Mutational Analysis of the *Sinorhizobium meliloti* Short-Chain Dehydrogenase/Reductase Family Reveals Substantial Contribution to Symbiosis and Catabolic Diversity

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The short-chain dehydrogenase/reductase (SDR) family is one of the largest and most ubiquitous protein families in bacterial genomes. Despite there being a few well-characterized examples, the substrate specificities or functions of most members of the family are unknown. In this study, we carried out a large-scale mutagenesis of the SDR gene family in the alfalfa root nodule symbiont *Sinorhizobium meliloti*. Subsequent phenotypic analysis revealed phenotypes for mutants of 21 of the SDR-encoding genes. This brings the total number of *S. meliloti* SDR-encoding genes with known function or associated phenotype to 25. Several of the mutants were deficient in the utilization of specific carbon sources, while others exhibited symbiotic deficiencies on alfalfa (*Medicago sativa*), ranging from partial ineffectiveness to complete inability to form root nodules. Five of the mutants had both symbiotic and carbon utilization phenotypes. These results clearly demonstrate the importance of the SDR family in both symbiosis and saprotrophy, and reinforce the complex nature of the interaction of *S. meliloti* with its plant hosts. Further analysis of the genes identified in this study will contribute to the overall understanding of the biology and metabolism of *S. meliloti* in relation to its interaction with alfalfa.

The rapid expansion of the known bacterial genome sequences, from both cultivated and uncultivated organisms, continues to radically alter microbiological research. Much effort is now expended toward the identification of the functions of novel genes that are discovered in the genome sequences, because this knowledge contributes toward the full understanding of the biology of the corresponding organisms.

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The well-characterized α -proteobacterium *Sinorhizobium meliloti*, whose genome sequence was made available in 2001 (Galibert et al. 2001), is a versatile model system for this type of research. There are several examples of the *Rhizobiales* order of the α -proteobacteria that form intimate interactions with eukaryal host cells, as pathogens or symbionts (Williams et al. 2007). Of these, *S. meliloti* forms alfalfa (*Medicago sativa*) root nodules in a symbiotic relationship during which atmospheric nitrogen (N_2) is converted to ammonia for the benefit of the plant. Such symbiotic N_2 fixation is of ecological importance, because it represents a major input of fixed N into ecosystems. It is also important for agricultural systems, relieving the need for economically and ecologically expensive nitrogen fertilizers.

S. meliloti cells are capable of using a variety of carbon substrates for growth, including sugars, sugar alcohols, amino acids, organic acids, and phenolics (Aneja and Charles 1999; Arias and Martinez-Drets 1976; Charles and Aneja 1999; Charles and Finan 1991; Dunn 1998; Finan et al. 1991; Gage and Long 1998; Gosselin et al. 2001; Graham 1964; Irigoyen et al. 1990; Johnson et al. 1966; Parke and Ormston 1984; Parker and Graham 1964); this capacity to utilize a wide spectrum of substrates probably contributes to cell survival and proliferation in the nutritionally complex soil and rhizosphere environments. Several candidates for contributing to the catabolic capacity of *S. meliloti* are found within the short-chain dehydrogenases/reductases (SDR) superfamily of proteins (Jörnvall et al. 1999; Kallberg et al. 2002a), also known as the tyrosine dependent oxidoreductases. The SDR enzymes carry out NAD(P)(H)-dependent oxidation and reduction reactions on specific compounds, and have the N-terminal coenzyme binding motif GXXXGXXG associated with the Rossmann fold and the catalytic Ser-Tyr-Lys triad that incorporates the YXXXXK motif (Filling et al. 2002; Kallberg and Persson 2006; Kallberg et al. 2002b; Persson et al. 2003). Although members of this family are easily identified based on phylogenetic and sequence characteristics, this identification does not predict the substrate specificity of the individual enzymes. Experimentally based knowledge of the substrates corresponding to orthologous SDR subclasses is required for the interpretation of genomics-enabled gene expression studies and microbial community analyses.

The *S. meliloti* genome contains 77 genes that are catalogued within the Pfam adh_short family (PF00106) (Finn et al. 2006), but only a few of these genes are of known function. SMb21010 (*bdhA*) encodes D-3-hydroxybutyrate dehydro-

genase (EC 1.1.1.30) as the first gene in an operon that also encodes the two subunits of a xanthine oxidase (Aneja and Charles 1999). SMc03878 encodes acetoacetyl-CoA reductase (*phbB*) (EC 1.1.1.36) (Aneja et al. 2004; Tombolini et al. 1995) and shares an operon with SMc03789, encoding acetyl-CoA acetyltransferase (EC 2.3.1.9). SMA0854 (*nodG*) and SMc00572 (*fabG*) encode redundant 3-oxoacyl-ACP reductase (EC 1.1.1.100) enzymes that are involved in fatty acid elongation (Lopez-Lara and Geiger 2001). This is particularly relevant to the provision of fatty acids required for synthesis of the lipochitin oligosaccharide nodulation signal molecule.

