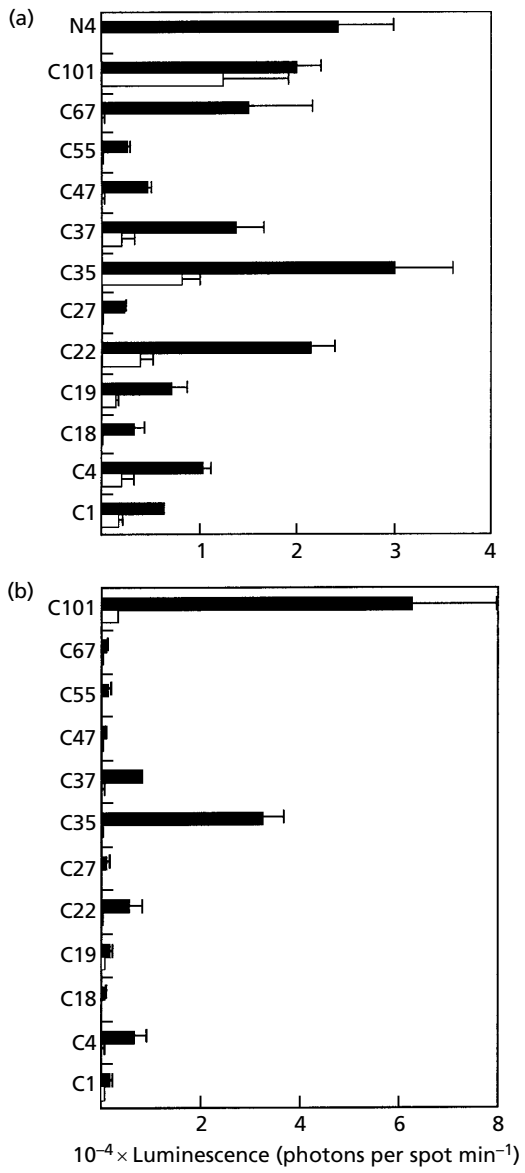

Fig. 5. Quantification of luciferase activity of fusions induced by C-deprivation. (a) Luminescence of the C-deprivation-induced strains and strain N4 (N-deprivation-induced) under C-deprivation conditions; (b) luminescence of the C-deprivation-induced strains under N-deprivation conditions. Luminescence was measured after 24 h of deprivation. The data are a representative sample of three repeated experiments, with measurements of three individual spots for each strain. Error bars indicate the standard error within a single experiment ($n = 1$). ■, Deprivation conditions; □, non-deprivation conditions.

some cases (i.e. strain C101; Fig. 5a) is significant and reproducible in multiple independent assays. Southern blot analysis of these 12 strains showed single hybridizing bands for 11 of 12 strains, indicating a simple Tn*5*-1063 transposition event (data not shown). The strain with multiple hybridizing bands was not analysed further.

The C-deprivation-induced *luxAB* gene fusions were cloned from the genome of the tagged strains and a partial DNA sequence of the locus flanking the transposon was determined, as described above. All of the tagged loci were found to contain significant ORFs (see Methods). The amino acid sequences deduced from the primary DNA sequence were compared to sequences in the non-redundant protein database at GenBank (Table 2).

The partial DNA sequence of the tagged locus of mutant strain C18 was found to share a high degree of similarity with several ferredoxin reductase genes. The highest similarity was observed with the rhodocoxin reductase *thcD* gene of *Rhodococcus* sp. (Nagy *et al*., 1995) and the 4-methyl-*o*-phthalate reductase/dehydrogenase *mopA* gene of *Burkholderia cepacia* (Saint & Romas, 1996). A slightly higher similarity value ($P = 5{\cdot}9 \times 10^{-19}$) was found with an unknown ORF from *Mycobacterium tuberculosis* (Philipp *et al*., 1996). The observed similarity was particularly significant in the carboxy-terminal region, due to the presence of a highly conserved FAD-binding consensus sequence motif [TXXXX(**I/V**)(**F/Y**)**A**(**A/V/I**)**GD**; Fig. 6] characteristic of FAD-binding oxidoreductases (Eggink *et al*., 1990). Therefore, it is likely that the locus tagged in strain C18 encodes an FAD-binding oxidoreductase, possibly involved in energy scavenging during C-deprivation.

