Proteome Analysis of Differentially Displayed Proteins As a Tool for the Investigation of Symbiosis

Siria H. A. Natera, Nelson Guerreiro, and Michael A. Djordjevic

Plant-Microbe Interaction Group, Research School of Biological Sciences, Australian National University,
P.O. Box 475, Canberra City, Australia, 2601
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Two-dimensional gel electrophoresis was used to identify differentially displayed proteins expressed during the symbiotic interaction between the bacterium *Sinorhizobium meliloti* strain 1021 and the legume *Melilotus alba* (white sweetclover). Our aim was to characterize novel symbiosis proteins and to determine how the two symbiotic partners alter their respective metabolisms as part of the interaction, by identifying gene products that are differentially present between the symbiotic and non-symbiotic states. Proteome maps from control *M. alba* roots, wild-type nodules, cultured *S. meliloti*, and *S. meliloti* bacteroids were generated and compared. Over 250 proteins were induced or up-regulated in the nodule, compared with the root, and over 350 proteins were down-regulated in the bacteroid form of the rhizobia, compared with cultured cells. N-terminal amino acid sequencing and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry peptide mass fingerprint analysis, in conjunction with data base searching, were used to assign putative identity to nearly 100 nodule, bacterial, and bacteroid proteins. These included the previously identified nodule proteins leghemoglobin and NifH as well as proteins involved in carbon and nitrogen metabolism in *S. meliloti*. Bacteroid cells showed down-regulation of several proteins involved in nitrogen acquisition, including glutamine synthetase, urease, a urea-amide binding protein, and a PII isoform, indicating that the bacteroids were nitrogen proficient. The down-regulation of several enzymes involved in polyhydroxybutyrate synthesis and a cell division protein was also observed. This work shows that proteome analysis will be a useful strategy to link sequence information and functional genomics.

Additional keywords: differential protein display, nodulin.

The symbiotic interaction between members of the genera *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Azorhizobium* and plants of the Leguminosae (legume) family results in the formation of nitrogen-fixing root nodules. Genes from both the plant and these rhizobia play a role in the establishment and maintenance of this interaction (Bladergroen and Spaink 1998; Schultze and Kondorosi 1998), in which the plant supplies reduced carbon to the bacteroid (the

nodule-residing form of the bacteria) in exchange for fixed nitrogen. During this interaction many changes can also occur in the metabolism of the rhizobia as part of the occupation of this new environment as a result of the micro-aerobic environment present in the nodule.

The main changes in *Rhizobium* metabolism between the cultured cells and the bacteroids are the shift from sugar metabolism to C₄-dicarboxylic acid metabolism and the shift from ammonium assimilation to ammonium secretion. The switch to the use of C₄-dicarboxylic acids means that bacteroids generally do not contain or express the transporters for sugars such as sucrose. Bacteroids also appear to be nitrogen proficient and, in some cases, some amino acid auxotrophs (including glutamate auxotrophs) are still able to form functional nodules. Ammonium transporters expressed in cultured cells are required for nitrogen scavenging but are repressed in the bacteroid (Day and Copeland 1991; Udvardi and Day 1997).

S. meliloti nodulates *Melilotus alba* and other legumes including *Medicago truncatula* and *M. sativa*. The *S. meliloti* strain 1021 has been proposed as a model microsymbiont and *M. truncatula* as a model legume (Cook 1999). However, *M. alba*, a small-seeded, autogamous, and diploid legume with a small genome and a short stature, has also been recognized as a potentially useful system. This is because *M. alba* is predicted to have many of the same characteristics and patterns of gene expression as *M. sativa* (Kneen and LaRue 1988), which is tetraploid, and a series of nodulation mutants that are blocked at various stages of bacterial infection and nitrogen fixation have been identified (Utrup et al. 1993).

Recent studies of the changes in gene expression between the cultured and bacteroid states in *S. meliloti*, *R. etli*, *B. japonicum*, and *Rhizobium* sp. strain NGR234 were conducted to gain a further understanding of how the microsymbiont alters its metabolism to establish and maintain the symbiotic interaction with the host plant (Gage and Long 1998; Girard et al. 1996; Oke and Long 1999; Perret et al. 1999). Girard et al. (1996) studied global transcription patterns of pSym from *R. etli* and found that, during symbiosis, transcription of almost all the genes on this symbiotic plasmid was induced. However, they also showed that poor carbon and nitrogen sources, flavonoids, and low oxygen concentrations also led to pSym gene expression. Oke and Long (1999) also studied the expression of *S. meliloti* genes during symbiosis with a novel promoter trap procedure to identify genes that were expressed in the nodule prior to the establishment of nitrogen fixation.

Corresponding author: Michael A. Djordjevic; Fax: 61 2 62490754; E-mail: michael@rsbs.anu.edu.au

However, changes in mRNA expression levels do not necessarily correlate to changes in protein levels (Humphery-Smith et al. 1997).

The techniques of proteome analysis and differential protein display with two-dimensional gel electrophoresis (2-DE) have the power to monitor global changes that occur in the protein expression of a tissue or an organism, and have some advantages over other approaches commonly used for similar studies (Wilkins et al. 1996; Guerreiro et al. 1997). Indeed, Utrup and Norris (1996) used 2-DE differential display of *in vitro* translation products to identify nodulin and nodule-stimulated products from *M. alba* nodules formed by mutant strains of *S. meliloti*. However, most of these products were not characterized, with the exception of leghemoglobin, which was identified through the use of antibodies. Another example of the use of proteome analysis of symbiotic proteins came from Dainese-Hatt et al. (1999), who identified 38 proteins that were induced in *B. japonicum* by the transition from aerobic to anaerobic or micro-aerobic (2% oxygen) conditions.

To identify the contribution of the microbial and plant proteins to the nodule proteome pattern, we have used a comparative proteome analysis strategy. The proteome pattern of nodules was compared with that of mock-inoculated roots and isolated bacteroid cells. In addition, to understand the changes that occur in microbial gene expression in bacteroids, the proteomes of bacteroids and cultured bacteria were also compared. Put together, these comparisons enable the synthesis and turnover of hundreds of proteins to be monitored under different conditions and for the individual contributions of the interacting organisms to be teased apart and assessed for alterations in protein expression.

The post-separation techniques of N-terminal sequencing and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) in combination with bioinformatics can be used to determine putative identity of many of the individual proteins present. N-terminal sequencing is somewhat slow and expensive compared with MALDI-TOF MS, which allows a high throughput of samples with rapid analysis times (Shevchenko et al. 1996). In this study, we have used N-terminal sequencing to identify a number of specific nodule proteins and MALDI-TOF MS analysis for a large-scale study of protein identification based on peptide mass fingerprint (PMF) homology.

The utility of PMF data is enhanced in organisms where the entire genome sequence has been determined. In this paper, we have used protein sequencing and PMF data of separated proteins to determine how useful this approach would be for identifying proteins of interest given that the currently available data bases at the time of searching contain limited entries for *S. meliloti* and *M. alba*. In the case of *S. meliloti*, 196 and 306 data base entries could be found in the recent updates of the Swiss-Prot and TrEMBL data bases, respectively. In addition, many of the TrEMBL entries are for open reading frames or hypothetical genes. The complete genome of *S. meliloti* 1021 is currently being sequenced by an international consortium and incomplete DNA sequence data is already available as part of the BLAST server at NCBI or as a incomplete chromosome BLAST server (see Materials and Methods for Web links). A result of this work has been the publication of high-resolution maps of the *S. meliloti* chromosome and pSyma plasmid (Capela et al. 1999; Barloy-Hubler et al. 2000).

The work described here contributes additional information to the proposed *S. meliloti* proteome map (Guerreiro et al. 1999) via PMF matching to complement the genome sequencing. In this paper, we have examined the proteome of root nodules of *M. alba* infected with *S. meliloti* 1021. We were able to effectively separate the contribution of the micro-symbiont to nodule protein content. We present, for the first time, the proteome of *M. alba* root and nodules and the bacteroid state of the model organism *S. meliloti*, using directly isolated proteins.

RESULTS

Comparison of proteome patterns.

Over 1,700 individual, abundant proteins were resolved in silver stained proteome maps of mock-inoculated root and root nodule tissue of *M. alba* (Fig. 1A and B), respectively, as determined by analysis of duplicate gels. Upon first inspection, the patterns appear to be different. However, comparison of the two patterns showed that at least 1,300 spots were resolved in identical patterns. Of the remaining proteins, over 250 were either induced or up-regulated in nodule tissue (yellow and green circled spots in Figure 1B) and 20 root proteins were down-regulated in nodule tissue (red circled spots in Figure 1A).

