Molecular characterization of the *bet* genes encoding glycine betaine synthesis in *Sinorhizobium meliloti* 102F34

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As a first step towards the elucidation of the molecular mechanisms responsible for the utilization of choline and glycine betaine (betaine) either as carbon and nitrogen sources or as osmoprotectants in *Sinorhizobium meliloti*, we selected a Tn5 mutant, LTS23-1020, which failed to grow on choline but grew on betaine. The mutant was deficient in choline dehydrogenase (CDH) activity, failed to oxidize [methyl-¹⁴C]choline to [methyl-¹⁴C]betaine, and did not use choline, but still used betaine, as an osmoprotectant. The Tn5 mutation in LTS23-1020 was complemented by plasmid pCHO34, isolated from a genomic bank of *S. meliloti* 102F34. Subcloning and DNA sequencing showed that pCHO34 harbours two ORFs which showed 60% and 57% identity with the *Escherichia coli* *betB* gene encoding betaine-aldehyde dehydrogenase (BADH) and *betA* gene encoding CDH, respectively. In addition to the homology with *E. coli* genes, the deduced sequence of the sinorhizobial BADH protein displays consensus sequences also found in plant BADHs. The deduced sequence of the sinorhizobial CDH protein shares only 21% identical residues with choline oxidase from *Arthrobacter globiformis*. The structural organization of the *betBA* genes in *S. meliloti* differs from that described in *E. coli*: (i) the two ORFs are separated by a 210 bp sequence containing inverted repeats resembling a putative rho-independent transcription terminator, and (ii) no sequence homologous to *betT* (high-affinity choline transport system) or *betI* (regulator) was found in the vicinity of the sinorhizobial *betBA* genes. Evidence is also presented that the *S. meliloti* *betBA* genes are not located on the megaplasmids.

Keywords: *bet* genes, glycine betaine synthesis, osmoregulation, *Sinorhizobium meliloti*

INTRODUCTION

Many bacterial and plant species accumulate glycine betaine (betaine; *N,N,N*-trimethylglycine) in response to salt stress or water deficit (Le Rudulier *et al.*, 1984; Csonka, 1989; Csonka & Hanson, 1991; Rhodes & Hanson, 1993). Much evidence indicates that betaine

acts as a non-toxic osmolyte which is highly compatible with metabolic functions at high cytoplasmic concentrations and contributes to turgor adjustment in cells subjected to osmotic stress (Yancey *et al.*, 1982; Somero, 1986; Warr *et al.*, 1988). These findings, and the prospect of genetically engineering water/osmotic stress resistance in beneficial bacteria and important plant crops, have focused considerable interest in research on betaine biosynthesis and transport (Le Rudulier *et al.*, 1984; Csonka & Hanson, 1991; McCue & Hanson, 1992; Rhodes & Hanson, 1993).

Aside from rare cases of *de novo* biosynthesis through unspecified pathways (Moore *et al.*, 1987; Gabbay-Azaria *et al.*, 1988), the production of betaine results

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Abbreviations: BADH, betaine aldehyde dehydrogenase; CDH, choline dehydrogenase.

The GenBank accession number for the sequence reported in this paper is U39940.

from the oxidation of choline by a two-step reaction with betaine aldehyde as the intermediate. Choline oxidation may be catalysed by three different enzymic systems involving one or two separate enzymes. A soluble choline oxidase (EC 1.1.3.17) is found in the Gram-positive bacteria *Arthrobacter pascens* and *Arthrobacter globiformis*, and in the fungus *Cylindrocarpon didymum* (Tani *et al.*, 1979; Rozwadowski *et al.*, 1991; Deshniun *et al.*, 1995). Higher plants utilize a choline monooxygenase (CMO) in combination with a betaine-aldehyde dehydrogenase (BADH, EC 1.2.1.8; Brouquisse *et al.*, 1989; Weretilnyk & Hanson, 1989). cDNA clones for the BADH of spinach (*Spinacia oleracea*), sugar beet (*Beta vulgaris*) and barley (*Hordeum vulgare*) have been characterized. Both gradual salinization and salt shock stimulate a several-fold increase in translatable mRNA containing these genes (Weretilnyk & Hanson, 1989; McCue & Hanson, 1992; Ishitani *et al.*, 1995). Some bacteria, such as *Pseudomonas aeruginosa* and *Escherichia coli*, use a membrane-bound choline dehydrogenase (CDH; EC 1.1.99.1) in conjugation with a cytosolic BADH (Nagasawa *et al.*, 1976; Landfald & Strøm, 1986).