The tagged locus of strain C27 shares significant similarity with the ribose transport *rbsA* genes of *E. coli* (Bell *et al*., 1986) and *B. subtilis* (Woodson & Devine, 1994), as well as the arabinose transport gene *araG* of *E. coli* (Scripture *et al*., 1987). The highest degree of similarity occurs in the amino-terminal region (Fig. 6), which includes an ATP-binding motif and membrane-spanning regions, potentially rendering *S. meliloti* more efficient at C uptake. Similarity was also found with the y4mk locus of *Rhizobium* sp. NGR234, which probably encodes an ABC transporter (Freiberg *et al*., 1997).

The tagged locus of strain C47 was found to be surprisingly similar to the *xoxF* ORF of *Paracoccus denitrificans*, which is part of an operon encoding cytochrome c553i (Ras *et al*., 1991; Fig. 6). Expression of the *P. denitrificans* ORF is induced by growth on choline as sole C source and this ORF has been predicted by Ras *et al*. (1991) to encode a quinoprotein dehydrogenase, based on sequence similarity with the *moxF* genes of *P. denitrificans* (Harms *et al*., 1987) and *Methylobacterium organophilum* (Machlin & Hanson, 1988).

In addition, 5′ upstream of the transposon insertion of the tagged locus in strain C37, similarity was found with an unknown ORF (ORF-f375; Blattner *et al*., 1997), which maps between approximately 65 and 68 minutes of the *E. coli* chromosome (Table 2). Although no similarities were found for the tagged loci of strains C4 and C22, sequence analysis revealed that the transposons were inserted into the same locus.

An analysis of the collection of 33 *S. meliloti* strains carrying gene fusions inducible by N- and C-deprivation for luciferase activity under different stress conditions,