We expected that many of the up-regulated proteins in nodule tissue were of bacterial origin. This was confirmed by comparing the proteome pattern of bacteroid proteins (Fig. 2B) with that of the nodule (Fig. 1B) and uninoculated root (Fig. 1A). This comparison showed that many proteins in nodules had a clear counterpart in bacteroid cells with respect to spot distribution, position, and coloring when stained with silver. An example of this was GroEL, which was identified in the nodule by N-terminal sequence homology (spot 12, Fig. 1B, and flanking isoforms) and in the bacteroid by PMF homology (spot x78, Fig. 2B; more analysis below). From this analysis, we concluded that over 180 of the up-regulated nodule proteins were from bacteroids (Fig. 2B) while another 70 appeared to be novel to nodule tissues and are potential new nodulins (Fig. 1B).

A comparison of bacteroid and cultured bacteria (both over 1,700 spots per gel) showed that a minimum of 350 proteins are down-regulated in the bacteroid, compared with cultured bacteria (red circled spots in Fig. 2A), and 130 proteins are up-regulated or induced in bacteroids (yellow and green circled spots, respectively, in Figure 2B).

Identification of nodule proteins by N-terminal sequencing.

Twenty-four nodule proteins, numbered in Figure 1B, were subjected to N-terminal amino acid sequencing. The homology of the protein sequences obtained to a non-redundant protein data base is shown in Table 1.

Six proteins (spots 2, 12, 14, 16, 21, and 22) were predicted to be bacterial on the basis of gel matching. This was confirmed by N-terminal sequence homology. These included spot 12, homologous to the molecular chaperone GroEL, spot 2, homologous to a nucleoside diphosphate kinase, and spot 16, homologous to a transcription elongation factor homologue. Spot 14, homologous to a 16-kDa, heat-shock protein from *Escherichia coli*, was reported to be up-regulated in

cultured *S. meliloti* at a late-exponential growth phase (Guerreiro et al. 1999). In addition, spot 21 showed homology to the TCA cycle enzyme, isocitrate dehydrogenase (ICD), which is required for effective nodules (McDermott and Kahn 1992). As expected, this spot was present in the nodule, bacteroid, and cultured bacteria. Spot 22 showed homology to enolase, an enzyme of the glycolytic pathway that is required by *S. meliloti* for growth on succinate (Finan et al. 1988).

Spots 3a, 4, 13, and 18 appeared to be plant-specific. Three of these proteins (spots 3a, 4, and 13) displayed homology to the late nodulin, leghemoglobin. Spot 18 did not display any significant homology to proteins in the data bases searched by both fasta3 and BLAST searches.

Spots 1, 6, 7, 10, and 19 were present in the nodule and bacteroid, but not in control roots or cultured *S. meliloti*, and are likely to be bacteroid specific. Spots 6 and 7, and spot 19, showed high homology to the nitrogenase genes *nifH* and *nifK*, respectively. Spot 1 appeared to be present in both the nodules and bacteroids, but not in roots or cultured *S. meliloti* cells. Spot 1 showed no homology to any proteins currently in the data bases when searched with a fasta3 search but showed homology to unspecified *S. meliloti* *fix* genes when a non-redundant BLAST search was performed (data not shown). The *fix* genes are required for nitrogen fixation and are primarily transcribed under low oxygen conditions found in the nodule (Fischer 1994). Spot 3b showed homology to a ferredoxin-like gene in the *nif* region from *Rhizobium* sp. strain NGR234. The location of this protein under spot 3a in the nodule makes it difficult to assess if this protein is also present in cultured bacteria and bacteroid but, given its identity, this is unlikely. Spots 8 and 10, which are also putatively classed as bacteroid-specific proteins, did not display any clear homology to proteins currently available in the data bases.

Of the predicted plant proteins sequenced, i.e., those present in both root and nodule gels (uncircled, numbered spots, Fig. 1B), homologies were found to (spot 5) calreticulin, an endoplasmic reticulum located calcium-binding protein (Coughlan et al. 1997), and the structural protein α -tubulin (spot 15). Spot 9 showed homology to a Bet v 1 protein from a family of plant pathogenesis-related proteins. Spot 17 showed 100% N-terminal homology to ascorbate peroxidase 2, an enzyme that uses ascorbate as an electron donor to prevent oxidative damage, especially in nitrogen-fixing legume nodules (Ross et al. 1999). Spot 24 showed homology to a protein disulfide isomerase precursor from *M. sativa* that is involved in the correct folding of proteins. Spots 11 and 23, which are putatively classed as constitutive plant proteins, did not display any clear homology to proteins currently available in the data bases.

PMF analysis of *S. meliloti* proteins.

A total of 500 proteins from either the bacteroid (nearly 100 proteins) or cultured *S. meliloti* cells (over 400 proteins) were examined by MALDI-TOF MS analysis to generate PMFs. Putative identities based on PMF homology are given in Tables 2, 3, 4, and 5. PMF matches to bacteroid proteins are shown in Tables 2 and 3 while matches to proteins from cultured *S. meliloti* cells are shown in Tables 4 and 5.

The 13 bacteroid proteins identified were further classified based on whether the levels present were the same as or different from that of the corresponding protein at the same gel

location in gels from cultured bacteria. Five bacteroid proteins appeared to be present at the same levels as in the cultured *S. meliloti* gels and were classed as constitutive (Table 2). These included malonyl-CoA decarboxylase, the molecular chaperone GroEL A, 30S ribosomal protein, NAD-dependent malic enzyme (ME), and betaine aldehyde dehydrogenase. Malonyl-CoA decarboxylase is involved in the malonate-metabolizing pathway that converts malonate to acetyl-CoA in the malonate-rich nodule and may play an important role in symbiosis (An and Kim 1998). Betaine aldehyde dehydrogenase catalyzes the conversion of betaine aldehyde to betaine, which may be used as either a carbon or nitrogen source or as an osmoprotectant in *S. meliloti* (Pocard et al. 1997). The other eight proteins identified from the bacteroid were up-regulated or induced, compared with their levels in cultured bacteria (Table 3). These included proteins with homology to NifD, DnaK, malate dehydrogenase (MDH), alcohol dehydrogenase (ADH), and four distinct spots with homology to the NifH protein.

Twenty-six proteins from cultured *S. meliloti* 1021 were tentatively identified on the basis of PMF homology and were classified as either constitutive (Table 4) or down-regulated, compared with levels in the bacteroid (Table 5). All but three of the proteins listed in Table 4 displayed homology to proteins from *S. meliloti* or closely related *Rhizobium* spp. and included three proteins with homology to 30S ribosomal protein S1 and two to GroEL C. Other proteins had homology to *S. meliloti* GroES C and a transcription factor homologue from *R. leguminosarum*, as well as proteins with homology to the PII protein, acetoacetyl-CoA reductase, and ADH. The other three proteins showed homology to *A. tumefaciens* proteins. Two of these had homology to elongation factor G and the other to elongation factor TU. Table 5 shows 14 proteins that were down-regulated in the bacteroid. These proteins showed homology to proteins involved in the regulation of carbon and nitrogen metabolism, a cell division protein, a protein involved in stationary phase survival, and a putative periplasmic iron-binding protein. Again, all proteins in this class showed homology to proteins from *S. meliloti* or closely related *Rhizobium* spp.

PMF of equivalent spots from cultured bacteria gels and bacteroid gels was used to confirm that the spots were in fact the same protein. For example, spot x89 from bacteroids is equivalent to spot z57 from the cultured bacteria and showed PMF homology to 30S ribosomal S1 protein. In the case of spots x70 and a15 from bacteroids, these spots were equivalent to spots 6 and 7 from the nodule, respectively. The PMFs of these matched to the same protein as that identified by N-terminal sequencing, namely, NifH.

Another four proteins, designated rm1, 11, 12, and 14, that were previously sequenced (Guerreiro et al. 1999), were also down-regulated in the bacteroid and are numbered in Figure 2A. Proteins rm1 and rm12 showed homology to a hypothetical amide-urea binding protein and enoyl-CoA hydratase, respectively, while rm11 and rm14 did not match to known proteins in a non-redundant protein data base.

S. meliloti data base matching in conjunction with PMF analysis.

The results of PMF searches against an incomplete *S. meliloti* 1X genome sequence data base followed by searches

Fig. 1. Two-dimensional gel electrophoresis separation of total protein from wild type *Melilotus alba* stained with silver nitrate. **A**, Roots collected 12 days after mock-inoculation. **B**, Nodules harvested 12 days after inoculation with *Sinorhizobium meliloti*. Total protein (100 μ g) was loaded in each case. Comparisons of root and nodule protein maps revealed differences shown where red circles indicate proteins down-regulated in the nodule, green indicates proteins likely to be bacterial in origin by comparison to Figure 2B, and yellow indicates novel nodule proteins. Numbered proteins correspond to proteins subjected to N-terminal sequencing (see Table 1). Proteins labeled “rm” were previously identified by Guerreiro et al. 1999.

against a *S. meliloti* chromosomal BLAST server are listed in Tables 6 to 9. These proteins were listed in separate tables due to the two-step procedure used to generate PMF matches (see Materials and Methods).