A few of the other SDR family members are sufficiently well conserved that their function is predicted with high confidence. SMc02322, which also contains an aldolase domain, is part of the rhamnose utilization operon. It has been shown previously that mutants of the *Rhizobium leguminosarum* ortholog are unable to catabolize rhamnose (Richardson et al. 2004). The substrate is probably a phosphorylated derivative of rhamnose. SMc02273 is predicted to encode a translationally fused version of a transcript that, in another wild-type isolate of *S. meliloti*, strain Rm41, is composed of several open reading frames (ORF) that are involved in K-antigen capsular polysaccharide synthesis (Kiss et al. 2001). SMc00326 and SMc00005 are predicted to encode enoyl ACP reductase enzymes (EC 1.3.1.9) involved in fatty acid biosynthesis.

In the study reported here, we set out to disrupt each of the *S. meliloti* genes that are predicted to encode SDR family members. Through phenotypic analysis of the resulting mutants, we have attempted to deduce the specific functions of the genes. To our knowledge, this is the first report of the attempted disruption of the complete complement of SDR-encoding genes in any organism.

RESULTS AND DISCUSSION

Identification of SDR-encoding genes in the *S. meliloti* genome.

Given the moderately high level of sequence conservation within the SDR family, primary screening of the 6.7-Mb genome by BLASTP (Altschul et al. 1990) was possible. The sequence of the previously well-characterized SDR D-3-hydroxybutyrate dehydrogenase (BdhA) of *S. meliloti* was used as query, and products with greater than 23% amino acid sequence identity to BdhA were considered as putative members of the SDR family. Further screening for the SDR-specific ADH_SHORT PROSITE consensus motif [LIVSPADNK] – x(9) – {P} – x(2) – Y – [PSTAGNCV] – [STAGNQCIVM] – [STAGC] – K – {PC} – [SAGFYR] – [LIVMSTAGD] – x – {K} – [LIVMFYW] – {D} – x – {YR} – [LIVMFYWG] [APTHQ] – [GSACQRHM] using PatScan (Hulo et al. 2006) identified a subset of 56 of these genes, while the Pfam *adh_short* family (PF00106) (Finn et al. 2006) based on this same motif identified 77 genes. Although SMA2019, SMc02040, and SMc02356 were not identified in the Pfam subset, they were included in the study because they showed the presence of the PROSITE consensus sequence. Interestingly, SMc02356 has been annotated as an ABC transporter even though it includes the SDR motif. SMc02273 and SMb20974, identified as SDR by Pfam, were not included in this study. SMc02273 appears to be a composite of proteins that contains multiple domains, of which one is an SDR domain. These domains are usually found encoded by individual genes organized as an operon in other organisms. If SMc02273 were functional, it would appear to be a multifunctional enzyme and not exclusively an SDR. SMb20974 does not have the PROSITE consensus sequence and appears to have a truncated SDR domain. Based on these analyses, 78 SDR-encod-

ing genes in the *S. meliloti* genome were retained for this study (Supplementary Table S1).

Protein structure studies have revealed the importance of a glycine-rich T-G-X₃-G-X-G motif and an N-N-A-G motif for the maintenance of the structure of the central β -sheet involved in coenzyme binding and in determining the direction of the reaction (Filling et al. 2002; Kallberg and Persson 2006; Kallberg et al. 2002b; Persson et al. 2003). These motifs are highly conserved and are not a part of the PROSITE consensus sequence. Moreover, the active site tetrad constituting the residues N-S-Y-K (part of the PROSITE consensus) is conserved in this group of proteins. Using multiple-sequence alignment facilitated by ClustalW (Ramu et al. 2003) to generate multiple sequence alignments (data not shown), we determined that the T-G-X₃-G-X-G motif was present in each predicted SDR, except in SMA1367, SMA0389, SMc00005, SMc00326, and SMc02040. Also, the NNAG motif was absent in SMA2019, SMA1629, SMc00326, SMc00005, and SMc02040. The active site residue asparagine was not found in SMA2019.

The 78 SDR-encoding genes are distributed on all three *S. meliloti* replicons, 21 on each of the megaplasmids pSymA and pSymB and 36 on the chromosome. Substrates for most of these SDR enzymes have yet to be determined. The predicted proteins range from 136 to 699 amino acids in length. The smallest SDR identified, SMA2019, has much of the N-terminal region absent when compared with the others. The longest SDR identified, SMc02322, has two other well-characterized Pfam domains: an aldolase domain and a domain corresponding to epimerase. The ortholog of SMc02322 has been studied in *R. leguminosarum* as part of the rhamnose utilization operon (Richardson et al. 2004). Three of the remaining SDR identified, *phbB*, *nodG*, and *bdhA*, have been previously characterized in *S. meliloti*, as indicated above.

Construction of SDR mutants.

Phenotypic analysis can be a very powerful approach to the determination of gene function. Therefore, in order to understand the role of the SDR-encoding genes in *S. meliloti*, each SDR gene was systematically mutated and the phenotype of each mutant was analyzed. RmP110, a derivative of Rm1021 in which a frameshift mutation in *pstC* is corrected (Yuan et al. 2006), was used as the parental strain. Our strategy was to create gene fusions in the gentamicin-resistant (Gm^r) reporter vector pTH1703 (Cowie et al. 2006). Because pTH1703 is unable to replicate in *S. meliloti*, a segment of each gene of interest was used to facilitate single homologous recombination events, thereby disrupting the gene.