In *E. coli*, betaine biosynthesis is controlled by the *betTIBA* genes. *betA* and *betB* code for CDH and BADH, respectively. *betT* codes for a proton-motive-force-driven, high-affinity transport system for choline, and *betI* for a repressor involved in the regulation of *bet* genes by choline (Lamark *et al.*, 1991). The expression of the *E. coli bet* genes is controlled by the osmotic strength of the environment, and, to a lesser extent, by the availability of choline, i.e., appreciable CDH and BADH activities are present only in cells grown under osmotic stress, and in the presence of choline (Landfald & Strøm, 1986; Styrvold *et al.*, 1986; Eshoo, 1988). It is noteworthy that betaine acts only as an osmotic stress protectant and cannot be catabolized by *E. coli* (Le Rudulier *et al.*, 1984; Landfald & Strøm, 1986).

In sharp contrast to *E. coli*, *Sinorhizobium meliloti* can utilize both choline and betaine as carbon and nitrogen sources (Bernard *et al.*, 1986; Smith *et al.*, 1988); an exogenous supply of choline strongly stimulates its rapid oxidation to betaine and the subsequent degradation of betaine via a series of demethylations. However, an increase in the osmotic pressure of the medium greatly reduces the catabolism of betaine so that accumulation is favoured (Bernard *et al.*, 1986; Smith *et al.*, 1988). These data strongly suggest that both choline and osmotic stress regulate the expression of the genes and/or activity of enzymes involved in betaine synthesis and catabolism in *S. meliloti*. They also serve to highlight the lack of information regarding the ability of various environmental factors to regulate the utilization of choline and betaine either for osmotic stress protection or for growth. As a first step towards the elucidation of the mechanism of such regulation, we report on the physiological characterization of *S. meliloti* LTS23-1020, a CDH-deficient mutant, and the functional complementation of the corresponding *betA*: Tn5 mutation using a genomic bank of *S. meliloti*

102F34. We also present the nucleotide sequence of the *sinorhizobial betBA* genes along with data indicating that these genes are chromosomally encoded in *S. meliloti*.

METHODS

Bacterial strains, plasmids and genomic bank. The bacterial strains and the plasmids used in this study are listed in Table 1. The genomic bank, made up of a *Bgl*II partial digest of *S. meliloti* 102F34 DNA cloned into pRK290 (Ditta *et al.*, 1980), was kindly provided by Dr Gary Ditta (University of California, San Diego, USA).

Media and growth conditions. Complex media used were LB (Sambrook *et al.*, 1989) for *E. coli* and MSY (Bernard *et al.*, 1986) for *S. meliloti* and *Agrobacterium tumefaciens* strains. Defined minimal media for *S. meliloti* were lactate-aspartate-salts (LAS; Bernard *et al.*, 1986) and S medium, which was identical to LAS in mineral content but was carbon- and nitrogen-free (Smith *et al.*, 1988). Choline, betaine aldehyde and betaine (Sigma) incorporated into minimal media were sterilized by filtration. Antibiotics were added at the following concentrations for *E. coli*: ampicillin (Ap), 50 µg ml⁻¹; kanamycin (Km), 30 µg ml⁻¹; streptomycin (Sm), 100 µg ml⁻¹; tetracycline (Tc), 20 µg ml⁻¹. For *S. meliloti*, the following concentrations were used: Km, 100 µg ml⁻¹; rifampicin (Rif), 25 µg ml⁻¹; Sm, 100 µg ml⁻¹; Tc, 5 µg ml⁻¹. 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) and isopropyl β-D-thiogalactopyranoside (IPTG) were used at 0.02% and 63 µM, respectively. Unless otherwise indicated, *S. meliloti* and *Ag. tumefaciens* were grown aerobically at 30 °C and *E. coli* at 37 °C. Inocula were grown overnight in complex media and washed in S medium prior to inoculation (3%, v/v). Bacterial growth was monitored spectrophotometrically at 420 nm.