Five up-regulated or novel bacteroid proteins are listed in Table 6. Three proteins in Table 6 showed homology to a small heat-shock protein while another protein showed homology to adenylosuccinate synthetase, which is involved in the de novo biosynthesis of AMP. The other potentially novel bacteroid protein (spot a20) showed a match to a hypothetical protein when a *S. meliloti* data base search was conducted but, when a general protein data base was searched, showed homology to MDH (see Table 3).

Of the eight constitutive proteins listed in Table 7, the homology of two proteins (spots x89 and y15 matching 30S ribosomal protein and ME, respectively) agreed with a more general data base search (see Tables 2 and 7). Six other proteins identified included a hypothetical protein (spot a19) and the beta subunit

of glutamyl-tRNA amidotransferase (spot x64), which is involved in ATP-requiring binding of glutamyl-tRNA for translation of nucleic acid sequence into proteins. Spot y9 showed homology to adenylosuccinate lyase, which is involved with the de novo synthesis of purines. In *B. subtilis*, this protein regulates the activity of glutamyl-tRNA synthetase and, as such, the interaction between these two enzymes may play a role in coordinating purine metabolism and protein biosynthesis (Gendron et al. 1992). Spot y2 showed PMF homology to an aminopeptidase P that is involved in the degradation of small, bioactive peptides. Recently, Barloy-Hubler et al. (2000) showed that a large proportion of the pSyma of *S. meliloti* contains genes involved in the metabolism of small molecules. Spot y14 showed homology to an ATP-dependent protease that may be involved in the degradation of short-lived regulatory and abnormal proteins. Spot y20 showed homology to a single-strand binding protein that is essential for replication of the chromosome and is also involved in DNA recombination and repair.

Table 1. N-terminal sequence of proteins isolated from *Melilotus alba* nodules^a

Nodule protein characteristics			Characteristics of matching protein from data base			
Spot no.	M _r /pI	N-terminal sequence	Protein (% identity)	Organism	M.w./pI ^b	Swiss-Prot access. no.
1	22,000 / 5.47	TYKTILLVVGISXEDDLRA	Unknown			
2	17,100 / 6.06	AIERTFSMIK	Nucleoside diphosphate kinase [EC 2.7.4.6] (90%)	<i>Escherichia coli</i>	15,332 / 5.55	P24233
3a ^c	13,800 / 6.37	QFTEVQEALVQSGAE	Leghemoglobin (71%)	<i>Psophocarpus tetragonolobus</i> (asparagus bean)	15,551 / 5.90	P27199
3b ^c	13,800 / 6.37	AFTIVQSQCTNC	Ferredoxin-like gene in the NIF-region (67%)	<i>Rhizobium</i> sp. strain NGR234	6,950 / 6.13	Q53204
4	13,900 / 6.62	GFTEKQETLVNS	Leghemoglobin I (92%)	<i>Vicia faba</i> (broad bean)	15,400 / 6.34	P02232
5	53,100 / 4.37	SVFFEERFE	Calreticulin (100%)	<i>Ricinus communis</i> (castor bean)	47,500 / 4.38	P93508
6	33,100 / 5.75	AALRQIAFYG	Nitrogenase reductase [EC 1.18.6.1], NifH (100%)	<i>Sinorhizobium meliloti</i>	32,023 / 5.44	P00460
7	33,100 / 5.62	AALRQIAFYG	Nitrogenase reductase [EC 1.18.6.1], NifH (100%)	<i>S. meliloti</i>	32,023 / 5.44	P00460
8	25,900 / 4.41	[S/M]RAVL(A)F(I)H	No good match			
9	16,400 / 5.03	GVFTFNDEHV	L1R18B protein, Bet v 1 family (78%)	<i>Lupinus luteus</i> (yellow lupine)	16,600 / 5.35	P52779
10	30,300 / 5.50	[N/D]IRQEEKGTE	No good match			
11	26,000 / 6.20	NFVIAI[D/V]YPAXRP	No good match			
12	54,200 / 5.10	AAKEV	GroEL	<i>S. meliloti</i>	57,673 / 5.11	P35469
13	14,300 / 6.24	GITQKQEALVNGSWE	Leghemoglobin (80%)	<i>Vicia faba</i> (broad bean)	15,875 / 5.90	P93849
14	18,000 / 6.52	MRHFDSPPLY	16-kDa heat-shock protein (80%)	<i>E. coli</i>	15,774 / 5.57	P29209
15	46,700 / 5.15	MRESIRIGFG	α-tubulin (100%)	<i>Pisum sativum</i> (garden pea)	49,653 / 4.92	P46259
16	20,000 / 4.90	VDKVPMTQGG	Transcription elongation factor homolog (80%)	<i>R. leguminosarum</i>	17,444 / 4.88	O68546
17	28,000 / 5.85	GKSYPVTSADQKAVE	Ascorbate peroxidase 2 [EC 1.11.1.11] (100%)	<i>Glycine max</i> (soybean)	27,139 / 5.65	Q39843
18	17,600 / 5.80	VEDRKVGVAIDFSRNSN	Unknown			
19	53,100 / 6.01	PQSAEKVLDH	Nitrogenase component I, NifK [EC 1.18.6.1] (89%)	<i>Rhizobium</i> sp. strain NGR234	57,302 / 5.81	P19067
20	48,400 / 5.25	Mixture of proteins - no clear sequence				
21	44,600 / 6.28	AKIKVANPVV	Isocitrate dehydrogenase [NADP] [EC 1.1.1.42] (90%)	<i>Sphingomonas yanoikuyae</i>	45,483 / 5.17	P50215
22	44,300 / 4.84	TAIDIIGREILDSR	Enolase [EC 4.2.1.11] (67%)	<i>Nitrosomonas europaea</i>	46,240 / 4.70	O85348
23	41,000 / 4.84	XDATLTIESWRNDDLA	No good match			
24	39,600 / 5.40	DDVVVLSEDNFEKEV	Protein disulfide isomerase precursor [EC 5.3.4.1] (87%)	<i>M. sativa</i> (alfalfa)	40,492 / 5.44	P38661

^a *M. alba* nodule proteins were characterized with N-terminal sequencing. Spot numbers were assigned arbitrarily and correspond to those marked in Figure 1B. A combination of proteins including those novel to the nodule and proteins likely to be bacterial in origin were chosen for analysis as well as proteins apparently present in both root and nodule. M_r and pI were estimated by gel migration relative to standards. When sequence was ambiguous, the alternative residues are indicated in brackets and all permutations were used for data base searching.

^b Theoretical molecular weight and pI values as calculated from the amino acid sequence.

^c Two independent amino acid sequences were called from the same area on the gel.

Twenty-eight proteins from cultured *S. meliloti* 1021 cells were putatively identified on the basis of PMF matching to the *S. meliloti* data base (Tables 8 and 9). Fourteen of these proteins were down-regulated in bacteroids while one protein was up-regulated (Table 8). The down-regulated proteins showed homology to several different types of periplasmic binding proteins, proteins involved in cobalamin and cysteine biosynthesis, a protease precursor, MDH, several hypothetical proteins, and a probable σ^{54} modulation protein that would be expected to be involved with the regulation of σ^{54} activity. This initiation factor is responsible for the expression of the nitrogen fixation proteins (*nif* operon), *glnA* encoding GSI protein, and *dctA* for dicarboxylate transport. Thirteen proteins were apparently constitutively expressed in bacteroids and cultured cells (Table 9).

PMF as a method of isoform identification.

PMFs provided a quick and economical method of identifying potential protein isoforms. Of the 400 bacterial proteins examined, 10% were clearly isoforms since they gave near identical PMF patterns (not shown). Protein isoforms often formed a closely bunched horizontal train and, less commonly, a vertical train. Examples of isoforms are indicated by the

boxed areas in Figure 2A. Clear isoforms of the PII protein, the chaperone GroEL, glutamine synthetase (GS), 30S ribosomal protein, heat-shock proteins and NifH were identified in either the cultured state or in the bacteroid. In some cases, isoforms of particular proteins were positioned near to each other but had significantly different molecular masses. An example of this is indicated in the black, circled area in Figure 2A.

