APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2001, p. 3728–3731 Vol. 67, No. 8
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.8.3728–3731.2001

Isolation and Regulation of *Sinorhizobium meliloti* 1021 Loci Induced by Oxygen Limitation

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Received 29 January 2001/Accepted 30 May 2001

Eleven *Sinorhizobium meliloti* 1021 loci whose expression was induced under low oxygen concentrations were identified in a collection of 5,000 strains carrying Tn*5-1063* (*luxAB*) transcriptional reporter gene fusions. The 11 Tn*5-1063*-tagged loci were cloned and characterized. The dependence of the expression of the tagged loci on the FixL/FixJ oxygen-sensing two-component regulatory system was examined. Three of the loci were found to be dependent upon *fixL* and *fixJ* for their expression, while one locus showed a partial dependence. The remaining seven loci showed *fixL*- and *fixJ*-independent induction of expression in response to oxygen limitation. This suggests that in *S. meliloti*, additional regulatory system(s) exist that respond either directly or indirectly to oxygen limitation conditions.

Bacteria in soil environments are continuously exposed to changing environmental conditions, including oxygen limitation (23). Oxygen levels in the soil fluctuate with time, often resulting in microaerobic and anaerobic microsites (15). In the rhizosphere, the soil adjacent to and influenced by the plant root system, root and microbial respiration can result in oxygen levels lower than those found in bulk soil (4). Microorganisms have developed mechanisms to sense and adapt to environmental changes, such as oxygen limitation. These responses may be important for the persistence of and competition between microorganisms in the soil.

The aerobic soil bacterium *Sinorhizobium meliloti* encounters oxygen limitation conditions in two different ecological niches: while in a free-living state in the soil or when in symbiotic association with the legume alfalfa (*Medicago sativa*) inside microaerobic nitrogen-fixing root nodules. Microaerobic conditions inside legume nodules are necessary to maintain nitrogen fixation activity due to the oxygen sensitivity of the enzyme nitrogenase. *S. meliloti* coordinates the expression of genes required for nitrogen fixation and for respiration inside nodules via a two-component regulatory system, FixL/FixJ, that senses microaerobic conditions and controls target genes accordingly (3, 6).

Towards the goal of examining the importance of the oxygen limitation response for *S. meliloti* survival in the soil environment, we have initiated a characterization of the molecular response of free-living *S. meliloti* to oxygen limitation using a gene reporter system. The involvement of FixL/FixJ in the regulation of the oxygen limitation response was also examined.

Isolation of *S. meliloti* loci expressed in response to oxygen limitation. A previously described (16) collection of 5,000 *S. meliloti* 1021 strains containing transposon Tn*5–1063* insertions was screened for strains that luminesced when oxygen levels became limiting. Tn*5–1063* functions as a transcriptional reporter system as it contains promoterless *luxA luxB* genes which encode the enzyme luciferase (24). The collection was screened by first spotting the strains onto duplicate plates containing solid GTS medium (12) lined with a filter membrane as previously described (16). The duplicate plates' contents were incubated at 28°C for 36 h followed by the incubation (28°C) of one plate's contents with atmospheric oxygen concentrations (~21%) and the incubation of the second plate's contents in an airtight jar under continuous flushing with an ~1% oxygen gas mixture. The gas mixture was generated by mixing nitrogen and compressed air using a Multigas System (Hotpack, Philadelphia, Pa.) and was monitored periodically using an oxygen electrode (Microelectrodes Inc., Londonderry, N.H.). After 6 to 8 h of incubation, the strains were examined for luciferase activity using a photonic camera system as described previously (16). A total of 11 strains containing Tn*5-1063* fusions that showed significantly increased luminescence after incubation under 1% oxygen, compared to incubation under 21% oxygen, were identified (Fig. 1). The loci tagged in these strains have been designated *loe* (low-oxygen expressed).

Molecular characterization of the *loe* strains. A Southern analysis using pRL1063a (24) as probe revealed that the 11 *loe* strains each contained a single Tn*5–1063* fusion and that the location of each fusion was distinct (data not shown).

The 11 Tn*5–1063* fusions were excised from genomic DNA and cloned, and at least 200 bp of the DNA sequence flanking each end of the Tn*5–1063* insertions was determined as described previously (5). The sequence obtained for each locus was assembled using the program Sequencher (Gene Code Corporation, Ann Arbor, Mich.) and compared to GenBank databases using the gapped BLASTX and BLASTN programs (2). The results are summarized in Table 1.

