Symbiotic Induction of Pyruvate Dehydrogenase Genes from *Sinorhizobium meliloti*

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Genes coding for components of the pyruvate dehydrogenase (PDH) multienzyme complex (PDHc) from *Sinorhizobium meliloti*, the alfalfa symbiont, have been isolated on the basis of their high expression in symbiotic bacteria. The E1p component, PDH, is encoded by two genes, *pdhA* α (1,047 bp) and *pdhA* β (1,383 bp), a situation encountered in the α -proteobacteria *Rickettsia prowazekii* and *Zymomonas mobilis* as well as in some Gram-positive bacteria and in mitochondria. *pdhA* α and *pdhA* β precede *pdhB* (1,344 bp), which encodes the E2p component, dihydrolipoamide acetyltransferase, of the PDHc. No gene encoding the E3 component, lipoamide dehydrogenase, was found in the immediate vicinity of *pdhA* and *pdhB* genes. *pdhA* α , *pdhA* β , and *pdhB* likely constitute an operon. Here, we provide evidence that *pdhA* expression is induced in the symbiotic stage, compared with free-living conditions. We demonstrate that symbiotic expression of *pdhA* genes does not depend on the *fixLJ* regulatory cascade that regulates nitrogen fixation and respiration gene expression in symbiotic *S. meliloti* cells. Induction of *pdhA* expression could be obtained under free-living conditions upon the addition of pyruvate to the culture medium. Induction by pyruvate and symbiotic activation of *pdh* gene expression take place at the same promoter.

Sinorhizobium meliloti is an α -proteobacterium that lives either as a free-living cell in the soil or as an intracellular symbiont plant of alfalfa and closely related legume species. The bacteria establish symbiosis by eliciting the formation of nodules on plant roots, in which they differentiate into bacteroids capable of fixing atmospheric nitrogen into ammonia to the benefit of the host plant (Mylona et al. 1995). Rhizobia are strict aerobes that make up the energy required for nitrogen fixation by operation of a tricarboxylic acid (TCA) cycle coupled to oxidative phosphorylation. To cope with the low ambient oxygen concentration of the nodule (10 to 30 nM O₂), rhizobia synthesize a specialized oxidase complex with high affinity for oxygen (Preisig et al. 1993). Expression of the corresponding *fixNOQP* operon is itself under O₂ control via a master two-component regulatory system, *fixLJ*, that also

controls nitrogenase synthesis in *S. meliloti* (David et al. 1988). In contrast to free-living bacteria and to bacteria at the early stages of plant colonization, nitrogen-fixing *S. meliloti* cells heavily depend on dicarboxylic acids (succinate, malate, and fumarate) as carbon sources. These compounds are provided by the plant partner and are translocated to the bacteroid by the action of two permeases located in the peribacteroid membrane and in the bacteroid membrane (McRae et al. 1989). The bacterial enzyme is a high affinity permease encoded by a single gene, *dctA*, that is required for entry of dicarboxylic acids in bacteroids. Accordingly, an *S. meliloti* *dctA* mutant is strictly unable to perform symbiotic nitrogen fixation (Fix⁻) (Engelke et al. 1987).

Because succinate, fumarate, and malate are intermediates of the TCA cycle, operation of the cycle requires anaplerotic synthesis of acetyl-CoA. It was proposed that acetyl-CoA is mainly generated in bacteroids from the combined activities of malic enzymes and pyruvate dehydrogenase (PDH; Driscoll and Finan 1993). So, PDH was postulated to be a key enzyme of carbon metabolism in bacteroid. However, genes coding for the PDH complex (PDHc), which catalyzes the irreversible oxidative decarboxylation of pyruvate to acetyl-CoA, have not been identified so far in *S. meliloti* or in other rhizobia.

In the course of a screening for *S. meliloti* genes highly expressed under symbiotic conditions, we have isolated an expressed sequence tag (EST) whose sequence analysis suggested that it encodes subunit E1p of the PDHc. Here we report on the isolation, genetic organization, and regulation of the *S. meliloti* *pdh* genes. Three genes have been found that encode the E1p and E2p components of the PDHc. Subunit E1 is encoded by two genes that are orthologues of genes found in other α -proteobacteria, in some Gram-positive bacteria, and in mitochondria. We show a preferential *pdhA* expression in the symbiotic stage, compared with free-living conditions, even when C₄-dicarboxylates were used as sole carbon source. We provide evidence that *pdhA* symbiotic expression is not regulated by oxygen and does not depend on the nitrogen status of the nodule. We have found that *pdh* expression can be induced by pyruvate in free-living cultures.

RESULTS

PDH cDNA isolation from *S. meliloti* bacteroids.

The development of N₂-fixing nodules requires the concerted expression of many bacterial and plant genes. On the bacterial side, most of the genes identified so far are involved

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in nitrogenase synthesis, the enzyme complex responsible for the reduction of N_2 into ammonia, or in energy production. These genes are under the control of oxygen, as their expression can be induced ex planta under microoxic conditions (Soupène et al. 1995). To identify other symbiotically important genes, we looked for genes that are highly expressed in bacteroids, compared with free-living oxic and microoxic bacteria. The selected method was the RAP (RNA arbitrarily primed) PCR (polymerase chain reaction) fingerprinting technique (Welsh et al. 1992; Wong and McClelland 1994). This method can be used to detect and clone transcripts that are differentially expressed in cells subjected to different environmental conditions. Briefly, total RNAs are reverse transcribed from an arbitrary primer into a first strand of cDNA. The second cDNA strand is synthesized with a second arbitrary primer and amplified by PCR. Products separated on a sequencing gel are compared, and differentially expressed cDNAs can be cloned.

We have compared gene expression patterns obtained with RNAs isolated from *S. meliloti* in free-living oxic, microoxic, and symbiotic conditions. This approach allowed us to isolate several ESTs differentially expressed during symbiosis (D. Cabanes, P. Boistard, and J. Batut, *manuscript in preparation*).

The present paper focuses on the characterization of one of these ESTs, 221 bp in size, that was predominant under symbiotic, compared with free-living oxic and microoxic condi-

tions, and whose sequence analysis revealed a similarity with the *pdhA* gene family.

Structure of *pdh* genes from *S. meliloti*.

The full-length *pdhA* gene was isolated by screening a genomic library of *S. meliloti* (kindly provided by T. Arcondéguy), with the 221-bp EST as a probe. From a selected cosmid clone, 3.9 kb of nucleotide sequence was determined on both DNA strands around the original EST with a primer-walking approach; relevant portions are presented in Figure 1. Several open reading frames (ORFs) were predicted from this nucleotide sequence with both the GLIMMER program (Salzberg et al. 1998) and NCBI-BLASTX comparisons against the NCBI-nr data base. Three ORFs exhibited significant similarities with components of the PDHc. The PDHc catalyzes the oxidative decarboxylation of pyruvate, the transfer of an acetyl moiety to CoA, and the reduction of NAD^+ (pyruvate + CoA + NAD^+ $\rightarrow$ acetyl-CoA + NADH + CO_2) by the combined action of three enzymes: PDH (E1p), dihydrolipoamide acetyltransferase (E2p), and lipoamide dehydrogenase (E3) (Patel and Roche 1990).