DISCUSSION

Differential display of proteins with 2-DE has facilitated the analysis of the contribution of both symbiotic partners to the proteins present in the nodule. The two different post-separation techniques of N-terminal sequencing and MALDI-TOF MS PMF matching were used to assign putative identity to proteins of interest and have provided information for over 100 proteins from the bacterium and the plant. The magnitude of the alteration in bacterial metabolism in the change from the cultured (in vitro) to the symbiotic state was demonstrated by the up-regulation of over 130 proteins in the bacteroid and by the down-regulation of at least 350 proteins found in the cultured state. This represents changes in nearly 20% of the observable rhizobial proteome and a major proportion of the

Table 2. Peptide mass fingerprint (PMF) matching of *Sinorhizobium meliloti* proteins isolated from bacteroids and present in both bacteroids and cultured cells^a

Protein characteristics		Characteristics of matching protein from data base					
		No. peptide matches / no. PMFs	Sequence coverage (%) ^b	Protein match	Organism	M.w./pI ^c	Swiss-Prot access. no.
x74	51,800 / 6.17	7 / 178	18.2%	Malonyl-CoA decarboxylase	<i>Rhizobium trifolii</i>	51,416 / 7.77	Q9ZIP6
x78	55,500 / 5.10	15 / 119	35.4%	GroEL A	<i>S. meliloti</i>	57,673 / 5.00	P35469
x89	59,600 / 5.21	17 / 123	41.2%	30S ribosomal protein S1	<i>S. meliloti</i>	62,640 / 5.24	P14129
y15	73,500 / 5.86	25 / 56	46.9%	NAD-malic enzyme [EC 1.1.1.39]	<i>S. meliloti</i>	82,865 / 5.55	O30807
y33	52,500 / 5.60	7 / 41	19.1%	Betaine aldehyde dehydrogenase [EC 1.2.1.8]	<i>S. meliloti</i>	51,522 / 5.19	P54222

^a *S. meliloti* bacteroid proteins expressed equally in cultured bacteria were characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. Spot numbers were assigned arbitrarily and correspond to those marked in Figure 2B. M_r and pI were estimated by gel migration relative to standards.

^b Indicates percentage of the data base matched protein that was made up of the matching PMFs.

^c Theoretical values as calculated from the amino acid sequence.

Table 3. Peptide mass fingerprint (PMF) matching of novel or up-regulated *Sinorhizobium meliloti* bacteroid proteins^a

Protein characteristics		Characteristics of matching protein from data base					
		No. peptide matches / no. PMFs	Sequence coverage (%) ^b	Protein match	Organism	M.w./pI ^c	Swiss-Prot accession no. ^d
a15	32,500 / 5.62	8 / 29	34.7%	NifH, nitrogenase reductase [EC 1.18.6.1]	<i>S. meliloti</i>	32,024 / 5.44	P00460
a20	37,000 / 6.02	5 / 30	26.6%	Malate dehydrogenase [EC 1.1.1.37]	<i>Rhizobium leguminosarum</i> (bv. <i>viciae</i>)	33,590 / 5.54	O33525
x63	62,300 / 4.90	5 / 176	11.7%	DnaK	<i>S. meliloti</i>	69,072 / 4.94	P42374
x67	51,500 / 5.50	10 / 148	25.4%	NifD, nitrogenase molybdenum-iron protein alpha chain [EC 1.18.6.1]	<i>Rhizobium</i> sp. strain NGR234	56,944 / 5.52	P19066 0.5 Da
x70	32,500 / 5.75	12 / 160	25.9%	NifH	<i>S. meliloti</i>	32,024 / 5.44	P00460
x71	32,600 / 5.86	11 / 175	37%	NifH	<i>S. meliloti</i>	32,024 / 5.44	P00460 0.5 Da
x94	35,600 / 6.08	12 / 202	48.5%	Alcohol dehydrogenase [EC 1.1.1.1]	<i>S. meliloti</i>	36,235 / 5.68	O31186
y21	33,900 / 5.92	11 / 35	35.7%	NifH	<i>S. meliloti</i>	32,024 / 5.44	P00460

^a *S. meliloti* bacteroid proteins were characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. Spot numbers were assigned arbitrarily and correspond to those marked in Figure 2B. M_r and pI were estimated by gel migration relative to standards. "a" spots were cut from polyvinylidene difluoride (PVDF) membrane while all other spots were taken from colloidal Coomassie-stained gels.

^b Indicates percentage of the data base matched protein that was made up of the matching PMFs.

^c Theoretical values as calculated from the amino acid sequence.

^d Data base searches were done with a mass tolerance of ± 0.1 Da unless otherwise indicated.

abundant cellular proteins. However, changes in protein levels alone do not imply changes in function, as many enzymes, for example, are regulated by post-translational modifications. It is possible that a post-translational modification, such as phosphorylation, may change in-gel migration of a protein spot such that a protein classified as down-regulated may in fact still be present elsewhere on the gel. Identification of such cases will be possible once a comprehensive analysis of the entire proteome is carried out.

N-terminal sequencing identified a mixture of plant and bacterial proteins among those proteins found in the nodule. While some of the proteins in nodules were shown to be bac-

terial in origin, the origin of other nodule-specific proteins (Fig. 1B) could not be predicted by gel observation alone. These proteins may represent abundant nodule proteins of plant origin such as the late nodulin, leghemoglobin, which was identified within the nodule. The 2-DE migration pattern showed multiple leghemoglobin isoforms that correlate well with the eight isoforms of leghemoglobin identified in effective *M. alba* nodules by Utrup and Norris (1996) with immunoprecipitation. The absence of leghemoglobin and the lack of plant protein matches to the bacteroid sample are indicative of the purity of the bacteroid preparation. However, the presence of plant proteins in the bacteroid preparation

Table 4. Peptide mass fingerprint (PMF) matching of proteins from cultured *Sinorhizobium meliloti* cells and also present in bacteroids^a

Protein characteristics		Characteristics of matching protein from data base					
Spot no.	<i>M_r</i> /pI	No. peptide matches / no. PMFs	Sequence coverage (%) ^b	Protein match	Organism	<i>M.w</i> /pI ^c	Swiss-Prot access. no.
y41	53,700 / 5.40	17 / 61	44.5%	60-kDa chaperonin, C (GroEL C)	<i>S. meliloti</i>	58,101 / 5.41	P35471
y77	74,100 / 5.45	11 / 57	24.3%	Elongation factor G	<i>Agrobacterium tumefaciens</i>	78,045 / 5.13	P70782
y78	75,000 / 5.41	9 / 62	21.6%	Elongation factor G	<i>A. tumefaciens</i>	78,045 / 5.13	P70782
z56	60,700 / 5.25	7 / 19	16.5%	30S ribosomal protein S1	<i>S. meliloti</i>	62,640 / 5.24	P14129
z57	60,400 / 5.21	11 / 32	28.7%	30S ribosomal protein S1	<i>S. meliloti</i>	62,640 / 5.24	P14129
z58	60,700 / 5.17	9 / 26	21.0%	30S ribosomal protein S1	<i>S. meliloti</i>	62,640 / 5.24	P14129
z88	54,600 / 5.33	5 / 16	15.9%	GroEL C	<i>S. meliloti</i>	58,101 / 5.41	P35471
1-20	20,800 / 4.88	5 / 23	33.5%	Transcription elongation factor homologue	<i>Rhizobium leguminosarum</i>	17,445 / 4.88	O68546
1-24	13,000 / 5.34	5 / 21	55.4%	GroES C	<i>S. meliloti</i>	11,188 / 5.87	P35474
1-27	12,400 / 5.41	6 / 20	65.2%	Nitrogen regulatory protein PII	<i>S. meliloti</i>	12,281 / 5.26	Q52905
2-13	36,400 / 5.79	8 / 20	37.4%	Alcohol dehydrogenase [EC 1.1.1.1]	<i>S. meliloti</i>	36,235 / 5.68	O31186
2-18	43,200 / 5.42	5 / 20	25.8%	Elongation factor TU (EF-TU)	<i>A. tumefaciens</i>	42,627 / 5.18	P75022

^a Proteins from *S. meliloti* grown in culture and equally expressed in bacteroids were characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. Spot numbers were assigned arbitrarily and correspond to those marked in Figure 2A. *M_r* and pI were estimated by gel migration relative to standards.

^b Indicates percentage of the data base matched protein that was made up of the matching PMFs.

^c Theoretical values as calculated from the amino acid sequence.