The *loe-1* fusion was shown to be located within the *fixN* gene of *S. meliloti. fixN*, the first gene of the *fixNOQP* operon, encodes a subunit of the cbb_3-type cytochrome *c* oxidase that

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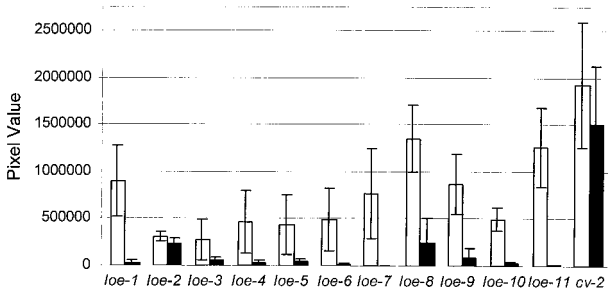

FIG. 1. Induction of expression of the *loe* fusions after 6 h of incubation under decreased oxygen concentrations. Shown are the average levels of luminescence generated by the *loe* fusion strains on solid medium when incubated for 6 h with 1% oxygen (white bars) or 21% oxygen (black bars). The *cv-2*::Tn*5-1063* fusion is expressed at both oxygen concentrations; therefore, the strain harboring the *cv-2* fusion was included to serve as a positive control in these studies. Shown atop each bar is the standard deviation observed in four or five independent trials. The pixel values correlate to the level of luminescence observed for each strain and were obtained using the University of Texas Health Science Center, San Antonio, Image Tool program. A standard *t* test of the data indicated that the difference observed between the two conditions for each *loe* fusion strain was significant.

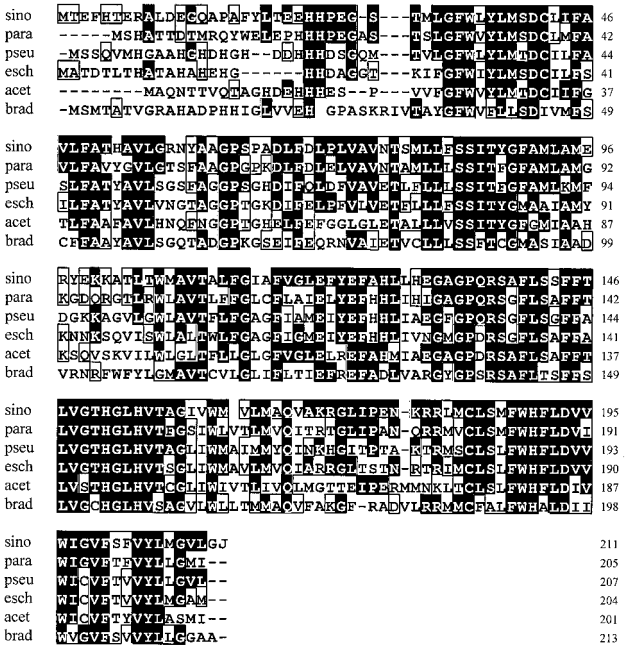

FIG. 2. Amino acid sequence comparison among ubiquinol oxidase subunit III homologs. The amino acid sequences aligned with the predicted polypeptide encoded by the *loe-8* and *loe-9* loci from *S. meliloti* (sino) include QoxC from *P. denitrificans* (para), CyoC from *P. putida* IH-2000 (pseu); CyoC from *E. coli* (esch); ubiquinol oxidase subunit III precursor from *A. aceti* (acet); and bo-type ubiquinol oxidase chain III from *B. japonicum* (brad). Sequence accession numbers for the homologs are C54759, BAA76358, C42226, BAA02482, and JC5901, respectively. Shaded regions correspond to amino acids identical to those found in the *S. meliloti* polypeptide. Boxed regions correspond to amino acid similarities and were determined using the PAM250 matrix. Dashes indicate gaps introduced by the computer program to maximize the alignment.

has been shown in *Bradyrhizobium japonicum* to be required for bacterial respiration inside soybean nodules (17, 18). The induction of *fixN* gene expression from *S. meliloti* in free-living microaerobic cultures had been demonstrated previously (6). Therefore, the identification of this locus in our screen indicated that our protocol was suitable for the isolation of loci expressed in response to oxygen limitation.

The *loe-8* and *loe-9* fusions were found to be within the same locus 474 bp apart. The predicted protein encoded by this locus was similar to subunit III of a family of ubiquinone oxidases conserved in a variety of microorganisms. A protein sequence alignment of representatives from this family was generated using the program Pile-up (Genetics Computer Group, Madison, Wis.) and is shown in Fig. 2. The *S. meliloti* sequence demonstrated 68, 61, 59, 51, and 47% amino acid identity to oxidases from *Paracoccus denitrificans*, *Pseudomonas putida* IH-2000, *Escherichia coli*, *Acetobacter aceti*, and *B. japonicum,* respectively. Based on the observed degree of amino acid sequence similarity and the fact that the regions of identity span the entire length of the proteins, it is likely that we have identified an ubiquinol oxidase subunit III protein from *S. meliloti*.