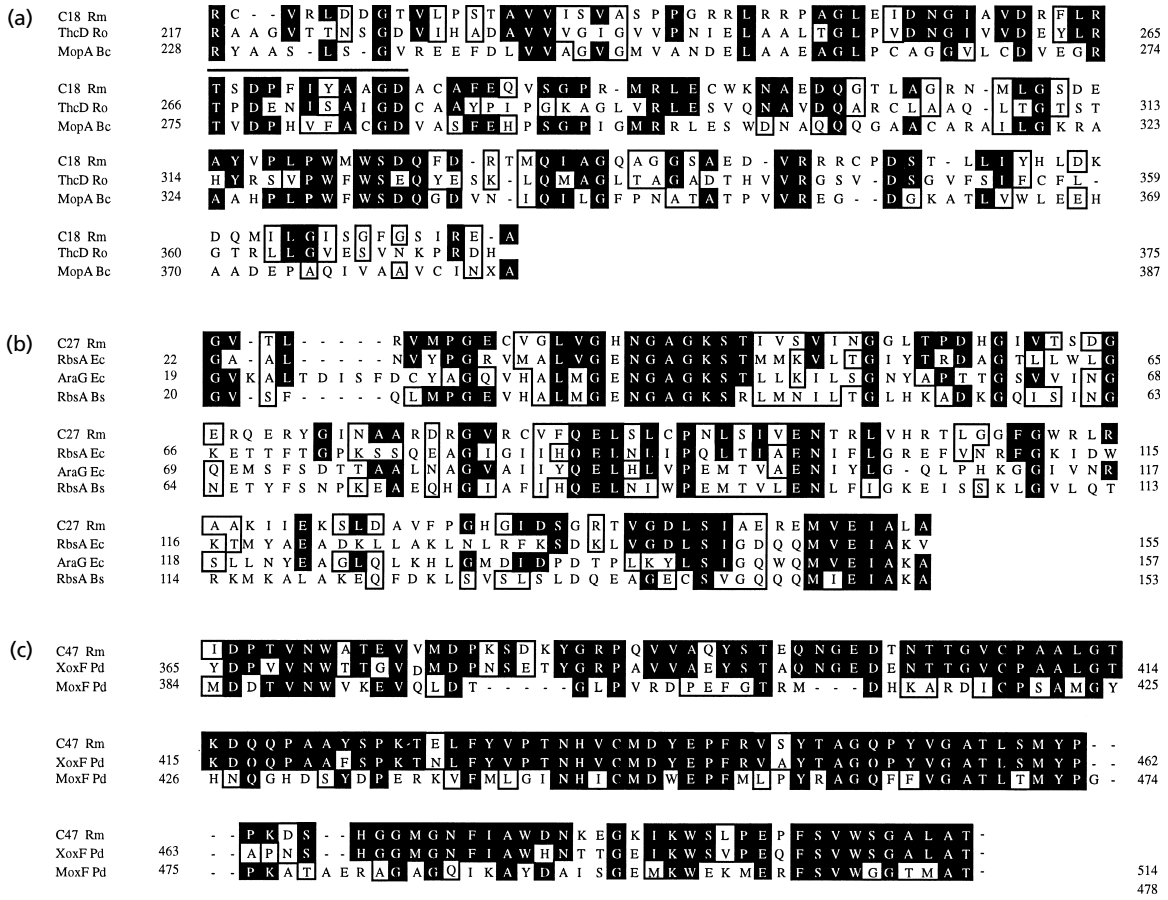

Fig. 6. Similarity of the amino acid sequence deduced from selected Tn*5*-1063-tagged ORFs induced by C-deprivation. (a) Amino acid sequence similarity of the putative protein product of the Tn*5*-1063-tagged ORF of strain C18 (C18 Rm) to rhodocoxin of *Rhodococcus* sp. (ThcD Ro) and a reductase of *B. cepacia* (MopA Bc). The solid bar above the sequence indicates a conserved FAD-binding domain. (b) Similarity of the putative protein product of the Tn*5*-1063-tagged ORF of strain C27 (C27 Rm) to ribose transport proteins from *E. coli* (RbsA Ec) and *B. subtilis* (RbsA Bs) and to an *E. coli* arabinose transport protein (AraG Ec). (c) Similarity of the putative protein product of the Tn*5*-1063-tagged ORF of strain C47 (C47 Rm) to pyrroloquinoline quinone (PQQ)-dependent dehydrogenase protein from *P. denitrificans* (XoxF Pd) and methanol dehydrogenase from *P. denitrificans* (MoxF Pd). Identical amino acids are shown in white on black and conserved amino acid residues are boxed (based on the PAM250 matrix). Dashes indicate gaps introduced to maximize alignment. Amino acid residue coordinates are given on each line.

revealed that six loci were inducible by both N- and C-deprivation conditions (N4, C4, C22, C35, C37 and C101; Fig. 4). Under N-deprivation conditions, the luciferase activity of strain C101 was found to be highly enhanced whilst strains C37, C22 and C4 showed a small, yet significant level of luciferase activity.

Competitive nodulation ability of *S. meliloti* Tn*5*-1063-tagged strains

All strains were tested for their symbiotic properties on the host plant *M. sativa* (alfalfa). Except for strains N112 (tagged *exoY* gene) and N149 (tagged *exoF* gene; see above), all strains were able to nodulate the plant and the nodules displayed a significant level of nitrogenase activity (Table 1).

To examine the effect of the transposon insertions in the Tn5-1036-tagged strains in competition with strain 1021 on infection of the host plant, nodule occupancy experiments were carried out (see Methods). Strains C18, C27 and C101 were chosen and co-inoculated with the reference strain 1021 at a 1:1 ratio onto alfalfa plants. The presence of mutant versus reference bacteria in mature 5-week-old nodules was investigated by plating bacteria re-isolated from nodules on selective plates. The mutant strains examined were not found to be out-competed by the reference strain. In fact, in three consecutive experiments, strain C101 appeared to be more capable of occupying the nodule then the reference strain (data not shown). Therefore, the C-deprivation-induced, Tn5-1063-tagged loci in these strains do not appear to negatively impact the competitive ability of the mutant strains to nodulate alfalfa plants.

DISCUSSION

Until recently, stress-induced gene expression in bacteria has been examined primarily using 2D-PAGE of differ-

entially expressed gene products (Nyström *et al*., 1990; Groat *et al*., 1986; Spector *et al*., 1986; Givskov *et al*., 1994). These analyses have revealed collections of proteins that are induced or specifically enhanced under stress conditions.

Here, a genetic approach to identify nutrient-deprivation-induced genes is described, and mutants have been simultaneously created to elucidate the role of these genes in responding to stress and persistence in the environment. *S. meliloti* strain 1021 was selected for our studies because it is amenable to transposon Tn*5* mutagenesis and it engages in a highly specific symbiotic interaction with its plant host (alfalfa), which allows a comparative study of the role of nutrient-deprivation-induced genes in the bulk soil, the rhizosphere and the infection process, as well as within infected plant cells (nodule occupancy).

The isolation and preliminary characterization of 33 N- and/or C-deprivation-induced *S. meliloti* genes, using Tn*5*-1063-generated *luxAB* reporter gene fusions is reported. Although occasionally two independent Tn*5*-1063 insertions in the same locus were found, we do not claim to have isolated all loci inducible by nutrient-deprivation since a collection of 5000 Tn*5*-1063 insertion mutant strains is too limited to ensure genome saturation. To achieve this goal, a collection of about 43000 Tn*5*-1063 carrying strains would be necessary (based on the *S. meliloti* genome size of 6·5 Mb, a mean bacterial gene size of 1 kb and a random distribution pattern of Tn*5* insertions in the genome).