The predicted first and second ORFs were 1,047 and 1,383 bp in length, respectively. Translated sequences from these ORFs showed high similarity with the α and β subunits of the heterotetrameric E1p component of known PDH complexes (Figs. 2–4). The first ORF, named *pdhA α* , encodes a polypeptide of 348 amino acids, corresponding to a protein of 38 kDa,

Fig. 1. Relevant portions of the nucleotide sequence of the *Sinorhizobium meliloti* *pdhA $\alpha\beta$* and *pdhB* genes. Deduced amino acid sequence is shown below nucleotide sequence. Putative ribosome-binding sites (RBS) are shown in bold. Transcriptional start site upstream of *pdhA α* is marked with an arrow. Putative p-independent transcriptional terminator is shown. Primers used for polymerase chain reaction, reverse transcription-polymerase chain reaction, and primer extension reactions are indicated. Accession number of the sequence is AF190792.

with highest sequence identity to the E1 α subunit of the PDHc of two other α -proteobacteria, *Zymomonas mobilis* (59% identity) and *Rickettsia prowazekii* (52% identity), and eukaryotic mitochondria (*Homo sapiens*, 51% identity; *Arabidopsis thaliana*, 48% identity). The *S. meliloti* E1 α predicted polypeptide contains the thiamine pyrophosphate (TTP)-binding site

(residues 172 to 202) involved in binding metal ion, and conserved in TTP-binding enzymes (Hawkins et al. 1989) (Fig. 2).

The start of the second ORF, named *pdhA β* , was assigned 18 bp downstream of the *pdhA α* gene. *pdhA β* encodes a polypeptide of 460 amino acids, corresponding to a protein of 49 kDa with highest sequence identity (58%) to the E1 β

ZM						MAKATQ	DSNRPHKADV	16
SM						MAPRKS	ASVSSRKTA	16
HS			M	RKMLAAVSRV	LSGASQKPAS	RVLVASRNFA	NDAT...FEI	38
SC	MLAASFQRQP	SQLVRLGAV	LRTPTRIHV	RTMATLKTTD	KKAP.EDIEG	SDTVQIELPE		59
AT				MALSRL	SSRSNIITRP	FSAAFSRLIS	TDTTPITIE	36
RP								
ZM	GSAIPNHDLP	PIPGRYHADR	ELLEEFYRRM	LMIRRFEEER	CQLYGLCLIA	GFCHLYIGQE		76
SM	KPAKDFAGG	TIA...EFSK	EDDLKAYREM	LLIRRFEEKA	CQLYGMGFIG	GFCHLYIGQE		73
HS	KKCDLHRL	GPPVTTVLTR	EDGLKYRMM	QTVRRMELKA	DQLYKQKIIR	GFCHLCDGQE		98
SC	S.SFESYMLE	PPDLSYETSK	ATLLQMYKDM	VIIRRMEMAC	DALYKAKKIR	GFCHLSVGQE		118
AT	SLPFTAHLCD	PPSRSVESSES	QELLDEERTM	ALMRRMEIAA	DSLYKAKLIR	GFCHLYDGOE		96
RP	MD	IKPEKYKPTK	EYIKSEKDM	LLLRRFEEKC	CQLYGMCKIG	GFCHLYIGQE		52
ZM	AVAVGLQAAAL	QPGRDSVITG	YREHGHMLAY	GIDPKIVMAE	LTGRASGISH	GKGGSMHMF	S	136
SM	AVVVGMOAL	KEG.DQVITG	YRDHGHMLAC	GMSARGVMAE	LTGRRGGLSK	GKGGSMHMF	S	132
HS	ACCVGLEAGI	NPT.DHLITA	YRAHGFTFTR	GLSVREILAE	LTGRKGCCAK	GKGGSMHMYA		157
SC	AIAVGLENAI	TKL.DSIITS	YRCHGFTFMR	GASVKAVLAE	LMGRRAGVSY	GKGGSMHLYA		177
AT	AVAIGMEAAI	TKK.DAIITA	YRDHCIFLGR	GGSLHEVFSE	LMGRQAGCSK	GKGGSMHLYK		155
RP	AV.IISAVAMI	KKKGDSTITS	YRDHAHIILA	GTEPKYVLAE	LMGRATGCSK	GKGGSMHLED		111
TTP-binding domain								
ZM	TEHKFFGGNG	IVGAQVPLGA	GLAFAHKYRN	DGGCSAAYFG	DGSANOGQVY	EAYNMAALWK		196
SM	KEKHFFGGHG	IVGAQVSLGT	GLAFANRYRG	NDNVSLAYFG	DGAANOGQVY	ESFNMAALWK		192
HS	K...NFYGGNG	IVGAQVPLGA	GLALACKYNG	KDEVCLTLYG	DGAANOGQIF	EAYNMAALWK		215
SC	P...GFYGGNG	IVGAQVPLGA	GLAFAHQYKN	EDACSFTLYG	DGASNOGQVF	ESFNMAKLWN		235
AT	KESFFYGGHG	IVGAQVPLGC	GLAFAQYKN	EEAVTFALYC	DGAANOGOLF	EALNISALWD		215
RP	IPNKFFYGGHG	IVGAQVPIGT	GLAFAEKYNG	TNNICETFLC	DGAVNQGQVY	EAFNMAASLWG		171
ZM	LPVIVFIENN	GYAMGTSTOR	ANAHTALSER	GAGFGIPALV	VDGMDVLEVR	GAATVAVDWV		256
SM	LPVIYIENN	RYAMGTSVSR	ASAOTDFSQR	GASEFGIPGYQ	VDGMDVRAVK	AAADEAVEHC		252
HS	LPICIFICENN	RYGMGTSVER	AAASTDYKRR	GDE...IPGLR	VDGMDILCVR	EATRFAAAYC		273
SC	LPVVFICENN	KYGMGTAASR	SSAMTEYFKR	GOY...IPGLK	VNGMDILAVY	QASKFAKDWC		293
AT	LPAILVICENN	HYGMGTAESR	AAKSPSYKRR	GDY...VPGLK	VDGMDAFAVK	OACKFAKQHA		273
RP	LPIVYIENN	EYSMGTSVAR	STFMCDLYKK	GESFGIRGFQ	LDGMDFEEMY	NGTKQVAEYV		231
ZM	QAGKGPIILE	MKTYRYRGHS	MSDP.AKYRS	REEVNDMKEN	HDPLDNLKKD	LFAAG.VPEA		314
SM	RSKGKPIILE	MLTYRYRGHS	MSDP.AKYRS	KDEVQKMRSE	HDPIEQVKAR	LTDKGWATED		311
HS	RSKGKPIILE	LOTYRYRGHS	MSDPGVSYRT	REEIQEVRSK	SDPIMLLKDR	MVNSNLASVE		333
SC	LSGKGPVILE	YETRYRGCHS	MSDPGTTYRT	RDEIQHMRSK	NDPIAGLKM	LIDLCIATEA		353
AT	LE.KGPIILE	MDTYRYRGHS	MSDPGSTYRT	RDEISGVRQE	RDPIERIKKL	VLSHDLATEK		332
RP	RENSEFPVILE	VKTYRYRGHS	MSDP.AKYRS	KEEVEKYK.E	RDITLVRIREI	ILDNKYATEA		289
ZM	ELVKLDEDIR	QOVKEAADFA	EK...APLPAD	EELYTNILVG	KY.....			354
SM	ELKQIDKEVR	DIVADSADFA	QS...DPEPDV	SELYTDILL.				348
HS	ELKEIDVEVR	KEIEDAAQEA	TA...DPEPPL	EELGYHIYSS	DPPFEVRGAN	QWIKFKSVS.		390
SC	EVKAYDKSAR	KYVDEQVELA	DAAPPPEAKL	SILEEDVYVK	GTETPTLRGR	IPEDTWDFKK		413
AT	ELKDMKEIR	KEVDDAIAKA	KDCPMPEP..	SELETNVYVK	GFGTESFGPD	RKEVKASLP.		389
RP	DLKATIEQSVR	EIIKVAVEES	EN...SELPPE	DELYTEIYV.				326
ZM								
SM								
HS								
SC	QGFASRD							420
AT								
RP								

Fig. 2. Alignment of amino acid sequences of the E1 α components of pyruvate dehydrogenase (PDH) complexes from *Arabidopsis thaliana* (AT), *Homo sapiens* (HS), *Rickettsia prowazekii* (RP), *Saccharomyces cerevisiae* (SC), *Sinorhizobium meliloti* (SM), and *Zymomonas mobilis* (ZM). Amino acids conserved in at least three out of the six sequences are shaded. Functional domains identified in homologous proteins are indicated above the amino acid sequences. EMBL accession numbers of sequences used: AT, AAD39331; HS, CAA36933; RP, CAA14723; SC, CAA50657; ZM, CAA73384.

subunit of the *Z. mobilis* PDHc (Fig. 3). Like its *Z. mobilis* homologue, the *S. meliloti* E1 β component contains an extension at the N terminus (residues 2 to 77), identified as a lipoyl domain. This lipoyl domain contains a highly conserved ly-

sine residue (K 43) that is the potential lipoylation site, and the P(S/A)LSPTM (residues 8 to 14) sequence motif (Fig. 3).