To construct the mutants, pTH1703 or pVIK112 carrying the ORF fragments were mobilized from *Escherichia coli* DH5 α into RmP110 by triparental mating and transconjugants selected using streptomycin and gentamicin for pTH1703 and streptomycin and neomycin for pVIK112. Cointegrates resulting from a single recombination event will have two incomplete copies of the gene. One copy will be the intact, promoterless copy of the gene and the second copy will be the truncated version of the gene, with the promoter transcriptionally fused to a reporter gene. Mutations in *bdhA* (SMb21010) were made using both pVIK112 and pTH1703, and, in both cases, yielded the expected D-3-hydroxybutyrate utilization phenotype. Such mutations are expected to be polar on downstream genes in the same operon.

Of the 78 genes identified as SDR, 3—*bdhA* (SMb21010), *nodG* (SMA0854), and *phbB* (SMc03878)—have been characterized previously. Of the remaining SDR, we were not able to recover *fabG* (SMc00572) mutants, although an *S. meliloti fabG* mutant has been reported (Miller-Williams et al. 2006). Thus, each of the 78 genes identified as SDR have been mutated.

Single-crossover mutagenesis in *S. meliloti* using vectors such as pTH1703 has proven to be extremely reliable (Cowie et al. 2006). To ensure that this was the case in our hands, 14 representative mutants were screened by polymerase chain reaction (PCR) to confirm whether recombination resulting in the mutation had occurred in the target gene. For this purpose, a primer upstream from the start codon of the gene of interest (forward primer) was used in a PCR reaction with a primer designed from *gus* or *gfp* on pTH1703 (reverse primer) (Supplementary Table S2). In each case, the obtained amplification product confirmed the desired recombination event. Having confidence in the nature of the mutants, the collection was further characterized in terms of role in substrate utilization and symbiosis.

SDR-encoding genes are required for utilization of specific carbon sources.

Given the importance of oxidation reactions in catabolic pathways, we expected that at least some of the SDR mutants would be unable to utilize specific carbon sources to support growth. Each of the SDR mutants was screened on 81 different substrates (Table 1) as 0.1% sole carbon source on rhizobium mineral medium (RMM) agar. The list was compiled based on published reports on specific carbon source utilization by *S. meliloti* (Charles and Finan 1991; Graham 1964; Parker and Graham 1964). Whenever available, mutants constructed in each of the two orientations in the integration vector were screened.

Single colonies of the mutants were streaked for single isolates and the plates were incubated at 30°C for up to 4 weeks, during which time they were scored for growth by comparison with the growth of RmP110. Each screen was performed three times, and each strain exhibiting a phenotype was further confirmed by testing fresh RmP110 transductants of the mutations for the same phenotype.

From the growth pattern on the different sole carbon sources, only one of the genes, SMc00778, appears to be required for prototrophy. The SMc00778 mutants were unable to grow on any of the carbon sources except proline. This suggests that a SMc00778 mutation results in proline auxotrophy, because this mutant was unable to utilize any of the carbon sources unless the media was supplemented with proline. As shown in Table 2, 18 other SDR genes were required for growth on specific carbon sources.

The phenotype of some of the SDR mutants confirmed previously reported results or predictions. The SMc02322 mutants are not able to utilize rhamnose and, from previously published results, it is known that this region is equivalent to the region responsible for rhamnose catabolism in *R. leguminosarum* (Richardson et al. 2004). The inability of the SMc01500 mutants to utilize sugar alcohols is consistent with the location of the gene in a region annotated to be involved in sorbitol metabolism (Galibert et al. 2001).

The SMb20210 mutant showed very weak growth on most substrates and lies adjacent to a series of genes predicted to be

Table 1. Carbon sources for short-chain dehydrogenase/reductase mutant strain screening

Pentoses and derivatives	α -Galactosides	Amino acids and derivatives
D-ribose	Melibiose	L-alanine
2-Deoxy-D-ribose	Raffinose	L-lysine
D-xylose		L-ornithine
D-lyxose	β -Galactosides	L-proline
D-arabinose	Lactose	Trans-4-hydroxy-L-proline
L-arabinose	Lactulose	Glycyl-L-proline
Methyl- β -D-xylopyranoside		L-serine
D-ribono- γ -lactone	α -Glucosides	L-homoserine
L-rhamnose	Trehalose	Glutamine
	Turanose	γ -Aminobutyric acid
Hexoses and derivatives	Maltose	Alanineamide
D-glucose	Sucrose	Glycine-aspartate
L-glucose	Maltotriose	Alanine-glycine
D-galactose	Dextrin	Glutamate-glutamate
D-fructose	Inulin	
D-mannose		Nucleotides and derivatives
D-tagatose	β -Glucosides	Uridine
D-fucose	Gentiobiose	Thymidine
L-fucose	Cellobiose	Adenine hemisulfate
Palatinose	Salicin	
Arbutin		Organic acids
D-galactono- γ -lactone	Sugar alcohols	D-malic acid
Methyl- α -D-galactopyranoside	Sorbitol	L-malic acid
Methyl- β -D-galactopyranoside	Adonitol	Fumaric acid
N-acetyl-D-glucosamine	D-mannitol	Sodium succinate
N-acetyl-D-galactosamine	Meso-erythritol	Monomethyl succinate
3-O-methyl-D-glucopyranose	Galactitol	Citric acid
	Maltitol	DL-bromosuccinic acid
	Inositol	Pyruvic acid
	Glycerol	Aminovaleric acid
	D-arabitol	Formic acid
	L-arabitol	Lactic acid
	Alcohols	
	Ethanol	
	Propanol	
	Sugar phosphates	
	Glucose-1-phosphate	
	Fructose-6-phosphate	