Table 5. Peptide mass fingerprint (PMF) matching of proteins from cultured *Sinorhizobium meliloti* cells that were down-regulated in the bacteroid^a

Protein characteristics		Characteristics of protein from data base					
Spot no.	<i>M_r</i> /pI	No. peptide matches / no. PMFs	Sequence coverage (%) ^b	Protein match	Organism	<i>M.w</i> /pI ^c	Swiss-Prot access. no.
b25	36,900 / 6.17	6 / 25	23.2%	Glutamine synthetase [EC 6.3.1.2] (fragment)	<i>Rhizobium</i> sp. strain USDA 1177 (fragment)	32,614 / 5.58 (fragment)	Q9Z3J6
b26	35,600 / 6.18	9 / 30	43.7%	Glutamine synthetase [EC 6.3.1.2] (fragment)	<i>Rhizobium</i> sp. strain USDA 1177 (fragment)	32,614 / 5.58 (fragment)	Q9Z3J6
b27	36,500 / 6.07	6 / 46	33.1%	Malate dehydrogenase [EC 1.1.1.37]	<i>R. leguminosarum</i> bv. (viciae)	33,590 / 5.54	O33525
b40	34,500 / 5.81	6 / 15	22.3%	UDP-glucose 4-epimerase [EC 5.1.3.2]	<i>S. meliloti</i>	36,113 / 5.50	P26503
y70	28,200 / 5.27	6 / 35	27.0%	Survival protein SURE	<i>S. meliloti</i>	27,772 / 5.17	O08248
y92	32,300 / 4.79	8 / 59	32.8%	Putative periplasmic iron-binding protein	<i>S. meliloti</i>	34,055 / 4.83	Q9Z3S7
z9	65,800 / 5.24	14 / 73	31.7%	Cell division protein ftsZ homologue 1	<i>S. meliloti</i>	62,975 / 5.06	P30327
z19	28,300 / 4.65	8 / 30	46.1%	Chain 1: PhoD	<i>S. meliloti</i>	29,897 / 4.71	Q52907
1-26	11,500 / 5.37	5 / 20	57.1%	Nitrogen regulatory protein PII	<i>S. meliloti</i>	12,281 / 5.26	Q52905
1-32	11,500 / 6.22	5 / 20	79.0%	Urease gamma subunit [EC 3.5.1.5]	<i>S. meliloti</i>	11,128 / 5.77	P42887
1-52	26,400 / 6.47	6 / 17	41.1%	Acetoacetyl-CoA reductase [EC 1.1.1.36]	<i>S. meliloti</i>	25,370 / 6.15	P50205
1-53	27,700 / 6.48	7 / 17	41.5%	3-hydroxybutyrate dehydrogenase [EC 1.1.1.30]	<i>S. meliloti</i>	27,179 / 6.07	O86034
2-19	49,000 / 5.44	5 / 22	20.3%	Glutamine synthetase I [EC 6.3.1.2]	<i>S. meliloti</i>	52,035 / 5.20	Q59747
2-73	55,100 / 5.93	10 / 18	34.0%	Phosphoenol pyruvate carboxykinase (ATP) [EC 4.1.1.49]	<i>S. meliloti</i>	58,120 / 5.57	P43085

^a Proteins from *S. meliloti* cells grown in culture were characterized by MALDI-TOF MS analysis. Spot numbers were assigned arbitrarily and correspond to those marked in Figure 2A. *M_r* and pI were estimated by gel migration relative to standards. "b" proteins were taken from polyvinylidene difluoride (PVDF) blots while the other spots were taken from colloidal Coomassie-stained gels.

^b Indicates percentage of the data base matched protein that was made up of the matching PMFs.

^c Theoretical values as calculated from the amino acid sequence.

cannot be discounted because of the reduced sensitivity of cross-species matching when considering PMF matching alone (Cordwell et al. 1995). NifK and NifH, which are components of the bacterial nitrogenase enzyme complex, were also identified and their relative abundances in bacteroid and nodule preparations were similar (Figs. 1B and 2B and Table 1).

Proteins involved in nitrogen metabolism.

Several proteins involved in nitrogen metabolism and regulation were identified on the basis of PMF homology. Two forms of the PII protein were identified with a reduction in one form at the protein level found in the bacteroid. Although clear differences in molecular mass and pI are observed, the spectra for the two proteins (spots 1-26 and 1-27, Fig. 2A) were very similar (spectra not shown). In *S. meliloti*, the PII protein is involved in both nitrogen metabolism and nodule development (Arcondéguy et al. 1997). This intracellular signal transducer is required in the free-living state and is involved in the regulation of GS activity. PII is regulated by feedback inhibition by glutamine, covalent modification, namely, uridylation and regulation at the protein synthesis level. Under limited nitrogen conditions, PII is uridylylated and as a result GS, which is involved in ammonium assimila-

tion, is activated, while under high nitrogen conditions the reverse occurs. Thus, the PII protein may be an example of the different functional activities and roles of post-translationally modified protein isoforms. However, it was not possible to determine if these two proteins were post-translationally modified isoforms (uridylylated and non-uridylylated) from the spectra with the FindMod tool available from ExPASy.

The down-regulation of a PII isoform is consistent with the observation that three proteins with homology to GS were also found to be down-regulated in the bacteroid. GS is generally important to nitrogen metabolism since glutamine is a precursor of many other metabolites and is part of the ammonium assimilation and amino acid biosynthesis pathways. Although *S. meliloti* contains three forms of GS, named GSI, GSII, and GSIII (de Bruijn et al. 1989), regulation of their expression and function is complex and dependent upon transcriptional and post-translational controls. Arcondéguy et al. (1996) showed that the activity of GSI was regulated by adenylation and that there was a reduction in the amount of GS protein present in bacteroid cells. GSII is not expressed in *S. meliloti* bacteroids (de Bruijn et al. 1989) and GSIII is not normally expressed in the presence of GSI or GSII (Shatters et al. 1993). Swiss-Prot predicts the molecular weights for the three

Table 6. Putative identity of *Sinorhizobium meliloti* 1021 bacteroid proteins present at altered levels, compared with cultured cells^a

Peptide characteristics			MassLynx results		BLAST search results		
Spot no.	<i>M_r</i> /pI	Status ^b	Matches / missed cleavages (sequence coverage [%])	Clone	Protein	Identity (%)	Hit features (% query / % clone covered)
a17	17,900 / 6.24	Novel	7 / 4 (45)	491005A07.x1	Small heat shock protein	45	79 / 70
a20	37,000 / 6.02	Novel	9 / 4 (58)	423012D12.x1	Hypothetical	90	85 / 100
							Malate dehydrogenase <i>Rhizobium leguminosarum</i>
x77	17,800 / 6.53	Up	7 / 4 (45)	491005A07.x1	Small heat shock protein	45	79 / 70
y10	17,900 / 6.28	Novel	7 / 4 (45)	491005A07.x1	Small heat shock protein	79	79 / 70
y22	42,700 / 5.80	Up	5 / 0 (36)	423037G10.x1	Adenylosuccinate synthetase	92	78 / 36

^a Proteins from *S. meliloti* 1021 bacteroids were characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. The peptide mass fingerprints generated were used to interrogate a 1X *S. meliloti* sequence data base that had been translated in all six reading frames (see Materials and Methods). The matching putative open reading frame was then used to search an *S. meliloti* chromosome BLAST server. Spot numbers were assigned arbitrarily and correspond to those marked in Figure 2B. *M_r* and pI were estimated by gel migration relative to standards.

^b Status: Novel - novel to source, Up - up-regulated in source.

Table 7. Putative identity of *Sinorhizobium meliloti* 1021 bacteroid proteins constitutively expressed in cultured and bacteroid cells^a

Peptide characteristics		MassLynx search results		BLAST search results			Swiss-Prot search result
Spot no.	<i>M_r</i> / pI	Matches / missed cleavages (seq. coverage [%])	Clone	Protein	Identity (%)	Hit features (% query / % clone covered)	Protein - Organism
a19	24,000 / 4.85	5 / 0 (48)	423110A01.x1	Hypothetical	91	100 / 79	
x64	53,500 / 4.96	9 / 1 (51)	423112A04.x1	Glutamyl-tRNA amidotransferase subunit B	92	100 / 46	
x89	60,200 / 5.21	7 / 2 (44)	423121D08.x1	30S ribosomal protein S1	93	98 / 39	30S ribosomal protein S1 <i>S. meliloti</i>
y2	60,800 / 5.60	5 / 0 (40)	423069D10.x1	Aminopeptidase P	82	98 / 31	
y9	47,200 / 6.43	6 / 2 (40)	423087H05.x1	Adenylosuccinate lyase	87	98 / 53	
y14	80,100 / 5.78	7 / 1 (51)	423047G06.x1	ATP-dependent protease (HSP)	84	97 / 22	
y15	73,500 / 5.86	6 / 2 (31)	423110G01.x1	NAD-malic enzyme	93	100 / 29	NAD-malic enzyme <i>S. meliloti</i>
y20	19,600 / 5.85	5 / 0 (73)	423078E07.x1	Single-strand binding protein	51	100 / 62	

^a Proteins from *S. meliloti* 1021 bacteroids were characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. The peptide mass fingerprints of the proteins generated were used to interrogate a 1X *S. meliloti* sequence data base that had been translated in all six reading frames (see Materials and Methods). The matching putative open reading frame was then used to search an *S. meliloti* chromosome BLAST server. Spot numbers were assigned arbitrarily and correspond to those marked in Figure 2B. *M_r* and pI were estimated by gel migration relative to standards.

different forms of *S. meliloti* GSs as 52,035, 36,966, and 48,481 for GSI, GSII, and GSIII, respectively. Therefore, it seems likely that spot 2-19 is GSI while the other two spots (b25 and b26) are GSII, based on molecular mass similarities.