The third predicted ORF (1,344 bp) started 13 bp after *pdhA β* . The *S. meliloti* *pdhB* gene encodes a protein of 447

Lipoyl domain										
ZM	MAIELKMPAL	SPTMEEGTLT	RWLVEGDSI	KAGEILAEIE	TDKAIIMEFEA	VDEGVITKIL	60			
SM	MPVEILMPAL	SPTMEEGTLT	KWLKNEGDKV	SSGDVIAEIE	TDKATMEVEA	VDKGTIGKLL	60			
HS										
SC										
AT										
RP										
ZM	IPEGSENVKV	GTAIAYLGTD	ANDVTLEGAS	AETKAEESAP	VASPAKTEAA	ALEEAATPSL	120			
SM	IAAGTEGVKV	NTPIAVLLQD	G.EAASDIDS	MKTEAPK.AE	TPKPAAAEAP	AASAAPVAAQ	118			
HS						MAA VSGLVRRPLR	13			
SC						MFSRLPTSL ARNVARRAPT	19			
AT						MLGILRQRA IDGASTLRRT	19			
RP										
ZM	GKV.INSAP	IPEGTEFFQO	TLREALRDAM	AEMMRDRDV	FVMGEEVAEY	QGAYKVTQGL	179			
SM	PKADVPSDPA	IPAGTEMATM	TVREALRDAM	AEMMRANEDV	FVMGEEVAEY	QGAYKVTQGL	178			
HS	EVSGLLKRRF	HWTAPAAALQV	TVRDAINOQM	DEELERDEKV	ELLGEEVAQY	DGAYKVSRLG	73			
SC	SFVRPSAAAA	ALRFSSTKTM	TVREALNSAM	AEELDRDDDV	ELIGEEVAQY	NGAYKVSRLG	79			
AT	RFALVSARSY	A...AGAKEM	TVRDALNSAI	DEEMSADPKV	FVMGEEVGOY	QGAYKITKGL	76			
RP		MQI	TVREALRDAM	QEEMLRDEKV	FVIGEEVAEY	QGAYKVTQGL	73			
ZM	LOEFGARRVV	DTPISEYGEFS	GIGVGAAMEG	LRPVIEFMTM	NFSMQAIDHI	INSAAKTHYM	239			
SM	LOEFGARRVV	DTPITEHGFA	GVGVGAAMTG	LRPIVEFMTF	NFAMQAIDQI	INSAAKTHYM	238			
HS	WKKYGDKRII	DTPISEMGEA	GIJAVGAAMAG	LRPICEFMTE	NFSMQAIDQV	INSAAKTHYM	133			
SC	LDRFGERRVV	DTPITEYGET	GLAVGAALKG	LKPIVEFMTSE	NFSMQAIDHV	VNSAAKTHYM	139			
AT	LEKYGPPEVV	DTPITEAGET	GIGVGAAYAG	LKPVVEFMTF	NFSMQAIDHI	INSAAKSNYM	136			
RP	LEQFGSKRVI	DTPITEYGEA	GLAVGAAFAG	LRPIVEFMTF	NFAMQAEDHI	VNSAAKTHYM	103			
ZM	SGGQVRCPIV	FRGPNGAAPR	VGAOHTONFG	PWYAAVPGLV	VLAPYDAIDA	KGLLKAARIS	299			
SM	SGGQMGAPIV	FRGPNGAAR	VAAQHSOCYA	AWYSHIPGLK	VVMPYTAADA	KGLLKAARID	298			
HS	SGGLQVPPIV	FRGPNGASAG	VAAQHSOCFA	AWYGHCPGLK	VVSPWNSEDA	KGLIKSAIRD	193			
SC	SGGTQKCOMV	FRGPNGAAVG	VGAQHSODFS	PWYGSIPGLK	VLVPYSAEDA	RGLLKAARID	199			
AT	SAGQINVPPIV	FRGPNGAAAG	VGAQHSQCYA	AWYASVPGLK	VLAPYSAEDA	RGLLKAARID	196			
RP	SGGQVRCPIV	FRGPNGAASR	VAAQHSQNYT	ACYSHIPGLK	VVAPYSAEDH	KGLMLTAIRD	163			
ZM	DDPVVFLECE	LLYGKTFDVP	K...MDDFVL	PIGKARTIRE	GKDVTVISYS	IGVSFALTAA	356			
SM	PNPVIFLENE	ILYGOSFEVP	K...LDDEFVL	PIGKARTIHR	GKDATLVSEF	IGMTYAIKAA	355			
HS	NNPVVFLENE	LMYGVPFEFP	PEAQSKDFLI	PIGKAKIERO	GTHITVVSHS	RFVGHCLEAA	253			
SC	PNPVVFLENE	LLYGSEFEIS	EEALSPEFTL	PYKAKIERE	GTDISIVTYT	RNVQFSLEAA	258			
AT	PDPVVFLENE	LLYGSEFEIS	EEALDSSECL	PIGKAKIERE	GKDVTVITFS	KMVGFALKAA	256			
RP	DNPVIFLENE	ILYGHSEFDVP	DIIEP....I	PFSKAKILKE	GSNVTIVTFS	IQVKIALDVV	219			
ZM	EALAKE.GID	AEVIDLRLTLR	PLDKETILOS	LAKTNRIVTV	EDGWPFVCSIS	SEIAAIAEME.	414			
SM	AELEAQ.GID	VEIIDLRLTIR	PMDLETVIES	VKKTGRILTV	EEGYPOSSVG	TEIATRVMO.	413			
HS	AVLSKE.GVE	CEVINMRTIR	PMDMETIEAS	VMKTNHLTV	EGGWPOFGVG	AEICARIMEG	312			
SC	EILQKKYGV	AEVINLRSIR	PLDTEAIKKT	VKKTNHLITV	ESTEPSPFGVG	AEIVAQVMES	318			
AT	EKLAE.GIS	AEVINLRSIR	PLDRATINAS	VKKTNHLITV	EEGEPQHGVC	AEICASVVE.	314			
RP	NILQND.NID	CELIDLRTIK	PLDTSIES	VKKTNRLTV	EEGWFEAGVG	ASIASIVMK.	277			
ZM	EGFDNLDAPV	LRTVNADTPT	PYAENLEKKG	LVNPEAIIIEA	VRKVCYRK.		462			
SM	QAFDYLDAPV	LTIAAGKDVPM	PYAANLEKLA	LPNVAEVVDA	VKAVCYK..		460			
HS	PAENFLDAPV	VRVTGADVPM	PYAKILEDNS	IPQVKDIIFA	IKKTLNI..		359			
SC	EAFDYLDAPV	QRTVGADVPT	PYAKELEDEFA	EPDTPTIKKA	VKEVLSIE.		366			
AT	ESESYLDAPV	ERTAGADVPI	PYTANLERLA	LPOIEDIVRA	SKRACYSRK		363			
RP	EAFDYLDAPV	ETVSGKDVPL	PYAVNLEKLA	MPSANDTIEA	VKKVCYYSI		326			