involved in pyrroloquinoline quinone (PQQ) synthesis. This is interesting given that PQQ is a cofactor for quinoprotein dehydrogenases. Mutants of two other SDR genes, SMc02486 and SMa0719, exhibited generally poor growth on most carbon sources. Of these, SMc02486 lies in a region that includes several genes encoding enzymes involved in the TCA cycle (Dymov et al. 2004). The growth phenotype observed for the mutant may be due to TCA cycle defects; however, determination of this requires further experimentation. Mutation of the pSymA gene SMa0719, lying adjacent to predicted arginine transport and catabolism genes, results in growth deficiency in all tested carbon sources except glucose, malate, succinate, fructose, D-lyxose, and D-xylose. A rationale for this phenotype is not readily apparent.

Three SDR-encoding genes from different regions of the chromosome, SMc01204, SMc02034, and SMc02356, are required for utilization of ribonic- γ -lactone. SMc01204 is predicted to have an N-terminal transmembrane domain and SMc02356 appears to be part of an ABC transporter system. SMc02034 is located adjacent to an ABC transporter system. Little is known about bacterial catabolism of this carbon source, which is presumably freely interconvertible with D-ribose via reduction or oxidation. Further study of these mutants should help to uncover the significance of ribonolactone metabolism in *S. meliloti*.

In the same region as SMc02034, there is an interesting cluster of four other SDR-encoding genes: SMc02037, SMc02039, SMc02040, and SMc02041. Only one of these, the downstream SMc02041, exhibited a carbon utilization phenotype. It was unable to utilize monomethyl succinate. Further investigation is required to determine the role of this SDR-rich cluster of genes.

The SMb21159 gene lies immediately upstream of a putative β -N-acetylhexosaminidase-encoding gene, SMb21160. Fusions to the nearby ABC transporter gene, SMb21151, indicated that its expression was induced by galactosamine. Rather than having a phenotype on galactosamine-containing media, SMb21159 mutants exhibited growth deficiency on glucose-1-phosphate and succinate. We do not yet understand the metabolic basis for this growth phenotype; however, it could sug-

gest a pathway for galactosamine catabolism that includes a glucose-1-phosphate intermediate.

SMb20492 and SMb20493 lie adjacent to each other in the genome and are transcribed in the same direction, with SMb20493 situated upstream of SMb20492. Downstream of these SDR are genes annotated to be involved in sugar transport, a carbohydrate kinase, and a D-fucose phosphate aldolase (*fucA2*). This is consistent with the inability of the SMb20492 and SMb20493 mutants to utilize fucose. The inability of these mutants to utilize succinate, monomethyl-succinate, leucine, and lysine perhaps reflects a role in a pathway common to utilization of fucose and these carbon sources, or might be due to common auxiliary requirements of these catabolic pathways.

The genes SMb20073 and SMb20076 are divergently expressed from each other, with SMb20073 being immediately upstream of an ABC transporter gene cluster. The first gene in this cluster, SMb20072, was induced by myo-inositol (Mauchline et al. 2006); however, our screens did not reveal a phenotype on myo-inositol for these two SDR mutants but, rather, indicated deficiency in utilization of D-fucose, lysine, leucine, and proline. Mutants of another pSymB-encoded SDR, SMb20750, are unable to grow on ornithine and leucine. This is located in a region that has previously been suggested to be involved in the catabolism of branched-chain amino acids isoleucine, leucine, and valine (Charles and Aneja 1999; Miyamoto et al. 2003).

The weak growth of the SMb20409 mutants on galactitol and D-mannose is consistent with the recent finding that the adjacent gene, SMb20410, encoding part of an ABC transporter system, is induced by galactitol (= dulcitol) and D-mannose (Mauchline et al. 2006). This is also consistent with the previously reported dulcitol growth phenotype for deletions of this region (Charles and Finan 1991). The weak growth of the SMb21111 mutants on D- and L-fucose is consistent with the location of this gene as a distal member of an operon that has been found to be induced by both D- and L-fucose (Mauchline et al. 2006).

As indicated above, transductants of each of the mutants were tested to negate the chance that a phenotype may be due to an unknown second site mutation. As a further check on the

Table 2. Short-chain dehydrogenase/reductase mutant strains showing a free-living or symbiotic phenotype