In bacteroids, the gamma subunit of urease, which as part of the urea cycle catalyzes the conversion of urea to NH_3 , was also down-regulated, as was the urea-amide binding protein, *rm1*. The down-regulation of proteins involved in the uptake and metabolism of urea or other similar compounds is consistent with the shift from ammonium assimilation to ammonium export and the idea that bacteroids are nitrogen proficient (Kahn et al. 1998).

Proteins involved in carbon metabolism.

Non-symbiotic bacteria metabolize sugars such as sucrose but evidence suggests that metabolism switches to C_4 -dicarboxylic acids such as malate and succinate in the nodule. As a result, it would not be surprising to see changes in the levels of proteins involved in these different parts of the carbon metabolic cycle.

Several enzymes of the TCA cycle were identified, including MDH and IDH. MDH was identified in both cultured and bacteroid forms of *S. meliloti* 1021 although the isoelectric points appeared to differ slightly. Unfortunately, because *S. meliloti* MDH is not represented in Swiss-Prot, it is difficult to tell what the difference between the two forms analyzed might be. The spectra obtained are largely identical and the FindMod tool available from ExPASy suggested that the bacteroid form may contain one methylated site while the form from cultured

cells may contain four methylation sites (data not shown). However, it is not clear whether the proteins analyzed are the same but with modifications, or are actually different forms of the enzyme. IDH, which was identified in the nodule and appeared to be present in bacteroids and cultured *S. meliloti* cells, may act as the source of reductant for nitrogen fixation, as IDH activity in bacteroids isolated from *P. sativum* nodules was found to be highest when nitrogen fixation was at a maximum (Kurz and LaRue 1977).

A protein with homology to NAD-dependent ME was also identified. ME catalyzes the formation of pyruvate from malate, a C_4 -dicarboxylic acid. C_4 -dicarboxylic acids are generally thought to be the reduced carbon source supplied to the bacteroids by the plant (Day and Copeland 1991). Both NADP- and NAD-dependent forms of ME have been identified in *S. meliloti*, although NADP-dependent ME is not required for nitrogen fixation (Driscoll and Finan 1996). The NAD-dependent form of ME, which we identified from *S. meliloti* bacteroids, is speculated to be the more important form during nitrogen fixation. Day and Copeland (1991) speculated that, together with MDH, NAD-ME could provide an effective means of generating the acetyl-CoA and oxaloacetate required for the TCA cycle.

Two proteins with PMF homology to ADH were also identified. This enzyme catalyzes the conversion of aldehydes or ketones into alcohol under anaerobic conditions, generating NAD^+ required for glycolysis. Interestingly, one protein (spot 2-13) appeared to be present in both the cultured and bacteroid forms of the bacteria, while another (spot x94) appeared to be

Table 8. Putative identity of proteins from cultured *Sinorhizobium meliloti* 1021 cells present at altered levels, compared with bacteroids^a

Peptide characteristics			MassLynx results		BLAST search results			Swiss-Prot search result
Spot no.	M_r / pI	Status ^b	Matches / missed cleavages (seq. coverage [%])	Clone	Protein	Identity (%)	Hit features (% query / % clone covered)	Protein - Organism
b4	17,500 / 5.32	Down	8 / 4 (55)	423112F07.x1	Hypothetical	90	85 / 100	Malate dehydrogenase <i>R. leguminosarum</i>
b27	36,500 / 6.07	Down	5 / 0 (63)	423012D12.x1	Malate dehydrogenase	81	100 / 48	
y68	34,400 / 5.38	Down	6 / 0 (41)	423056B01.x1	O-acetylserine sulfhydrylase A	95	97 / 70	
y87	35,500 / 5.91	Down	6 / 0 (32)	423019B02.x2	Cobalamin biosynthesis (COBS)	99	99 / 63	
y88	34,100 / 5.94	Down	6 / 0 (56)	423036G01.x1	Hypothetical	32	82 / 44	
z10	46,900 / 4.79	Down	7 / 0 (52)	423085D04.x1	Periplasmic sorbitol-binding protein	100	100 / 47	
z20	40,900 / 4.88	Down	8 / 1 (60)	423085D04.x1	Periplasmic sorbitol-binding protein	100	100 / 47	
z35	58,000 / 5.71	Down	5 / 1 (30)	423106B05.x1	Periplasmic dipeptide-binding protein	26	96 / 40	
z93	58,000 / 4.78	Down	5 / 1 (32)	423001E01.x1	Protease precursor	79	96 / 38	
1-5	43,400 / 4.42	Down	6 / 2 (60)	423074B12.x1	ABC transporter periplasmic-binding protein	98	99 / 50	
1-48	19,700 / 6.53	Up	7 / 4 (45)	491005A07.x1	Small heat shock protein	45	79 / 70	
2-60	41,400 / 6.26	Down	6 / 0 (39)	423110C09.x1	Hypothetical	23	76 / 61	
2-94	27,200 / 5.88	Down	5 / 1 (40)	423043D06.x1	Probably σ^{54} modulation protein	93	100 / 91	

^a Proteins from *S. meliloti* 1021 cells grown in culture were characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. The peptide mass fingerprints of the proteins were generated and used to interrogate a 1X *S. meliloti* sequence data base that had been translated in all six reading frames (see Materials and Methods). The matching putative open reading frame was then used to search an *S. meliloti* chromosome BLAST server. Spot numbers were assigned arbitrarily and correspond to those marked in Figure 2A. M_r and pI were estimated by gel migration relative to standards.

^b Status: Down - down-regulated in source, Up - up-regulated in source.

up-regulated in the bacteroid. These proteins had almost identical PMF spectra (not shown), but it is unclear from the available data what post-translational modifications may be present. In *S. meliloti*, the gene encoding ADH (*adhA*) has been mapped to the 1.4-Mb megaplasmid, pSyma (Barloy-Hubler et al. 2000). However, Willis and Walker (1998) found that *S. meliloti adhA* mutants retained some ADH activity, suggesting that ADH is encoded by more than one gene. This may account for our observation of two proteins with homology to ADH.

Two proteins with homology to enzymes involved in polyhydroxybutyrate (PHB) synthesis, acetoacyl-CoA reductase, and 3-hydroxybutyrate dehydrogenase, were down-regulated in the bacteroid. PHBs act as a carbon sink (Walshaw et al. 1997) and can account for up to 98% of lipids extracted from rhizobia. Although the presence of PHB varies in the bacteroids of different rhizobia species, the down-regulation of the enzyme levels found here agrees with the observation that wild-type *S. meliloti* bacteroids do not contain PHB (Kahn et al. 1998).

Heat shock and cell division proteins.

Although changes in levels of proteins with homology to several heat-shock proteins and a cell division protein were identified, these proteins are probably altered as a consequence of symbiosis and not as a cause. Barloy-Hubler et al. (2000) found that these types of proteins, with the exception of those encoded by *groESLa*, did not appear to be encoded by pSyma on which genes involved in nodulation and nitrogen fixation are located.

The chaperonin DnaK, which is the major prokaryotic form of the heat-shock protein, was found to be up-regulated in the bacteroid. This protein is required for correct protein folding and is induced under stress conditions (Münchbach et al. 1999). Several small heat-shock proteins (spots 14, 1-48, a17, x77, and y10) were also up-regulated or induced in nodules. The process of infection and adaptation to the endophytic environment could be a stressful event for the bacteria (Brewin 1998) or, alternatively, these proteins could be involved in the folding and stabilization of many of the nodule-specific proteins, as has been found for NodD by GroEL C (Ogawa and Long 1995). The up-regulated or induced expression of DnaK and other heat-shock proteins in the bacteroid is consistent with this hypothesis.