Fig. 3. Alignment of amino acid sequences of the E1 β components of pyruvate dehydrogenase (PDH) complexes from *Arabidopsis thaliana* (AT), *Homo sapiens* (HS), *Rickettsia prowazekii* (RP), *Saccharomyces cerevisiae* (SC), *Sinorhizobium meliloti* (SM), and *Zymomonas mobilis* (ZM). Amino acids conserved in at least three out of the six sequences are shaded. Functional domains identified in homologous proteins are indicated above the amino acid sequences. The P(S/A)LSPTM motif and the lipoylation site are boxed. EMBL accession numbers of sequences used: AT, AAA52225; HS, CAA40924; RP, CAA14724; SC, CAA85184; ZM, CAA73385.

amino acids with a predicted molecular mass of 46 kDa that exhibited significant sequence similarity with the dihydrolipoamide acetyltransferase (E2p) component of the PDHc from many different species (Fig. 4). *S. meliloti* E2p shows high

identity to the sequence of E2p components of *Z. mobilis* (42%), *R. prowazekii* (42%), and mitochondrial dihydrolipoamide acetyltransferases (*A. thaliana*, 41%; *Saccharomyces cerevisiae*, 38%). *S. meliloti* E2p has the characteristic

Lipoyl domain						
ZM	MSIEVKMPAL	SPTMTEGTLA	KWLKKEGDAV	KAGDILAEIE	TDKATMEFEET	VDAGIIAKIE 60
SM	MPINITMPAL	SPTMEEGNLA	KWLKKEGDKV	KSGDVIAEIE	TDKATMEVEEA	VDEGTIVAKIV 60
RP	MPKILMPAL	SPTMREGNLA	RWLKKEGDKV	NPGEVIAEIE	TDKATMEVES	VDEGILAKII 60
HS	PHMQVLLPAL	SPTMTMGTVQ	RWEKKVGEKL	SEGDLIAEIE	TDKATIGFEV	QEEGYLAKIL 244
AT	PHQEIIMBSL	SPTMTEGNIA	RWLKKEGDKV	APGEVLCEVE	TDKATVEMEC	MEEGYLAKIV 148
SC	EHTIIGMPAL	SPTMTQGNLA	AWTKKEGDQL	SPGEVIAEIE	TDKAQMDFEF	QEDGYLAKIE 92
ZM	VPEGSNTAV	GQVIAVMAEA	GEDVSQVAAS	ASSQISEPSE	KADVAQKETA	DSETISIDAS 120
SM	VPAGTEGVKV	NALIAVIAAE	GEDVATAAKG	GNGAAGAVPA	PKP...KETA	ETAPAAAPAP 117
RP	IPONSONVPV	NSLIAVLSEE	GEDKADIDSE	IA	ONNSVSLSLK	102
HS	VPEGTRDVP	GTPLCIIVEK	EADISAFADY	RPTEVTDLKP	QVP...PPTPE	PVAAPPTPO 302
AT	KAEGSKIEQV	GEVIAITVED	EEDIGKFKDY	TPSSTADAAP	TKA...EPTPA	PKKEEKVKQP 206
SC	VPEGTKDIPV	NKPIAVYVED	KADVPAFKDF	KLEDGSGDSK	TST...KAQPA	EPQAEKKQEA 150
Subunit binding domain						
ZM	LDKAISNAGY	GNKTENMTAS	YQEKAGRIKA	SPLAKRLAKK	NHVDLKQVSG	SGPHGRIIKA 180
SM	AAAPAPQAA	...APASAP	ADGEGKRIFS	SPLARRLAKE	AGIDLSAIAG	SGPHGRVVKK 173
RP	TDATLKKS	...NDSITNVEG	IKHDSNKIFA	SPLAKRLAKI	GDIRLENVQ	SGPHGRIVKQ 159
HS	PLAPTPSAP	...CPAT	PAGPKGRVFEV	SPLAKRLAVE	KGIDLTOVK	KGPDGRITKK 355
AT	SSPEPKAS	...KPST	PP.TGDRVFA	SPLAKRLAED	NNVPLSDIEG	TGPEGRIVKA 258
SC	PAEETKTS	...PEAKKSD	VAAPQCRIFA	SPLAKTIALE	KGISLKDVRG	TGPRGRITKA 206
ZM	DIEAEVTVG	...ANQA.SSNPS	VSTPEVSGK	IT	HDTPHNSIKL	SNMRRVIARR 228
SM	DVETAVSGGA	AKPA.GAPAA	APAPATLAKG	MSEDAVLKLF	EPGSYELVPH	DGMRKTIARR 232
RP	DI.....	...L.SYDSS	TSSNKIVYR		DTEEYRSVEN	NNIRKIIARR 196
HS	DIDSEFV	PS	KVAPAPAAVV	PPTGPGMAPV	PTGVFTDIP	SNIRRVIAQR 403
AT	DIDEYL	AS	S...GKGATA	KPSKSTDSKA	PALDYVDIPH	SOIRKVTASR 303
SC	DIESYLEKSS	KQSSQTSAA	AATEAAATSS	TTAGSAPSPS	STASVEDVPE	STMRSIIGER 266
ZM	LTESKQNTIPH	IYLTVDVOMD	ALIKLESELN	ESLAVON...	...IKISVNDML	TKAQAALAKA 284
SM	LVESKQNTIPH	FYVSVDCELD	ALMALRAQLN	AAAPEKDGKP	VYKLSVNDMV	IKALALALRD 292
RP	LLESKQNTIPH	FYLSIECNVD	KLLDVREDIN	KSFSEDK...	VTKISVNDEI	ILAVAKALQE 253
HS	LMOSKQNTIPH	YYLSIDVNMG	EVLLVRKELN	KILE...GR...	...SKISVNDEI	IKASALACLK 458
AT	LAFSKQNTIPH	YYLTVDTCVD	KLMALRSQLN	SFKEASGG...	...KRISVNDIV	VKAALALARK 360
SC	LLQSTQGLPS	YIVSSKISIS	KLLKLRQSLN	ATAN...DK...	...YKLSINDIL	VKAITVAARR 321
ZM	TPNVNVAFD	...GDOMLOFSQ	ADISVAVSVE	GGLITPILKO	ADTKSLSALS	VEMKELIARA 342
SM	VPDANVSWT	...DONMVKHKK	ADVGVAVSIP	GGLITPIVRO	AELKSLSALS	NEMKDLGKRA 350
RP	VPNANASWS	...EDATRYNN	VDISVAVSIP	NGITVPIVRO	ANKKNIELS	REMKTILKRA 311
HS	VPEANSSWM	...DTVIRONHV	VDVSVAVSTP	AGLITPIVEN	AHKGVEITIA	NDVVSILATKA 516
AT	VPOCNSSWT	...DDYIROFKN	VNINNAVQTE	NGLYVPVVKD	ADRKGLSTIG	EEVRLIAOKA 418
SC	VPDANAYWLP	...NENVIRKFKN	VDVSVAVATP	TGLLTPIVKN	CEAKGLSOLS	NEIKELVKRA 381
ZM	REGRLQPOEY	OGGTSSISNM	G.MFGIKQFN	AVINPPQASI	LAIGSGERRP	WV...IDDAI 398
SM	KERKLPKEEY	HGGTTAVSNM	G.MMGVKDFA	AVVNPPHATI	LAVGAGEDRV	VV...RNKEM 406
RP	KDNKLTPIEF	OGGGFTISNL	G.MYGIKNFN	AIINTPQSCI	MGVGASTKRA	IV...KNDQI 367
HS	REGKLOPHEF	OGGTFTISNL	G.MFGIKNFS	AIINPPQACI	LAIGASEDKL	VP...ADNEKGF 574
AT	KENSLKPEDY	EGGTFTVSNL	GGPEGIKQFC	AVVNPPQAAI	LAVGSAEKRV	VP...GNGPDQF 477
SC	RINKLAPEEF	OGGTICISNM	GMNNAVNMFT	SIINPPQSTI	LATATVERVA	VEDAAAENG 441
Catalytic site						
ZM	TIATVATITG	SFDHRVIDGA	DAAAEFMSAFK	HLVERKPLGIL	AQ	440
SM	VIANVMTVTL	STDHRQVDGA	LGAELLAAFK	RYIENPMGML	V.	447
RP	IIATIMDVTL	SADHRVIDGA	VSAEFLASFK	RFIENPVLML	I.	408
HS	DVASMMSVTL	SCDHRVVDGA	VGAQWLAEFR	KYLEKPITML	L.	615
AT	NEASYMPVTL	SCDHRVVDGA	IGAEWLKAFFK	GYIENPKSML	L.	518
SC	SFDNQVTITG	TFDHRVIDGA	KGAEEFMKELK	TVIENPLEML	L.	482