Mutant ORF ^a	Gene	Carbon utilization deficiency ^b	Symbiotic phenotype
SMa0719*	...	All except glucose, DL-malic acid, succinate, fructose, D-lyxose, D-xylose	Fix ^{+/-}
SMb20073	...	D-fucose, lysine, leucine, proline	Fix ⁺
SMb20076	...	D-fucose, lysine, leucine, proline	Fix ⁺
SMb20210*	...	All except glucose, DL-malic acid, succinate, fructose, D-lyxose, D-xylose, D-ribose	Fix ⁻
SMb20214	...	None	Fix ^{+/-}
SMb20409	...	Galactitol, D-mannose	Fix ⁺
SMb20492	...	D-fucose, succinate, monomethyl-succinate, leucine, lysine	Nod ⁻
SMb20493	...	D-fucose, succinate, monomethyl-succinate, leucine, lysine	Fix ^{+/-}
SMb20750	...	D-fucose, glucose-1-phosphate, leucine, ornithine	Fix ⁺
SMb21010	<i>bdhA</i>	None (D-3-hydroxybutyrate deficiency confirmed as control)	Fix ⁺
SMb21111	...	D-fucose and L-fucose	Fix ⁺
SMb21159	...	Succinate, glucose-1-phosphate	Fix ⁺
SMc00005	<i>fabI1</i>	None	Fix ⁻
SMc00326	<i>fabI2</i>	None	Fix ⁻
SMc00778	...	Proline auxotroph	Fix ⁺
SMc01204	...	Ribonic- γ -lactone	Fix ⁺
SMc01500	<i>smoS</i>	Mannitol, maltitol, sorbitol, myo-inositol, meso-erythritol	Fix ⁺
SMc02034	...	Ribonic- γ -lactone	Fix ⁺
SMc02041	...	Monomethyl succinate	Fix ⁺
SMc02322	...	L-rhamnose	Fix ⁺
SMc02356	...	Ribonic- γ -lactone, monomethyl succinate	Fix ⁺
SMc02486*	...	All except glucose, D-lyxose, L-leucine, N-acetyl-D-glucosamine, inositol, N-acetyl-D-galactosamine, sorbitol, meso-erythritol, maltitol	Fix ⁻

^a ORF = open reading frame; * = shows poor growth on most substrates, except those indicated.

^b All growth phenotypes are in comparison to the wild type strain RmP110.

robustness of the screen, each of the SDR-encoding genes from the *S. meliloti* ORFeome (Schroeder et al. 2005) (kindly provided by I. Oresnik and T. Finan) were transferred into the Gateway-compatible expression vector pCO37 (Clark, *unpublished*). pCO37 clones containing the ORF of SDR showing a carbon utilization phenotype were then introduced into the corresponding mutant background. The resulting transconjugants were tested for complementation of the loss of function. All carbon utilization phenotypes could be complemented in this manner.

Symbiotic phenotype of SDR mutant strains.

The SDR mutants were screened under nitrogen-deficient conditions on alfalfa seedlings in both vermiculite containing Leonard assemblies (Leonard 1943) and in agar slants to determine the involvement of any of the SDR-encoding genes in

symbiosis. After 4 weeks, the plants inoculated with the parental strain RmP110 were healthy and green, with pink nodules, compared with the uninoculated control plants that were stunted, chlorotic, and devoid of nodules. Although inoculation with most of the SDR mutants yielded plants that were indistinguishable from the plants inoculated with the parental strain, a substantial number of the SDR mutant-inoculated plants exhibited clear deficiencies in symbiosis (Supplementary Table S3). This observation was reinforced by comparison of the shoot dry weights, and the nodules were further analyzed by light microscopy. For each mutant exhibiting a symbiotic phenotype, the mutation was reintroduced into RmP110 by transduction (Finan et al. 1984), and testing of the resulting transductants yielded the same results.

The screen revealed eight of the SDR-encoding genes to be required for the formation of normal, nitrogen-fixing nodules

Table 3. Distribution of orthologs of 25 *Sinorhizobium meliloti* short-chain dehydrogenase/reductase (SDR)-encoding genes in genomes of members of the Rhizobiales^a