Among bacteroid proteins that were apparently down-regulated was a cell division protein, ftsZ homologue 1, that putatively binds to and hydrolyzes GTP and is essential to the cell division process (Margolin et al. 1991). *S. meliloti* contains two *ftsZ* homologues that both map to the chromosome, but although the second homologue lacks the carboxy-terminal region conserved in other *ftsZ* proteins, overproduction of either inhibits cell division (Margolin and Long 1994). Overproduction of *ftsZ* resulted in branched and swollen cells, suggesting that these are the ways by which cell mass is increased in the absence of cell division (Latch and Margolin 1997). Although this protein appeared to be present at lower levels in the bacteroid than in cultured cells, we cannot infer what division status the bacteroids are in (i.e., lower *ftsZ* levels do not necessarily mean

Table 9. Putative identity of proteins from cultured *Sinorhizobium meliloti* 1021 cells constitutively expressed in cultured and bacteroid cells^a

Peptide characteristics		MassLynx results		BLAST search results			Swiss-Prot search result
		Matches / missed cleavages (seq. coverage [%])	Clone	Protein	Identity (%)	Hit features (% query / % clone covered)	Protein - Organism
Spot no.	<i>M_r</i> / pI						
y72	31,000 / 5.23	6 / 0 (45)	423123G06.x2	Hypothetical	87	100 / 61	
y91	32,500 / 4.84	8 / 3 (53)	423051A08.x1	Hypothetical	26	95 / 53	
z54	52,700 / 4.96	7 / 0 (45)	423021E03.x1	Glutamyl-tRNA amidotransferase subunit B	90	100 / 42	
z56	60,700 / 5.26	6 / 1 (43)	423121D08.x1	30S ribosomal protein S1	93	98 / 39	30S ribosomal protein S1 <i>S. meliloti</i>
z57	60,400 / 5.21	8 / 2 (49)	423121D08.x1	30S ribosomal protein S1	93	98 / 39	30S ribosomal protein S1 <i>S. meliloti</i>
z58	60,700 / 5.17	5 / 1 (35)	423121D08.x1	30S ribosomal protein S1	93	98 / 39	30S ribosomal protein S1 <i>S. meliloti</i>
z59	45,500 / 5.16	6 / 1	423085E05.x1	Histidinol dehydrogenase	100	97 / 48	
z89	53,200 / 5.31	6 / 0 (42)	491008F01.x1	Glutamyl-tRNA amidotransferase subunit B	91	100 / 48	
1.24	13,000 / 5.34	7 / 3 (54)	423063C06.x1	10 kDa chaperonin A	83	63 / 91	GroES C <i>S. meliloti</i>
1.49	22,900 / 6.62	7 / 1 (74)	423111D06.x2	Transcription anti-termination protein	91	100 / 80	
1.57	27,600 / 6.27	6 / 0 (46)	423097C03.x1	Oxidoreductase	31	98 / 86	
1.73	20,900 / 5.83	6 / 1 (74)	423078E07.x1	Single-strand binding protein	51	100 / 62	
2.20	48,300 / 5.39	8 / 2 (81)	423078C03.x1	Mannitol 2-dehydrogenase	98	98 / 39	
3.50	45,000 / 5.51	6 / 1 (40)	423034B11.x1	Hypothetical	91	98 / 42	
3.57	30,100 / 6.37	8 / 2 (50)	423047H05.x1	Hypothetical	99	100 / 69	

^a Proteins from *S. meliloti* 1021 cells grown in culture were characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. The peptide mass fingerprints of the proteins generated were used to interrogate a 1X *S. meliloti* sequence data base that had been translated in all six reading frames (see Materials and Methods). The matching putative open reading frame was then used to search an *S. meliloti* chromosome BLAST server. Spot numbers were assigned arbitrarily and correspond to those marked in Figure 2A. *M_r* and pI were estimated by gel migration relative to standards.

that cells are not dividing). In *E. coli*, levels of *ftsZ* transcription varied with the cell cycle with levels highest at about midcycle and lowest at division (Zhou and Helmstetter 1994). Earlier, Dai and Lutkenhaus (1992) found results of varying the relative levels of *ftsZ* and *ftsA* that suggested that the gene products may interact, and that it may be the ratio of *ftsZ* to *ftsA* that is critical in the control of cell division.

Other proteins.

Other proteins that were down-regulated in the bacteroid included several proteins with homology to periplasmic binding proteins (Tables 5 and 8). This is consistent with the finding of Gage and Long (1998), who identified a gene with protein sequence homology to a periplasmic oligopeptide-binding protein that was differentially expressed (decreased) when *S. meliloti* became an intracellular microsymbiont. The down-regulation of proteins involved in the uptake of sugars and small peptides is consistent with the shift in carbon and nitrogen metabolism outlined above.

We have also identified a protein with putative homology to a probable σ^{54} modulation factor and have classed it as being down-regulated in the bacteroid. Ronson et al. (1987) identified a *ntrA*-like gene whose gene product *ntrA* had homology to σ^{54} and is required for C_4 -dicarboxylate transport (*dctA*) and symbiotic nitrogen fixation (*nifA*). The down-regulation of the σ^{54} modulation factor observed here suggests that it may work as a negative regulator and, when absent, allows the σ^{54} transcription factor to function in gene transcription.

Oxygen regulation of protein synthesis.

During the symbiotic interaction the plant supplies the bacteroid with reduced carbon in exchange for fixed nitrogen as well as subjecting the bacteria to the changes in oxygen in the environment required for the functional activity of nitrogenase. In *B. japonicum*, proteins have been identified that change in expression levels as a result of the change in available oxygen concentration (Dainese-Hatt et al. 1999). Oxygen regulation could account for a proportion of the changes in protein levels observed in our gels.

Estimation of the extent of post-translational modification in *S. meliloti*.

MALDI-TOF MS analysis not only provided the likely identity of over 100 *S. meliloti* proteins but also confirmed that about 10% of the protein species analyzed were isoforms. For example, four forms of NifH were identified among the proteins novel to the bacteroid (compared with the bacteria). *S. meliloti* contains only one copy of the *nifH* gene (Corbin et al. 1982) and the presence of isoforms of the NifH protein has, to our knowledge, not previously been reported. Several isoforms of elongation factor G, the 30S ribosomal protein S1, small heat-shock protein, GroEL, and GS were also found. The extent of protein isoforms in rhizobia has not been previously established but the estimate of 10% of proteins being isoforms is consistent with a similar estimate of 18% made for *E. coli* (Link et al. 1997).

Membrane and other hydrophobic proteins are probably under-represented in 2-DE gels at the present time. Therefore, nodulins located in the membrane, such as nodulin 26, are unlikely to be identified by the method described in this paper due to their insolubility in the detergent used. However, recent

advances in 2-DE technology, particularly in areas such as development of improved detergents that are compatible with 2-DE and rehydration of the isoelectric focusing strip with the protein sample to be separated, are improving the chances of observing membrane proteins (Molloy 2000). Sample enrichment and sequential extraction methods will also help to improve the range of proteins detected by 2-DE proteome methods (Molloy et al. 1998) as will use of isoelectric focusing strips that cover wide pH ranges separated over longer distances (Görg et al. 1999).

Adding the results of our work to previously generated matches (Guerreiro et al. 1999), we now have information about the expression of over 100 proteins from *S. meliloti*. With the completion of the *S. meliloti* genome data base, the data generated here will help to provide a more complete picture of events occurring within this model microsymbiont during the transition from free-living to bacteroid states. In line with similar studies in yeast, we expect that confident matches for up to 90% of the PMF entries will be made once the complete *S. meliloti* genome sequence becomes available (Shevchenko et al. 1996).

MATERIALS AND METHODS

Chemicals.

All chemicals used were of the highest obtainable grade and are outlined in Guerreiro et al. (1997). MilliQ-quality water (Millipore, Bedford, MA) with resistance of greater than 18 M Ω .cm was used throughout.

Plant growth and inoculation.

M. alba seeds were obtained as a gift from A. M. Hirsch (University of California). Seeds were scarified and then surface sterilized in 70% vol/vol ethanol containing a 0.5% vol/vol Tween detergent for 5 min and then in commercial bleach (5% available chlorine present as sodium hypochlorite) for 15 min. After five rinses with sterile, distilled water, the seeds were placed on Bergensen's modified medium (BMM) plates (Bergensen 1961), covered with a thin layer of water agar (0.8% wt/vol in water), and placed at 4°C for 48 h in the dark to synchronize germination. The plates were then transferred into a 29°C incubator for 24 h in the dark to germinate. Germinated seedlings were transferred to Fåhræus medium plates (Fåhræus 1957) modified to contain 1.2 mM CaCl₂ and adjusted to pH 7.1. Five seedlings were transferred to each plate and grown in growth chambers with a 16 h night / 8 h day cycle at 20°C/25°C, respectively, 60% relative humidity, and a photon flux density of 160 $\mu\text{mol s}^{-1} \text{m}^{-2}$ with a combination of metal halide and fluorescent lighting. After 4 days, plants were inoculated at the root tip with 10 μl of either *S. meliloti* 1021 grown in liquid BMM containing between 10⁵ and 10⁶ bacteria, or liquid BMM only. After 12 days, nodules or sections of mock-inoculated roots (2 cm down from site of inoculation) were harvested and immediately frozen in liquid nitrogen.

Plant protein extraction.