Fig. 4. Alignment of amino acid sequences of the E2p components of pyruvate dehydrogenase (PDH) complexes from *Arabidopsis thaliana* (AT), *Homo sapiens* (HS), *Rickettsia prowazekii* (RP), *Saccharomyces cerevisiae* (SC), *Sinorhizobium meliloti* (SM), and *Zymomonas mobilis* (ZM). Amino acids conserved in at least three out of the six sequences are shaded. Functional domains identified in homologous proteins are indicated above the amino acid sequences. The P(A/S)LSPTM motif and the lipoylation site are boxed. EMBL accession numbers of sequences used: AT, AAD25602; HS, CAA68787; RP, CAA14979; SC, CAA60189; ZM, CAA63808.

multidomain structure of E2 components in which the amino-terminal lipoyl domain, the subunit-binding domain, and the C-terminal catalytic domain are separated by flexible linkers (Fig. 4). The carboxy-terminal domain contains the active-site motif S-X₅₁-H-X₃-DG common to all E2 enzymes (Russell and Guest 1991). The *S. meliloti* E2p component possesses a single lipoyl domain, like *Z. mobilis* E2p, with the characteristic P(S/A)LSPTM motif (residues 8 to 14), and lipoylation site (K 43).

An ORF was detected 137 bp upstream of *pdhA*, with sequence similarity (52%) to *R. prowazekii* ORF-782 of unknown function that, in this organism, is unlinked to *pdh* genes (Andersson et al. 1998). Numerous *pdh* operons have a *lpd* lipoamide dehydrogenase (E3) gene immediately downstream of E1p and E2p. However, no *lpd* gene was found in *S. meliloti*, immediately downstream of *pdhB*. Instead, an ORF starting 51 bp downstream of *pdhB* displayed sequence similarity to an arylesterase from *Agrobacterium radiobacter* (AAD02335).

The small distances separating *pdhAα*, *pdhAβ*, and *pdhB* (17 bp between *pdhAα* and *pdhAβ*, and 12 bp between *pdhAβ* and *pdhB*) suggest that these three genes might be co-transcribed. Direct evidence for co-transcription of the two *pdhA* genes was indeed obtained by reverse transcription (RT)-PCR analysis with two specific primers (A3 and A1; see Figure 1), and RNAs extracted from symbiotic bacteroids. This experiment demonstrated the existence of a *pdhAα*-*pdhAβ* co-transcript (data not shown). Downstream of *pdhB*, an inverted repeat followed by several U residues was identified (Fig. 1), and could act as a transcriptional termination signal.

As the *S. meliloti* genome contains many repeated genes (Fischer 1994), the number of *S. meliloti* E1p gene copies was determined by Southern blot analysis. Genomic DNA was isolated, digested with *Bam*HI, *Eco*RI, *Hind*III, *Sal*I, or *Sma*I, and hybridized with a *pdhA*-specific (A1-A2) probe. The presence of a single hybridization band in all cases was consistent

with E1p being encoded by unique genes in *S. meliloti* 8011 (data not shown).

***pdhAαβ* expression is induced in bacteroids, independently of the nitrogen fixation and respiration regulatory pathways.**

Originally, *pdhAαβ* genes were isolated in the course of screening for *S. meliloti* genes highly expressed under symbiotic conditions, compared with free-living conditions. To confirm that *pdhAαβ* expression was induced during symbiosis, RT-PCRs were performed on *S. meliloti* 211 total RNA, isolated from free-living oxic bacteria, free-living microoxic bacteria, and bacteroid 3 weeks after alfalfa inoculation by *S. meliloti* (see Materials and Methods). With specific primers synthesized from the *pdhA* sequence (A1 and A2; see Figure 1), RT-PCR products were subjected to Southern hybridization with the 191-bp A1-A2 product generated by PCR on *S. meliloti* total DNA. Data showed that *pdhA* gene transcripts accumulated at a high level in symbiotic conditions, compared with free-living conditions (Fig. 5A). To control the amount of RNA in the reaction, the constitutively expressed *S. meliloti* *hemA* gene (Batut et al. 1989) was in the same way subjected to the RT-PCR procedure with *hemA* specific primers (see Materials and Methods). No RT-PCR product was detected in controls lacking reverse transcriptase during cDNA synthesis, relative to background DNA contaminating the RNA preparations.

In *S. meliloti*, two master regulatory genes, *nifA* and *fixK*, control the expression of genes involved in nitrogen fixation and bacteroid respiration. These regulators are themselves under the control of the FixLJ two-component system that mediates oxygen signaling (David et al. 1988; Soupène et al. 1995). To test whether *pdhA* symbiotic induction was under this pathway control, RT-PCRs were performed on RNAs isolated from different *nif* and *fix* *S. meliloti* mutant strains. RNAs were extracted from bacteroids 2 weeks after alfalfa inoculation with wild-type (GMI 211), *fixK* (GMI 942), *nifA* (GMI 5601), and *fixJ* (GMI 5704) *S. meliloti* strains. No effect of the different mutations on *pdhA* symbiotic activation was detected (Fig. 5B). Similar data were obtained with a different set of primers (A1 and A3, Fig. 1; and data not shown).

Pyruvate induces *pdhA* expression in free-living cultures of *S. meliloti*.

To test the effect of various carbon sources on *pdhA* expression, RT-PCRs were performed on *S. meliloti* RNAs isolated from oxic or microoxic free-living cells grown in minimal medium containing different carbon sources (20 mM; see Materials and Methods). Cells grown in M9 minimal media with either mannitol or succinate as sole carbon source showed a low level of *pdhA* expression (Fig. 6A), much like cells grown in TY rich medium (Fig. 5A). No induction was observed in minimal medium supplemented with acetate (data not shown). By contrast, a large increase of the *pdhA* RNA level was observed when pyruvate was the sole carbon source (data not shown), or when pyruvate was added to minimal media containing mannitol or succinate (Fig. 6A). Thus, pyruvate was capable of inducing *pdhA* expression under free-living conditions. Similar results were obtained when the cells were grown under oxic or microoxic-STA conditions (data not shown).