SDR	Smed	RI	Re	MI	Bj	At	Oa	Bm
SMa0719	89%	...	...	...	41%	67%	...	...
	Smed_3585	...	...	...	blr1409	Atu5465	...	...
SMa0854	98%	80%	81%	86%	60%	79%	74%	73%
(<i>nodG</i>)	Smed_6193	RL1558	RHE_CH01443	mlr7850	blr4083	Atu1095	Oant_0571	BMEI1477
SMb20073	95%	78%	79%	...	...	62%	72%	...
	Smed_4064	pRL110421	RHE_PE00299	...	...	Atu5276	Oant_4304	...
SMb20076	87%	...	65%	...	...	...	...	...
	Smed_4061	...	RHE_CH02674	...	...	...	...	...
SMb20210	92%	48%	49%	...	66%	...	...	...
	Smed_3913	RL1854	RHE_PF00540	...	blr5937	...	...	...
SMb20214	64%	74%	...	62%	...	48%	...	...
	Smed_5531	RL4052	...	ml16686	...	Atu5444	...	...
SMb20409	93%	...	...	72%	...	...	...	...
	Smed_3714	...	...	mlr5248	...	...	...	...
SMb20492	...	67%	...	57%	48%	...	...	...
	...	RL3778	...	ml1982	blr0293	...	...	...
SMb20493	...	72%	70%	62%	60%	...	70%	...
	...	RL3779	RHE_CH03352	ml1981	bl14020	...	Oant_3448	...
SMb20750	91%	...	...	...	...	47%	...	...
	Smed_4244	...	...	...	...	Atu2417	...	...
SMb21010	92%	69%	67%	57%	66%	65%	58%	...
(<i>bdhA</i>)	Smed_4500	RL3569	RHE_CH03123	ml13632	blr1488	Atu2308	Oant_1122	...
SMb21111	96%	83%	84%	78%	52%	61%	79%	...
	Smed_4971	pRL110599	RHE_PE00431	ml14781	bl13914	Atu4466	Oant_031	...
SMb21159	91%	77%	76%	46%	...	73%	47%	46%
	Smed_4652	pRL110503	RHE_PE00386	mlr4150	...	Atu2597	Oant_3894	BMEI0543
SMc00005	98%	80%	80%	78%	76%	89%	80%	80%
(<i>fabI1</i>)	Smed_0512	RL1011	RHE_CH00938	mlr7458	blr2629	Atu0757	Oant_0535	BMEI1512
SMc00326	95%	85%	83%	75%	76%	82%	78%	77%
(<i>fabI2</i>)	Smed_3448	RL0118	RHE_CH00109	ml15565	blr0771	Atu0149	Oant_0741	BMEI1958
SMc00572	97%	89%	89%	88%	64%	89%	80%	81%
(<i>fabG</i>)	Smed_0745	RL1558	RHE_CH01443	mlr7850	blr4083	Atu1095	Oant_0571	BMEI1477
SMc00778	92%	68%	...	72%	...	...	...	...
	Smed_032	RL0975	...	mlr4172	...	...	...	...
SMc01204	87%	72%	...	...	...	...	...	...
	Smed_1269	RL3838	...	...	...	...	...	...
SMc01500	93%	52%	64%	78%	...	65%	64%	...
	Smed_2354	RL0650	RHE_PC00218	ml14921	...	Atu3164	Oant_3850	...
SMc02034	94%	57%	42%	42%	58%	43%	...	...
	Smed_2445	RL0645	RHE_CH02402	ml17334	blr3225	Atu1897	...	...
SMc02041	88%	48%	...	...	...	...	48%	...
	Smed_2462	RL1748	...	...	...	...	Oant_4068	...
SMc02322	94%	74%	74%	79%	...	74%	74%	...
	Smed_0223	pRL110415	RHE_PE00291	mlr5708	...	Atu3485	Oant_3572	...
SMc02356	94%	...	...	...	...	...	...	...
	Smed_5345	...	...	...	...	...	...	...
SMc02486	93%	70%	70%	68%	61%	...	49%	...
	Smed_2937	RL4430	RHE_CH03884	ml14297	bl10450	...	Oant_2512	...
SMc03878	94%	83%	82%	79%	74%	78%	...	...
(<i>phbB</i>)	Smed_3116	RL4620	RHE_CH04019	mlr3848	blr3725	Atu2770	...	...

^a Abbreviations are as follows: Smed, *Sinorhizobium medicae*; RI, *Rhizobium leguminosarum* bv. *viciae*; Re, *R. etli*; MI, *Mesorhizobium loti*; Bj, *Bradyrhizobium japonicum*; At, *Agrobacterium tumefaciens*; Oa, *Ochrobactrum anthropi*; Bm, *Brucella melitensis*. Only those matches above 40% are reported as potential orthologs. Only one *fabG/nodG* ortholog was detected in all except *S. medicae*, and these were designated as *fabG* in each case.

on alfalfa (Table 2). For these mutants, the phenotypes ranged from Nod⁻, in which no nodules were formed, to the formation of abnormal-looking nodules accompanied by evidence of complete or partial nitrogen starvation (Fix⁻ or Fix^{+/-}). The Fix⁻ phenotype corresponds to <25% shoot dry weight of the plants inoculated with the wild type strain, whereas Fix^{+/-} corresponds to 25 to 75% of the shoot dry weight of the positive control. The SMb20492 mutants were the only mutants that were unable to form nodules. Interestingly, mutation of the upstream gene SMb20493 did not abolish nodulation but instead yielded nodules that were white and more elongated than those formed by the parental strain, and the plants were only partially nitrogen starved. The nature of the relationship between the symbiotic phenotypes and the carbon utilization phenotype of these two mutants demands further study. The other mutations that resulted in partial nitrogen starvation were those in SMA0719, which exhibited a complex carbon utilization phenotype, and SMb20214, which had no carbon utilization deficiencies. Nodules formed by SMA0719 mutants were white and more elongated than the nodules formed by the parental strain, whereas the nodules formed by the SMb20214 appeared similar to those formed by the parental strain. Inoculation with four of the SDR mutants (SMb20210, SMc02486, SMc00005, and SMc00326) resulted in complete nitrogen starvation. Nodules formed by mutants of SMc00005 and SMc00326, both annotated as *fabI* orthologs encoding enoyl-ACP reductase enzymes (EC 1.3.1.9) involved in fatty acid biosynthesis, exhibited severe abnormalities. Roots inoculated by the SMc00005 mutants were thick and swollen, with very few lateral roots present, and nodules only formed very infrequently, whereas the nodules formed on the SMc00326 mutant-inoculated roots were very small and white. Light microscopy revealed that bacteria were not visible in the SMc00326 mutant nodules (data not shown). The nodules formed by SMb20210 mutants were white and elongated, whereas the nodules formed by SMc02486 mutants were smaller in size and more numerous. In both SMb20210 and SMc02486 mutant-induced nodules, the plant cells were found to be replete with bacteria (light microscopy, data not shown).

Distribution of SDR orthologs in other genomes.