Total cellular protein was extracted from 500-mg samples of roots or nodules. Extraction followed the method of Kamo et al. (1995) with some modifications. The samples were ground in liquid nitrogen and precipitated with cold (–20°C) 10% (wt/vol) TCA in acetone containing 0.07%

(wt/vol) dithiothreitol (DTT). After 1 h at -20°C , the sample was centrifuged at $35,000 \times g$ for 15 min at 4°C with a Sorvall SW-50.1 rotor (Du Pont, New Town, CT), which was used for all centrifugation steps. The precipitate was washed twice in acetone containing 0.07% (wt/vol) DTT with 30-min incubations at -20°C and centrifugation at $12,000 \times g$ for 15 min at 4°C for each wash. The samples were lyophilized for 5 min and immediately resuspended in 1 ml of lysis buffer (Guerreiro et al. 1997) by a combination of vortexing and sonication. Insoluble material was removed by centrifugation at $19,000 \times g$ for 15 min at 20°C . The protein concentration of the supernatant was determined by the Bradford dye-binding assay (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard.

Bacterial growth, bacteroid isolation, and protein extraction.

S. meliloti 1021 was used for all experiments and was grown at 28°C in BIII medium (Dazzo 1982) to early exponential phase (OD_{600} of 0.32). Cells were harvested and samples prepared for 2-DE (Guerreiro et al. 1997).

Bacteroids were isolated from *M. alba* nodules by the modified method of Day et al. (1989), with some additional unpublished modifications outlined here. Nodules (1.5 g fresh weight per extraction) were harvested 12 days after inoculation with *S. meliloti* and rinsed in ice-cold wash buffer containing 350 mM mannitol, 25 mM 2-[*N*-morpholino]ethanesulfonic acid (MES)-KOH (pH 7.0) and 3 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The peribacteroid units (PBUs) were isolated by the Percoll gradient method with proportional reduction in the volumes used. The 60–80% Percoll gradient band containing large, intact PBUs was resuspended in 12 ml of wash buffer by gentle inversion before being centrifuged in a Sorvall HB-4 rotor (Du Pont, New Town, CT) for 15 min at 5,000 rpm and 4°C . The supernatant was removed and the PBUs resuspended in 1 ml of wash buffer containing the proteinase inhibitors 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma, St. Louis, MO), 5 μM trans-exopoxysuccinyl-L-leucylamido-(4-guani-dino)butane (E64; Sigma) and 1 mM DTT. The sample was then vigorously vortexed for 2 min to rupture the PBUs. The mixture was transferred to a clean Eppendorf tube and centrifuged at 5,000 rpm (Eppendorf microfuge; Brinkman Instruments, Westbury, NY) for 5 min at 4°C to pellet pure bacteroids. Protein extraction from each bacteroid pellet was done by the method given in Guerreiro et al. (1997) with 1 ml of lysis buffer per pellet.

2-DE.

2-DE was done as previously described (Görg et al. 1995) with minor modifications (Guerreiro et al. 1997). Isoelectric focusing was done on 18 cm, linear pH 4 to 7, IPG strips (Amersham Pharmacia Biotech, Uppsala, Sweden). For analytical gels, an aliquot containing 100 μg of protein was loaded onto each IPG strip. For preparative gels, an aliquot containing 1 mg of protein was loaded onto each IPG strip. When multiple loadings were required, the isoelectric focusing run was restarted from the beginning after each loading.

Image processing and analysis.

Proteins on analytical 2-DE gels were visualized by silver staining (Rabilloud 1992) with the addition of 2.5 mM *N*-[2-

hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid], hemisodium salt (HEPES; Research Organic, Cleveland, OH) buffer to the silver nitrate solution (Rabilloud et al. 1994). The images were digitized at 600 dpi with a UMAX PS-2400X lamp scanner run under Photoshop 3.0 (Adobe, Mountain View, CA). The resulting images were transferred into the UNIX environment of an SUNW, Ultra 5 station (Sun Microsystems, Mountain View, CA) for analysis. With MELANIE 2.3 image analysis software (Bio-Rad, Hercules, CA) run under OpenWindows 3.6, protein spots (features) common to the compared gels were assigned as "landmarks." The gel images were automatically aligned and the remaining features matched between gels. In this way, features that were new or absent from a set of gels could be identified. On the basis of this gel pattern matching and subsequent quantification, proteins were tentatively assigned as constitutive or altered in expression. Quantification of the optical density (OD) of each feature over its area (vol) as a percentage of the total OD over the area of whole gel image (%vol) was used as a measure of changes in protein expression. When a difference of at least 2.5 times between features from the compared gels was found, this feature was marked as being differentially displayed in the figures. MELANIE 2.3 was also used to determine the relative molecular masses of the proteins by co-electrophoresis with the following standard proteins (Amersham Pharmacia Biotech): phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and lactalbumin (14.4 kDa).

Electroblotting, N-terminal sequencing, and MALDI-TOF MS analysis.

Electroblotting onto polyvinylidene difluoride membranes (PVDF) (Bio-Rad) was done (Guerreiro et al. 1997) with the exception of electrotransfer being performed at 0.9 mA/cm² for 90 min. Selected Coomassie (R-250)-stained spots were excised from dried membranes with scalpel blades and sent for either N-terminal sequencing or MALDI-TOF MS analysis. Spots for MALDI-TOF MS analysis were also taken from preparative gels stained for 24 h with a colloidal Coomassie stain modified from Neuhoff (Neuhoff et al. 1988). The composition of the colloidal Coomassie stain was obtained from the Australian Proteome Analysis Facility (Macquarie University, Sydney, Australia) 2-DE Manual and was as follows: 0.1% (wt/vol) Coomassie G-250 (Bio-Rad), 17% (wt/vol) $(\text{NH}_4)_2\text{SO}_4$, 3% (wt/vol) H_3PO_4 , 34% (vol/vol) methanol. The gel was destained in 1% (vol/vol) acetic acid.

N-terminal amino acid sequencing of PVDF protein spots was done on a PROCISE 494-01 sequencer system (Perkin-Elmer Applied Biosystems, Foster City, CA). The N-terminal sequences were used to interrogate data bases with Web-accessible search programs such as fastA and TfastA, available online from ANGIS, and fasta3, available online from the EMBL Outstation of the European Bioinformatics Institute (to identify homology to proteins already in protein and nucleic acid data bases. Data bases searched included SWISS-PROT, non-redundant (NR) protein and non-redundant (NR) nucleic acid data bases and Swall NR protein sequence data base plus SwissProt, TrEMBL and TrEMBLNew, respectively.

MALDI-TOF MS was used to determine the masses of the peptides generated following enzymatic digestion of an individual, isolated protein (Wheeler et al. 1996). These PMFs

were measured with a Micromass ToFSpec 2E Time of Flight Mass Spectrometer (Waters Corporation, Milford, MA) at the Australian Proteome Analysis Facility following tryptic digestion. These PMFs were then used to search protein and nucleic acid data bases with the PeptIdent program available online from ExPASy. This program compares the measured PMFs with computer-generated theoretical digests of the proteins in the data bases to look for possible matches based on PMF homology. Searches of Swiss-Prot and TrEMBL bacterial data bases were done for proteins derived from *S. meliloti* grown in culture whereas all data bases were searched for bacteroid proteins. Isoelectric point and molecular mass windows of ± 1.0 unit and $\pm 20\%$, respectively, were used. Initial searches were conducted with a mass accuracy of ± 0.1 Da but the accuracy was increased to ± 0.5 Da if no matches were found. Confidence in the PMF matches was based on multiple criteria including the species of the matching peptide, the difference in the number of matching peptides between the first and second matches, the number of PMF matches, number of missed cleavages, percent sequence coverage, and compatibility of isoelectric and molecular mass of the analyzed and data base protein (Wilkins and Gooley 1997).

***S. meliloti* chromosome BLAST server interrogation with MALDI-TOF MS-generated PMFs.**

PMFs generated by MALDI-TOF MS analysis of trypsin-digested proteins were also used to interrogate an incomplete *S. meliloti* 1X genome data base generated by Melanie Barnett and S. R. Long at the Department of Biological DNA Sequencing and Technology Center. The DNA sequences in this data base were translated in all six reading frames with software at the Australian Proteome Analysis Facility (Macquarie University, Sydney, Australia). The resulting protein sequences were then theoretically digested with trypsin to give predicted PMFs. PMFs of individual protein spots generated from MALDI-TOF MS analysis were then compared with these "theoretical" PMFs with the MassLynx version 3.4 browser (Micromass, Manchester, UK). The theoretical amino acid sequence from the open reading frames to which they matched was then used to interrogate the *S. meliloti* chromosome BLAST server available over the Web (links to both the BLAST server at NCBI and the *S. meliloti* chromosome BLAST server are available online from INRA). In this way, protein homology from the open reading frames could be linked to the PMF lists. The criteria for confidence in a match were similar to that described for direct PMF searches of non-redundant protein data bases. Although the results of the *S. meliloti* data base searches were viewed with a lower level of confidence than those of Swiss-Prot and TrEMBL bacterial data base searching because of the two-step matching process, in most cases the two searches gave the same match (see Tables 6 to 9). Agreement between the data bases is expected to improve as more *S. meliloti* sequence data become available.

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