Sequence analysis of the N-deprivation-induced tagged loci revealed significant similarities with genes involved in nitrate and nitrite assimilation (*nasA*, *nasD*, *nasE*), amino acid metabolism (*arcC*, *speB*, *dapA*) as well as amino acid transport (*braG*, *livH*). These data suggest that when *S. meliloti* experiences N-deprivation conditions, it induces the expression of genes/operons involved in scavenging N from nitrate, nitrite and amino acids. The *S. meliloti* C-deprivation-induced loci were found to include three genes whose deduced gene products contain regions that share significant similarity with functional domains found in FAD-binding oxidoreductases and a quinoprotein dehydrogenase, as well as a locus encoding a putative ribose ABC transporter. Thus it appears that during C-deprivation, *S. meliloti* induces the expression of genes that encode proteins involved in C-assimilation or transport, as well as energy scavenging. In the case of strain C18, where the tagged locus shows DNA sequence similarities with oxidoreductases, we cannot exclude the possibility that the induction of this locus is caused by the transfer to fresh medium after C-deprivation rather than C-deprivation *per se* and that the oxidoreductase may be involved in the generation of energy after starvation conditions were induced.

The identification of the exopolysaccharide synthesis genes *exoY* and *exoF* in our N-deprivation screen was not entirely surprising since exopolysaccharide biosynthesis has been shown to be increased by altering the N/C balance in the growth medium (Sutherland, 1979; Ozga *et al*., 1994). However, the two *exo–luxAB* fusions that were isolated provide novel tools to examine the control of exopolysaccharide gene expression.

One surprising observation was that the separate screening efforts for N- and C-deprivation-induced *S. meliloti* genes yielded only six loci that were induced by both nutrient-deprivation conditions. We made similar observations when analysing *P. fluorescens* genes induced by N- or P-deprivation using the Tn*5*-1063 mutagenesis system (Kragelund *et al*., 1995). These results contrast with observations made by Groat *et al*. (1986) using 2D-PAGE analysis of N-and C-deprivation-induced *E. coli* proteins. In that analysis, 24 of 48 N-deprivation-induced proteins were also found to be induced by C-deprivation.

Recently, Uhde *et al*. (1997) described four *S. meliloti* loci involved in stationary-phase survival. Partial DNA sequence analysis of these tagged loci revealed that one locus encoded a protein sharing similarity with an ABC-type transporter of *B. subtilis*. In our study, we found several potential ABC transporters (loci of strains N12, N110, C27), but none showed similarity with *B. subtilis* proteins and therefore possibly are distinct from the ones described by Uhde *et al*. (1997).

Rhizobial persistence and competition in the soil and rhizosphere are complex processes, the molecular basis of which is poorly understood. Mutations in selected nodulation (*nod*) genes, or in the N_2-fixation regulatory gene *nifA*, have both been found to cause a reduction in competitiveness and nodulation rates or nodule occupancy (Downie & Johnston, 1988; Sanjuan & Olivares, 1991). In addition, genes have been identified by Tn*5* mutagenesis which appear to affect nodule occupancy only (Onichshuk *et al*., 1994). Moreover, a proline dehydrogenase mutant has been described displaying an altered nodulation efficiency or nodule occupancy phenotype (Jimenez-Zurdo *et al*., 1995). The hypothesis that N- or C-deprivation-induced loci may play a role in competitiveness, as measured by nodule occupancy studies is not supported by the limited preliminary studies using three C-deprivation-induced loci reported here since none of the three mutant strains was outcompeted by the reference strain for nodule occupancy. Further competition studies in non-sterile soil with all of the available insertion mutants may shed further light on the role of individual genes in nodule occupancy and persistence in soil.

With regard to a second hypothesis underlying our research, namely that the regulatory circuits controlling nutrient-deprivation-induced gene expression in *Sinorhizobium* may be different from these reported for enteric bacteria, we have observed that selected N-deprivation-induced *S. meliloti* genes are not controlled by the canonical N-regulation system (*ntr*; Magasanik & Neidhardt, 1987) identified in enteric bacteria (A. Milcamps & F. J. de Bruijn, unpublished). Therefore, studies on nutrient-deprivation-induced gene expression

in non-enteric bacteria may reveal the presence of novel and distinct genetic control mechanisms responsible for environmental control of gene expression.

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