The genome sequences of numerous other α -proteobacteria are completed and available (Williams et al. 2007). We examined the distribution of orthologs of each of the 25 SDR-encoding genes for which phenotype or functions have now been associated. This analysis was limited to the very closely related *S. medicae* (unpublished) (GenBank accession number NC_009636); the other major root nodule bacteria *R. leguminosarum* (Young et al. 2006), *R. etli* (Gonzalez et al. 2006), *Mesorhizobium loti* (Kaneko et al. 2000), and *Bradyrhizobium japonicum* (Kaneko et al. 2002); the plant pathogen *Agrobacterium tumefaciens* (Wood et al. 2001); and the human intracellular pathogens *Ochrobactrum anthropi* (unpublished) (GenBank accession number NC_009667 to NC_009672) and *Brucella melitensis* (DeVecchio et al. 2002) (Table 3). Each SDR sequence was compared with the genomic data of these eight bacteria using BLASTP (Altschul et al. 1990) at the National Center for Biotechnology Information, and values above 40% amino acid sequence identity were reported as potential orthologs. Only three of the genes, the well-conserved *fabI* (SMc00005), *fabI2* (SMc00326), and *fabG* (SMc00572), were found in all eight of the genomes. The *fabI* gene, encoding enoyl-ACP reductase, is well known as the target of the popular antimicrobial agent triclosan (Sivaraman et al. 2004). The only other SDR-encoding gene with an ortholog in *B. melitensis* was SMb21159, which was present in all except *Bradyrhizobium japonicum*.

Although *O. anthropi* is acknowledged as a close relative of *Brucella* spp. (Williams et al. 2007), it shares a considerably greater number of SDR orthologs with *S. meliloti*. This could reflect its more versatile lifestyle, including association with nutrient-rich soil environments. It will be interesting to determine whether the *O. anthropi* orthologs of those SDR-encoding genes that have a link to symbiosis will be shown to have a similar involvement in pathogenesis.

As expected, most of the SDR in *S. meliloti* exhibited very high sequence identity with their orthologs in *S. medicae*, its closest relative with a very similar nodulation host range. Notable exceptions were the adjacent genes SMb20492 and SMb20493, both of which had no orthologs in *S. medicae* or *A. tumefaciens*. SMb20492 had orthologs in each of the other rhizobia except *R. etli*, and SMb20493 had orthologs in each of the rhizobia and also in *O. anthropi*. Considering the shared host range and recent evolutionary history of *S. meliloti* and *S. medicae*, the immediate question concerns what genes play the role of these missing genes in the *S. medicae* symbiosis. Interestingly, the best matches for SMb20492 were from *Burkholderia ambifaria* (ZP_01552904, unpublished) and *B. dolosa* (ZP_00983133, unpublished), both at 76% amino acid sequence identity. The best match for SMb20493 was from *Gluconacetobacter diazotrophicus* Pal5 (YP_001602914, unpublished), at 77% identity.

Intriguingly, two of the SDR, SMc01204 and SMc02356, were *Sinorhizobium* specific. They were found only in *S. meliloti* and *S. medicae*, and *S. meliloti* mutants of both of these genes were unable to utilize the same carbon source, ribonic- γ -lactone. It remains to be determined whether the utilization of this carbon source might hold special value in some aspect of their symbiotic interactions with the medic plants.

Concluding remarks.

Our work builds on previous pregenomic and post-genomic studies that revealed loci involved in symbiotic and catabolic functions (Charles and Finan 1991; Luo et al. 2005; Mauchline et al. 2006; Oresnik et al. 2000), and reinforces the stature of *S. meliloti* as a robust model organism for investigation of host-microbe interactions and bacterial cell biology and physiology. The collection of post-genomic experimental tools for *S. meliloti* is impressive, with platforms for transcriptional profiling (Barnett and Fisher 2006; Barnett et al. 2004; Rüberg et al. 2003), proteomics (Chen et al. 2003), and metabolomics (Barsch et al. 2004). Functional genomics activities are further supported by the availability of the Gateway-compatible ORFeome clone library (Schroeder et al. 2005) and signature-tagged transposon (Pobigaylo et al. 2006) and plasmid insertion or gene fusion (Cowie et al. 2006) mutant collections.

The results reported in this article confirm that members of the SDR family make significant contributions to the symbiotic and saprophytic life of *S. meliloti*. Although the symbiotic phenotypes are interesting in their own right and further investigations are sure to lead to insight into the symbiotic relationship, it will be equally interesting to establish links between catabolism of specific carbon sources and symbiosis. We still do not know the substrate specificities for most of the SDR enzymes; however, we have taken an important step toward gathering that knowledge. This will be particularly important in the context of provision of experimental validation of new molecular docking techniques aimed at the prediction of SDR substrate specificity (Favia et al. 2008). Finally, we note the striking number of pSymB-located genes identified in this study, which reinforces the role of pSymB in the auxiliary functions related to symbiosis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

S. meliloti RmP110, the wild type used in this study, is a derivative of Rm1021 with the point mutation in *pstC* corrected (Yuan et al. 2006). Culture methods in tryptone yeast (TY), Luria-Bertani, and RMM, and the concentration of antibiotics, were as described previously (Wang et al. 2006). Screening for carbon utilization phenotype was done on RMM (Broughton et al. 1986) supplemented with a single carbon source at a final concentration of 0.1%. The 81 carbon sources tested are listed in Table 1. Plasmids used in this study and the phage used for transduction are listed in Supplementary Table S4.

Plasmid construction and insertion mutagenesis.