Fig. 5. *pdhA* expression is induced under symbiotic conditions independently of *fixLJ* regulatory pathway. **A**, Autoradiographs of reverse transcription-polymerase chain reactions (RT-PCRs) of RNAs isolated from (lane 1) free-living oxic, (lane 2) microoxic (2% O₂), and (lane 3) stoppered tube assay (STA) (<1% O₂) *Sinorhizobium meliloti* bacteria grown in rich TY (tryptone-yeast) medium, and (lane 4) from symbiotic bacteroids 3 weeks after inoculation of alfalfa. *pdhA*: RT-PCR was performed with *pdhA*-specific primers (A1 and A2), and products hybridized with the *pdhA* (A1-A2) ³²P-labeled probe. *hemA*: RT-PCR was performed with specific primers of the constitutively expressed *hemA* gene to ensure that an equal amount of RNA was used in all reactions. **B**, Autoradiographs of RT-PCRs of RNAs isolated from symbiotic bacteroids 2 weeks after alfalfa inoculation with (lane 5) wild-type GMI 211, (lane 6) *fixK* GMI 942, (lane 7) *nifA* GMI 5601, and (lane 8) *fixJ* GMI 5704 *S. meliloti* strains.

The kinetics of induction by pyruvate were investigated by measuring *pdhA* RNA levels at different times following pyruvate addition. RNAs were extracted from free-living cells 0, 5, 15, 30, and 60 min after addition of pyruvate to minimal medium containing succinate as carbon source. As shown in Figure 6B, induction of *pdhA* expression was already detectable 5 min after pyruvate addition. After 15 min, *pdhA* transcripts leveled off. Hence, induction of *pdhA* expression by pyruvate is fast.

Levels of *pdhA* RNA reached in symbiosis or in free-living conditions upon addition of pyruvate were compared by RT-PCR. Experiments were performed on total RNAs extracted from free-living bacteria grown in M9 minimal medium supplemented with succinate or with succinate and pyruvate, and from bacteroids. High levels of *pdhA* transcripts were detected under the latter two conditions. However, *pdhA* RNA levels from bacteroids were consistently higher (Fig. 6C). Thus, induction by pyruvate in free-living conditions was only partial, compared with *pdhA* symbiotic activation.

Pyruvate induction and symbiotic activation take place at the same promoter.

The transcription start site of *pdhA* was mapped by primer extension analysis with RNAs isolated from free-living cells grown in minimal medium supplemented with succinate and pyruvate and from 3-week-old symbiotic bacteroids. For primer extension, a primer complementary to the 5' end of *pdhA* α coding region was used (A4; see Figure 1). The same elongation product was detected with RNAs from free-living bacteria induced by pyruvate and from symbiotic bacteroids. Transcription was shown to start 26 bp upstream of the putative start codon of *pdhA* (Figs. 7 and 1). The amount of the primer extension product generated was greater with RNAs isolated from bacteroids than with RNAs isolated from free-living cultures (Fig. 7). This is consistent with the *pdhA* α gene being more highly expressed in bacteroids than in free-living cultures (Fig. 6C). Existence of an additional transcription start site upstream or downstream of the one identified above was tested by RT-PCR experiments. RNAs were subjected to RT-PCR with different primer sets. With primers (A5 and A7; Fig. 1) surrounding the identified start site no product was detected, and with primers (A6 and A7; Fig. 1) downstream of the A4 primer no change was observed on the product levels (data not shown).

Altogether, these results indicate that pyruvate induction and symbiotic activation of *pdhA* take place at the same promoter.

DISCUSSION

Structure and genetic organization of *S. meliloti* *pdh* genes.

We have cloned three *S. meliloti* genes whose predicted protein products display high sequence identity with components of the PDHc from several organisms, including the α -proteobacteria *Z. mobilis* and *R. prowazekii*. The *S. meliloti* components identified have highest identity with subunits of *Z. mobilis*, from which the PDHc has been purified and biochemically characterized as such (Neveling et al. 1998b). Thus, the *S. meliloti* genes that we have isolated are likely orthologues of the *pdhA* α , *pdhA* β , and *pdhB* genes of *Z. mobilis*.

pdhA α and *pdhA* β code for the *S. meliloti* E1p component, suggesting a heterotetrameric E1 $\alpha_2\beta_2$ form, as described in the Gram-negative bacteria *Z. mobilis* (Neveling et al. 1998b) and

Fig. 7. Mapping of the *pdhA* transcription start site. Primer extension analysis with RNAs isolated from free-living *Sinorhizobium meliloti* bacteria grown in minimal medium with succinate (lane 1) before and (lane 2) 4 h after pyruvate addition, and (lane 3) from symbiotic bacteroids 3 weeks after alfalfa inoculation with *S. meliloti*. Products of extension reactions were fractionated alongside a sequence ladder generated with the A4 primer used for primer extension.

Fig. 6. Pyruvate activates *pdhA* expression. **A**, Reverse transcription-polymerase chain reaction (RT-PCR) products were derived from RNA isolated from free-living oxic bacteria grown in minimal medium with (lane 1) mannitol, (lane 2) mannitol and pyruvate, (lane 3) succinate, or (lane 4) succinate and pyruvate as carbon sources. RNAs were extracted 4 h after addition of pyruvate. Similar results were obtained after 16 h (data not shown). **B**, Kinetics of *pdhA* induction by pyruvate. RT-PCRs were performed on RNAs extracted from free-living oxic bacteria grown in minimal medium supplemented with succinate at different times (0, 5, 15, 30, 60 min) after pyruvate addition (lanes 5, 6, 7, 8, 9, respectively). **C**, *pdhA* expression analysis by RT-PCR on RNAs from free-living oxic bacteria grown in minimal medium with (lane 10) succinate, (lane 11) succinate plus pyruvate, or (lane 12) from symbiotic bacteroids 3 weeks after alfalfa inoculation with *Sinorhizobium meliloti*.

Thiobacillus ferrooxidans (Powles and Rawlings 1997), and also in some Gram-positive bacteria and in mitochondria. The *S. meliloti* E1 α component contains the conserved TTP-binding site found in E1-type enzymes. Interestingly, the E1 β subunit contains an amino terminus E1 β lipoyl domain, like *Z. mobilis* E1 β but unlike *R. prowazekii* E1 β . N-terminal lipoyl domains are usually found in E2p components, in which a covalently bound lipoyl group is essential for the coupling of the activities of the separate multienzyme components (Berg and de Kok 1997). Lipoyl domains are also found in the E3 component of multienzyme complexes from several species, but their role, as well as the role of the N-terminal lipoyl domain from *Z. mobilis* E1 β , has not yet been established.

The *pdhB* gene encodes the E2p component of the complex. As in all other E2p, *S. meliloti* E2p has a multidomain structure, with an amino-terminal lipoyl domain, a subunit-binding domain, and a carboxy terminal catalytic domain separated by flexible linkers. In PDHc, the number of E2p lipoyl domains varies from one to three, and the reason for this variation is unknown. In complexes from Gram-negative organisms, the number is usually two to three, but *S. meliloti* possesses only one E2p lipoyl domain.

Comparison of amino acid sequences shows that PDHc enzymes from the three α -proteobacteria *S. meliloti*, *Z. mobilis*, and *R. prowazekii* are evolutionarily clustered. Their E1p component is composed of two subunits, α and β , like the components of PDH complexes of some Gram-positive organisms and of mitochondria. These E1p components do not show homology to the E1p type found in *Escherichia coli* (CAA24740), *Alcaligenes eutrophus* (Q59097), *Mycobacterium tuberculosis* (CAA9466), or *Streptomyces coelicolor* (CAB51267). While E2p sequence analysis shows homology with the E2p proteins from eukaryotes and Gram-positive and Gram-negative bacteria, the three α -proteobacteria E2p showed highest sequence identity to the sequences of eukaryotic E2p (Neveling et al. 1998a). Moreover, N-terminal lipoyl domains attached to the three α -proteobacteria E2p and to the *S. meliloti* and *Z. mobilis* E1 β components display the P(S/A)LSPTM motif highly conserved in eukaryotic E2p lipoyl domain but absent in the E2p lipoyl domain of prokaryotes. Hence, PDHc enzymes from α -proteobacteria show overall higher sequence identity with mitochondrial PDHc than with other Gram-negative PDHc components.