The *loe-10* and *loe-11* fusions were positioned within two different loci whose predicted partial amino acid sequences showed weak similarity to a hypothetical protein of unknown function, ORF278 from *P. denitrificans* (23 and 28% identity for *loe-10* and *loe-11*, respectively) (8), and to each other (21% amino acid identity). The deduced protein sequences were also

TABLE 1. Sequence similarities for *loe* loci

Locus	Protein similarity[a]	Proposed function	% Identity/BLAST score	*E* value[b]
loe-1	FixN	High-affinity oxidase (cbb_3) subunit	100/362	2.0×10^{-99}
loe-2	HI1351	Hypothetical protein	28/42	0.006
loe-3	APE0766	Hypothetical protein	27/55	6.0×10^{-7}
loe-4	AsnB	Asparagine synthesis	35/68	7.0×10^{-11}
loe-5	NS			
loe-6	CphA	Cyanophycin synthesis	30/76	4.0×10^{-13}
loe-7	NS			
loe-8/*loe-9*	CyoC	Ubiquinol oxidase subunit	59/246	2.0×10^{-64}
loe-10	ORF278	Unknown	32/40	0.014
loe-11	ORF278	Unknown	27/45	5.0×10^{-4}

[a] GenBank matches identified at the protein level (BLASTX). NS, no significant similarity. Origin of similar proteins: FixN, *S. meliloti*; HI1351, *Haemophilus influenzae*; APE0766, *Aeropyrum pernix*; AsnB, *B. subtilis*; CphA, *Anabaena variabilis*; CyoC, *E. coli*; and ORF278, *P. denitrificans*.

[b] Significance of similarity at protein level, indicated by the *E* value.

weakly similar to that for ORF277 from *B. japonicum* (27% amino acid identity) (17) and to that for ORF277 from *Rhodobacter capsulatus* (28% amino acid identity), respectively (13).

The *loe-4* fusion was found to be in a locus whose predicted amino acid sequence was similar to that of asparagine synthetase from *Bacillus subtilis* (25). This family of asparagine synthetases uses preferably L-glutamine or alternatively ammonia as the amino group donor to generate L-asparagine from L-aspartate (11, 25).

The *loe-6* fusion was in a locus encoding a protein sharing similarity to cyanophycin synthetase from a number of cyanobacteria. Cyanophycin synthetase catalyzes the ATP-dependent polymerization of arginine and aspartate, generating cyanophycin (multi-L-arginyl-poly-L-aspartate) (20, 21). Cyanophycin is believed to function as a nitrogen reserve polymer (20) and has been shown to accumulate in the presence of a source of nitrogen under a variety of different growth conditions in cyanobacteria (1, 14, 22). The synthesis of cyanophycin under microaerobic and anaerobic growth conditions has also been suggested (1).

The loci identified by the *loe-5* and *loe-7* fusions encoded putative polypeptides lacking significant similarity with proteins in the databases, indicating that they are novel. The proteins predicted to be encoded by the *loe-2* and *loe-3* loci were found to share a low level of similarity to hypothetical proteins of unknown function.

Regulation of the *loe* fusions by *fixL* and *fixJ*. An important objective of this research was to determine if regulatory systems in addition to FixL/FixJ exist that help to mediate the physiological response of *S. meliloti* to changes in oxygen availability. Towards this goal, the luciferase expression patterns of the strains carrying the *loe* fusions were examined in genetic backgrounds where the oxygen regulatory genes *fixL* and *fixJ* were inactivated.

To generate *loe*::Tn*5*–*1063 fixL* or *fixJ* double mutants, it was necessary to first generate insertions within *fixL* and *fixJ* that contained a selectable marker different from the markers carried by Tn*5*–*1063*. Therefore, we obtained *S. meliloti* strains GMI5705 (*fixL*2.66::Tn*5*) and GMI5704 (*fixJ*2.3::Tn*5*) (6) and replaced the Tn*5* insertions in these strains with Tn*5-233* via homologous recombination using the method described by De Vos et al. (9). The exact replacement of Tn*5* with Tn*5-233* within *fixL* and *fixJ* in the resulting strains (FdB3463 and FdB3464) was verified by Southern analysis (data not shown.)