SDR gene DNA fragments (approximately 600 bp) were amplified by PCR from *S. meliloti* RmP110 genomic DNA with specific primers for each gene. The primers were defined on one end by the start codon. For Sma2019, the shortest gene in our collection, a fragment of 270 bp was amplified. All PCR was carried out using *Taq* DNA polymerase (MBI Fermentas) or *Pfu* DNA polymerase (Promega Corp.). PCR products were cloned into an intermediary vector pGEM-T Easy Vector (Promega Corp.) in the T7 proximal orientation. The inserts were then released by digesting with *NotI* or *EcoRI*, purified with GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), and ligated to *NotI*-digested and dephosphorylated pTH1703 (Cowie et al. 2006) or *EcoRI*-cut and dephosphorylated pVIK112 (Kalogeraki and Winans 1997), followed by *E. coli* DH5 α (Hanahan 1983) transformation. The orientation of the inserts in each of the pTH1703 clones was confirmed by PCR using the *gfp* and *gus* primers and the corresponding forward primer that was used in the original amplification. Both orientations were retained if available. The orientation of the inserts in the pVIK112 clones was determined by PCR using the pVIK112-lac primer and the corresponding forward primer. Only clones with inserts in the same orientation as the lac reporter gene were retained. For six of the genes (Sma0326, Sma0959, Sma1629, SMb20662, SMc01157, and SMc01955), PCR amplification products were not successfully obtained; therefore, we made use of clones from the *S. meliloti* ORFeome (Schroeder et al. 2005). The *S. meliloti* ORFeome consists of each of the 6,204 ORF identified in the genome, cloned into pMK2010. Using appropriate restriction enzymes, internal fragments of these genes were subcloned into pTH1703. A 393-bp fragment of *HaeIII*-digested Sma1629, a 343-bp fragment of *HincII*-digested Sma0959, a 687-bp fragment of *AluI*-digested Sma0326, and a 329-bp fragment of *BmgBI*-cut SMc01955 were ligated to *SwaI*-cut, dephosphorylated pTH1703. Similarly, 616- and 285-bp fragments of *EagI*-digested SMb20662 and SMc01157, respectively, were ligated to *NotI*-cut, dephosphorylated pTH1703. Resulting plasmid constructs were mobilized from DH5 α into wild-type strain *S. meliloti* RmP110 by triparental mating using *E. coli* DH5 α (pRK600) as helper (Finan et al. 1986). Transconjugants were isolated on TY agar medium containing streptomycin and gentamicin (for pTH1703) or streptomycin and neomycin (for pVIK112), and were subjected to at least two rounds of single-colony streak purification.

Plant assays.

Two-day-old alfalfa (*Medicago sativa* var. Iroquois) seedlings were transferred to Jensen's (Jensen 1942) nitrogen-free agar slants or Jensen's saturated vermiculite in modified Leonard assemblies (Leonard 1943), as previously described (Aneja et al. 2005). Each slant contained one seedling and five seedlings

were planted per assembly. The seedlings were kept in a growth chamber (16 h of light at 25°C and 8 h of dark at 20°C, light intensity 300 $\mu\text{moles m}^{-2} \text{s}^{-1}$). Two days following seedling transfer, inoculation was carried out with 5 ml (assemblies) or 0.1 ml (slants) of a 1:50 water dilution of a saturated TY culture. Plants were grown for 4 to 6 weeks and examined for nodulation phenotype and symptoms of nitrogen starvation indicative of ineffective symbiosis. Shoot dry weight of 4-week-old plants was determined after separating the root and shoot systems and drying at 65°C for at least 10 days. Shoot dry weight of five plants from each of the three pots per strain was measured. The values are presented as the average weight per group of five plants, with standard deviation calculated using data from three pots.

Light microscopy.

Nodules were fixed in 10% formalin for 24 h and dehydrated by washing in a gradient of ethanol (20%, 1 $\times$ for 10 min; 50%, 1 $\times$ for 10 min; 70%, 3 $\times$ for 10 min; 95%, 2 $\times$ for 10 min; and 100%, 2 $\times$ for 10 min). Thereafter, the nodules were washed in xylene/ethanol (1:1; 1 $\times$ for 10 min), xylene (2 $\times$ for 10 min), paraffin/xylene (1:1; 1 $\times$ for 10 min), and paraffin (2 $\times$ for 5 min) and embedded in paraffin for sectioning. Using a microtome, 5- μm -thick sections were obtained, were placed in water on a slide coated with albumin, and were dried on a slide dryer at 60°C for 48 h. The sections were washed in xylene (1 $\times$ for 5 min and 1 $\times$ for 3 min), 100% ethanol (1 $\times$ for 5 min and 1 $\times$ for 3 min), 70% ethanol (1 $\times$ for 5 min), 50% ethanol (1 $\times$ for 3 min), and distilled water (1 $\times$ for 1 min). They were stained in toluidine blue (pH 4.5) for 20 s, washed in distilled water (2 $\times$ for 1 min), rehydrated by washing in a gradient of ethanol (50%, 1 $\times$ for 3 min; 70%, 1 $\times$ for 5 min; and 100%, 1 $\times$ for 5 min and 1 $\times$ for 3 min), rinsed in xylene (2 $\times$ for 5 min), and mounted using permount (Fisher Scientific). The slides were air dried for 48 h and observed using a Nikon ALPHA-PHOT-2 YS2 light microscope at $\times 40$ and $\times 400$ magnification, and digital images were captured.

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