In the *S. meliloti* genome, *pdhA $\alpha\beta$* and *pdhB* are clustered as they are in other prokaryotic genomes, with the notable exception of *Z. mobilis* and *R. prowazekii*. We have shown the existence of a *pdhA α -pdhA β* co-transcript and the presence of a very short gap (12 bp) between the end of *pdhA β* and the beginning of *pdhB*. In addition, a potentially stable stem-loop structure that could function as a ρ -independent transcriptional terminator was detected immediately downstream of *pdhB* (Fig. 1). Altogether, these results suggest that the three *pdh* genes form a transcriptional unit in *S. meliloti*, with a transcription start site 26 bp upstream of the proposed *pdhA* transcriptional start codon.

Southern analysis of genomic DNA showed that a single gene very likely encodes E1p in the *S. meliloti* genome, in contrast to *Alcaligenes eutrophus*, that possesses two paralogous genes coding for PDH (Hein and Steinbuchel 1996).

In prokaryotes, the E3 component is shared by different enzymatic complexes, such as 2-oxoglutarate dehydrogenase

complexes (OGDHc) or acetoin dehydrogenase complexes (ADHc), in addition to PDHc. Accordingly, the corresponding *lpd* gene can be part of different gene clusters (de Kok et al. 1998). In some prokaryotes, such as *Bacillus subtilis* and *Staphylococcus aureus*, an *lpd* gene lies immediately downstream of *pdhB* (Neveling et al. 1998a). Instead, in *S. meliloti*, no *lpd* gene was found immediately downstream of *pdhB*, as described in *A. vinelandii* (Hengeveld et al. 1997) and *P. aeruginosa* (Rae et al. 1997).

Symbiotic role of *pdhA* induction.

C₄-dicarboxylic acids are the principal source of carbon supplied by the plant to N₂-fixing bacteroids within the root nodules (Ronson et al. 1981). The use of C₄-dicarboxylates as sole carbon source is dependent on the availability of acetyl-CoA. Bacteroids from alfalfa nodules lack phosphoenolpyruvate carboxykinase (PCK) activity (Finan et al. 1991). Thus, the pathway for formation of acetyl-CoA via oxaloacetate to phosphoenolpyruvate probably cannot operate in bacteroids. It was thus proposed that acetyl-CoA, which is essential for the TCA cycle operation, is generated via the combined activities of malic enzymes and PDH in bacteroids (Driscoll and Finan 1993). Accordingly, both malic enzymes and PDH activities have been detected in *S. meliloti* bacteroids (Driscoll and Finan 1993; Miller et al. 1991). In further support of this model, *S. meliloti dme* malic enzyme mutants induced root nodules that contained bacteroids that failed to fix nitrogen. We note that both *dme* expression and DME activity that generate pyruvate are not increased during symbiosis. *S. meliloti* has a second malic enzyme, TME, whose synthesis is repressed in bacteroids (Driscoll and Finan 1997). In this context, construction of a *pdh* mutant and assessment of its symbiotic phenotype will be interesting. Given the central role of acetyl-CoA in the metabolism, it is possible that a *pdh* mutation affects symbiotic interaction at different stages. Here we have shown that growth on C₄-dicarboxylates (succinate) as sole carbon source, under oxic or microoxic free-living conditions, results in weak *pdh* gene expression. By contrast, we have shown by both RT-PCR and primer extension that transcription of *pdhA* is enhanced under symbiotic conditions. Higher PDH synthesis could fulfill a so far unknown physiological need of the bacteroid, requiring an increase in acetyl-CoA synthesis. Acetyl-CoA has two major roles in the cell: (i) acetyl-CoA allows energy production upon oxidation in the TCA cycle; and (ii) acetyl-CoA is a precursor for fatty acid biosynthesis. Hence, an increase in acetyl-CoA synthesis could serve either function in bacteroids.

Alternatively, it is possible that high expression of *pdh* is not essential for bacteroid metabolism. Engineering an *S. meliloti* mutant with a low constitutive expression of *pdhA* in bacteroids may help answer this question.

Pyruvate regulation of *pdh* expression.

Studies of *pdhA* expression in *S. meliloti* cells grown on different carbon sources showed induction of *pdhA* expression by pyruvate. No induction was observed in minimal medium containing acetate or succinate. Thus, pyruvate was a specific inducer of *pdhA* expression in free-living conditions. Kinetics of *pdhA* induction by pyruvate showed that induction started to be detectable 5 min after pyruvate addition. In this time frame, it is unlikely that pyruvate was metabolized, so the fast

kinetics are in favor of pyruvate being the actual inducer molecule.

Induction by pyruvate was also observed in *E. coli* (Quail et al. 1994). In this case, a pyruvate-sensitive repressor, PdhR, regulates PDH gene expression (Quail and Guest 1995). PdhR represses *pdh* transcription by binding to a palindromic operator site. Repression is antagonized in a concentration-dependent manner by pyruvate as co-effector. Thus, it is possible that a *pdhR* homologue exists in *S. meliloti*, even though it is not immediately linked to *pdh* genes, contrary to what exists in *E. coli*. However, no sequence similarity with the *E. coli* PdhR binding site was detected in the *S. meliloti* *pdhA* promoter. Thus, the genetic circuit that mediates pyruvate induction in *S. meliloti* needs to be elucidated.

In *S. meliloti*, N₂-fixation gene expression (*nif* and *fix* regulons) is under the control of a regulatory cascade that mediates oxygen signaling via the FixLJ pair of regulatory proteins (Soupène et al. 1995). *pdhA* symbiotic activation does not depend on *nif* and *fix* gene cascade regulation, nor on oxygen status and nitrogen fixation, and thus differs from most of the *S. meliloti* genes studied so far. Interestingly, this indicates a new type of symbiotic regulation. *pdhA* transcription start site analysis showed that the same promoter was used under symbiotic conditions and in free-living cultures supplemented with pyruvate. In spite of pyruvate activation being less than symbiotic, these results suggest that regulation by pyruvate could be symbiotically relevant. Such a model would imply a high pyruvate level in bacteroids. This has not been measured to date. However, we note that a recently proposed alternative for dinitrogen transfer between the bacteroid and its plant host involves alanine (Waters et al. 1998). Alanine would be synthesized by alanine dehydrogenase, which converts pyruvate to alanine with ammonium and NADH ($\text{NADH} + \text{NH}_4^+ + \text{pyruvate} \rightarrow \text{L-alanine} + \text{NAD}^+ + \text{H}_2\text{O}$). So, we speculate that a high level of pyruvate in symbiotic bacteria could be required for ammonium assimilation and export. However, it is also possible that *pdhA* expression escapes pyruvate regulation in bacteroids. This would be the case if, for example, *pdhA* expression was under negative control, as in *E. coli*, and if the pyruvate-sensing repressor was, for some reason, inactive in symbiosis. Further characterization of *pdh* gene regulation will shed light on this question and may lead to the elucidation of the mechanisms regulating PDH genes during the symbiotic interaction with the host plant.

MATERIALS AND METHODS

Microbiological techniques.

All *S. meliloti* strains used in this study were isogenic with GMI 211 (*lac Sm^r*) (David et al. 1988). GMI 5601 (*lac nifAZ239::Tn5*) and GMI 5704 (*lac fixJ2.3::Tn5*) were constructed by David et al. (1988). GMI 942 was constructed by Foussard et al. (1997). *S. meliloti* strains were grown at 30°C in TY complex medium (Rosenberg et al. 1981) or in defined M9 media (Miller 1972) supplemented with 0.3 mM CaCl₂, 1 mM MgSO₄ and 2 µM biotin. Sterilized carbon sources were added at 20 mM final concentration. Microoxic conditions were achieved either as described by de Philip et al. (1990) (2% oxygen for 4 h), or with the stoppered tube assay (STA) described by Ditta et al. (1987) (<1% oxygen for 16 h). To test

induction of *pdhA* expression by pyruvate, *S. meliloti* strains were grown to OD₆₀₀ = 0.3 in M9 medium supplemented with 0.3 mM CaCl₂, 1 mM MgSO₄ and 2 µM biotin, and the appropriate carbon source (20 mM). Then, pyruvate (20 mM) was added and samples were collected after 4 or 16 h. For the kinetics experiments (shown in Figure 6B), strains were grown to OD₆₀₀ = 0.5 instead of OD₆₀₀ = 0.3.