By using protocols described in reference 10, phage ϕ M12 lysates of FdB3463 and FdB3464 were generated and used to transfer the *fixL*::Tn*5*–*233* and the *fixJ*::Tn*5*–*233* insertion mutations into the 11 *loe* fusion strains. The resulting double mutants were examined by Southern analysis of genomic DNA to ensure that the positions of the Tn*5*–*1063* and Tn*5*–*233* markers within the genome had been maintained (data not shown). The double mutants were then examined for changes in the expression of the *loe* fusions relative to the parent strains after a switch to oxygen limitation conditions. The expression of the *loe-1*, *loe-10*, and *loe-11* fusions was found to be dependent upon both *fixL* and *fixJ*, while *loe-7* fusion expression was found to be partially dependent upon *fixL* and *fixJ* (Fig. 3). *fixL*- and *fixJ*-dependent expression of *loe-1* (*fixN*) was the expected result, as it had been demonstrated previously in *S. meliloti* that *fixN* expression is controlled via the FixL/FixJ regulatory system (6). The remaining *loe* fusions demonstrated increased expression in a background of *fixL* and *fixJ*. This increase may have been an artifact of the luciferase reporter system and assay, since the positive control fusion, *cv-2*, also showed this increase. It was clear, however, that the induction of the expression of the remaining *loe* fusions (*loe-2*, *loe-3*, *loe-4*, *loe-5*, *loe-6*, *loe-8*, and *loe-9*) was independent of *fixL* and *fixJ*. These results support the idea that at least one additional regulatory system exists in *S. meliloti* that responds to low-oxygen conditions. Whether the regulator(s) responds to oxygen levels directly or indirectly (e.g., a change in redox or growth rate caused by oxygen depletion) remains to be determined.

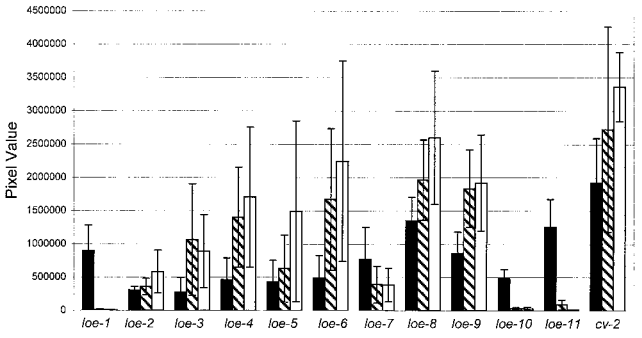

FIG. 3. The effect of *fixL* and *fixJ* mutations on the induction of expression of the *loe* fusions upon oxygen limitation. Shown are the average luminescence levels (pixel values) observed for each *loe* fusion strain (black bars) and its *fixL* (hatched white bars) and *fixJ* (white bars) derivatives when incubated under 1% oxygen. The standard of deviation for four or five independent trials is shown for each strain.

The symbiotic phenotype of the *loe* fusion strains. Because the physiological environment inside alfalfa nodules is microaerobic (0.03 μM oxygen) (3), we hypothesized that the loci identified in this study may have roles important in the formation of functional nitrogen-fixing nodules on alfalfa. To test this hypothesis, the 11 *loe* fusion strains were inoculated onto alfalfa seedlings and examined for nodule formation and for nitrogenase activity (via acetylene reduction assays) as described previously (16). All 11 *loe* fusion strains generated nodules with nitrogenase activity (data not shown) comparable to those for the reference strain *S. meliloti* 1021 (data not shown), suggesting that the loci disrupted by Tn*5*–*1063* in these strains were not essential for symbiotic nitrogen fixation. Although not essential, the *loe* loci may still be involved in symbiotic nitrogen fixation, since our assay may not have been sensitive enough to detect small decreases in nitrogenase activity. Lack of a nitrogen fixation phenotype may also be due to the presence of additional copies of the loci within the genome. For example, the finding that the strain carrying *loe-1*(Tn*5-1063* insertion within *fixN*) was symbiotically proficient was expected, as it had been noted in the literature that a second functional copy of *fixN* exists within the genome of *S. meliloti* (7, 19).

In conclusion, the approach presented here has clearly permitted the identification of loci affected by oxygen availability. In addition we have provided evidence for the existence of additional regulatory systems in *S. meliloti* that are responsive

either directly or indirectly to oxygen limitation conditions. Future studies in our laboratory will focus on isolating the genes involved in the regulation of the FixL/FixJ-independent *loe* loci and on examining a possible role for the regulatory genes in microbial persistence in the soil environment.

Nucleotide sequence accession numbers. The GenBank nucleotide sequence accession numbers for the *loe* loci are AF336863 (*loe-2*), AF336864 (*loe-3*), AF336865 (*loe-4*), AF336866 (*loe-5*), AF336867 (*loe-6*), AF336868 (*loe-7*), AF336869 (*loe-8, loe-9*), AF336870 (*loe-10*), and AF336871 (*loe-11*).

We thank G. Walker and P. Boistard for strains and U. Rossbach for computer support. We also thank C. P. Wolk and de Bruijn lab members for helpful suggestions and discussions.

This work was supported by grant DE-FG02–91ER20021 from the U.S. Department of Energy. J. Trzebiatowski was supported by National Research Service Award 5-F32-GM19412–03 from the National Institute of Health.

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