DNA methods.

Standard DNA techniques were as described previously by Sambrook et al. (1989). *S. meliloti* genomic DNA was prepared from free-living cells as described by Chen and Kuo (1993). For Southern blot analysis, 1 µg of genomic DNA was digested for 20 h (37°C) with *Bam*HI, *Eco*RI, *Hind*III, *Sal*I, or *Sma*I. Restriction fragments were separated on a 0.8% agarose gel, blotted onto a nylon membrane (Biodyne A transfer membrane; Pall, East Hills, NY) and hybridized to a *pdhA* (A1-A2) ³²P-labeled probe. Washing was carried out in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS (sodium dodecyl sulfate) at 42°C for 30 min.

A cosmid library was constructed by Tania Arcondeguy by ligation of a partial *Sau*3A digest of *S. meliloti* total DNA in the pLAFR3 cosmid vector. Screening of the library by colony hybridization was performed as recommended by Sambrook et al. (1989).

Sequence of the *pdh* region was determined on both DNA strands by a primer-walking approach with cosmid vector DNA as template. Cosmid DNA was prepared with the Qiagen plasmid purification kit (Qiagen, Hilden, Germany), with vigorous pipetting during the lysis and neutralizing steps. Cosmid was sequenced with the ABI PRISM Dye terminator cycle-sequencing ready reaction kit (Perkin-Elmer, Oak Brook, IL), with the ABI 373 automated sequencer (ABI, Columbia, MD). Two micrograms of DNA, 4 µl of Dye terminator mix, and 15 pmol of primer were mixed in a total reaction volume of 10 µl. This mix was denatured at 96°C for 5 min, followed by 90 thermal cycles (96°C 30 s, 50°C 30 s, 60°C 4 min). Excess Dye terminator was removed by filtration over Bio-gel P-10 (Bio-Rad Laboratories, Hercules, CA) with a MultiScreen filtration system (Millipore, Bedford, MA).

Sequence assembly was achieved with the Contig Assembly Program (Huang 1992). Data base searches were conducted through the NCBI web site with the BLAST2 package program (Altschul et al. 1997). ORFs were predicted with the GLIMMER program (Salzberg et al. 1998) and NCBI-BLASTX comparisons against the NCBI-nr data base.

Plant methods and preparation of endosymbiotic bacteria.

Medicago sativa cv. Gemini seedlings were aseptically grown on agar slants made with nitrogen-free Fahraeus medium. Three-day-old plants were inoculated with the different *S. meliloti* strains. Nodules (0.25 g) were harvested and immediately frozen in liquid nitrogen 2 weeks after inoculation in the case of Fix⁻ strains, and 3 weeks for Fix⁺ strains. Nodules were ground in liquid nitrogen with a mortar and pestle, and the powder was suspended in 2 ml of 50 mM Tris hydrochloride (pH 7.6), 500 mM mannitol. Debris were sedimented by centrifugation for 5 min at 800 × g. Bacteroids were pelleted by centrifugation of the supernatant for 5 min at 12,000 × g, and stored at -80°C if necessary.

RNA preparation.

The bacteria from a 25-ml culture ($OD_{600} = 0.5$) or from 0.25 g of nodules were resuspended and incubated for 10 min at 65°C in 2 ml of prewarmed lysis solution (1.4% SDS, 4 mM EDTA, 75 µg of proteinase K). Proteins were precipitated by adding 1 ml of NaCl 5 M at 4°C. Nucleic acids were precipitated from the supernatant by addition of 1 volume of isopropanol and the pellet was resuspended in nuclease-free water. DNA was eliminated by addition of 7.5 U of FPLC-pure RNase-free DNase I (Amersham-Pharmacia Biotech, Buckinghamshire, U.K.). RNAs were further extracted with phenol/chloroform/isoamylalcohol and chloroform/isoamylalcohol, then precipitated with ethanol. The RNA pellet was washed with 70% ethanol and resuspended in nuclease-free water. RNAs were quantitated by measuring the absorbency at 260 nm. Absence of contaminating DNA in the preparation was ensured by PCR amplification.

RT-PCR analyses.

The following oligonucleotides were used to evaluate gene expression:

For *pdhA* (see Figure 1):

A1 (sense primer) 5'-CGGAGCATGATCCG

A2 (reverse primer) 5'-TTGTCCCTCCCCGG

A3 (reverse primer) 5'-AGCCACTTGGAGAGCGTGC

A4 (reverse primer)

5'-TTGGCCGGCTTGGCAGCGGTCTTGC GGCTG

A5 (sense primer) 5'-TTTTCTTTCCCTATGGTAGC

A6 (sense primer) 5'-GCGAAAATCCGCGTCCG

A7 (reverse primer) 5'-ACGCCGGATCAACAGC

For *hemA* (accession no. P08080):

hemAr (reverse primer) 5'-GTCGATCGCGTTCTT

hemAs (sense primer) 5'-TGGATGGGCTGCATCA

First-strand cDNA synthesis took place in a 17-µl reaction volume. One microliter of total bacterial RNA (100 ng), 7.1 µl of DEPC (diethyl pyrocarbonate)-treated H₂O, and 1 µl (50 ng) of the reverse primer were heated at 70°C for 10 min and quickly chilled on ice. After brief centrifugation, 3.4 µl of the following 5× buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂), 2 µl of 0.1 M DTT (dithiothreitol), and 1 µl of a 25 mM dNTP solution were added and incubated at 42°C for 2 min. After addition of 1 µl (200 U) of MMLV (Maloney Murine leukemia virus) reverse transcriptase and thorough mixing, the reaction tube was incubated for 50 min at 42°C. Reverse transcriptase was inactivated by heating at 95°C for 5 min because heating at 70°C did not fully inactivate the enzyme.

For PCR amplification, 2.5 µl of 10× PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1 µl of 50 mM MgCl₂, 1 µl of dNTP mix (25 mM), 1 µl of reverse primer (50 ng), 1 µl of sense primer (50 ng), and 0.5 µl (2.5 U) of *Taq* DNA polymerase were added to the first-strand synthesis reaction mix. After gentle mixing, the reaction mixture was layered with 1 drop of silicone oil, heated to 94°C for 3 min, and submitted to 25 cycles of the sequence 94°C/30 s, 50°C/30 s, 72°C/1 min, followed by 1 cycle at 72°C/10 min. RT-PCR products were electrophoresed on a 2% agarose gel, blotted onto a nylon membrane (Biodyne A Transfer membrane; Pall, East Hills, NY) and probed with a ³²P-labeled DNA probe, internal to the gene of interest. Washing was carried out in 0.1× SSC, 0.1% SDS at 42°C for 30 min.

Mapping of the *pdhA* transcriptional start site.

The transcriptional start site of *pdhA* was mapped by primer extension as described previously (Batut et al. 1989). The purified A4 oligonucleotide was used to prime the action of the reverse transcriptase (Superscript II RT; Life Technologies, Rockville, MD). RNA-primer hybridization was performed at 45°C with 2.5 µg of RNA, and the extension reaction was run at 50°C. A dideoxy sequencing reaction of a DNA sample containing the *pdhA* promoter was generated with the A4 oligonucleotide as primer, and run in parallel to the primer extension reactions.

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