Enzyme and transport assays. CDH and BADH activities and choline uptake were assayed according to established procedures (Pocard *et al.*, 1989; Smith *et al.*, 1988). Radioactive [*methyl*-¹⁴C]choline (2.15 TBq mol⁻¹) was purchased from Amersham.

Characterization of the Bet phenotype in *S. meliloti*. Specific physiological and biochemical tests were used to study the ability of *S. meliloti* strains to oxidize choline into betaine (Bet phenotype). These tests included: (i) the utilization of choline, betaine aldehyde or betaine (20 mM) as the sole carbon and nitrogen sources, (ii) the assay of CDH or BADH activities in cellular extracts (Smith *et al.*, 1988), (iii) osmoprotection by choline or glycine betaine aldehyde (1 mM) in 0.5 M NaCl LAS medium and (iv) the metabolism of [*methyl*-¹⁴C]choline by osmotically stressed and unstressed cells (Bernard *et al.*, 1986; Smith *et al.*, 1988).

Conjugation and transposon mutagenesis. Triparental spot matings to introduce pRK290 and its derivatives from *E. coli* into *S. meliloti* were performed as reported by Ditta *et al.* (1980), using *E. coli* HB101(pRK2013) as the helper strain. The filter-mating was performed at an initial cell density of 10⁸ c.f.u. for each donor, recipient and helper strain (24 h, 30 °C, LB plates). Random Tn5 mutagenesis of *S. meliloti* 102F34rif was also performed according to this procedure, using pTJ7.1 as the Tn5 donor. Strains were filter-mated at a ratio of 1:2:2 of recipient to donor to helper. Approximately 40 000 Rif^r and Km^r double mutants were screened for growth in S medium containing either 1 g mannitol l⁻¹ and 1 g ammonium chloride l⁻¹ or 20 mM choline as the sole growth substrates. Mutants that did not grow on choline occurred at

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Reference/source
Strains		
<i>Sinorhizobium meliloti</i>		
102F34	Wild-type	Ditta <i>et al.</i> (1980)
102F34rif	Spontaneous Rif ^r derivative of 102F34	This study
LTS23-1020	102F34rif <i>betA</i> : : Tn5	This study
RCR2011	SU47, wild-type strain	C. Rosenberg*
1021	RCR2011str, Str ^r derivative of RCR2011	Meade <i>et al.</i> (1982)
<i>Agrobacterium tumefaciens</i>		
GMI9023	C58 cured of pAtC58 and pTiC58	Rosenberg & Huguet (1984)
At125	GMI9023 pRme1021b	Finan <i>et al.</i> (1986)
At128	GMI9023 pRme1021a	Finan <i>et al.</i> (1986)
<i>Escherichia coli</i>		
DH5 α	F ⁻ , <i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> (r _K ⁻ m _K ⁺) <i>recA1 endA1 gyrA96 thi-1 relA1</i>	Sambrook <i>et al.</i> (1989)
HB101	F ⁻ , <i>hsdS20</i> (r _B ⁻ m _B ⁻) <i>recA13 ara-14 proA2 lacY1 galK2 leuB6 xyl-5 mtl-1 supE44 rpsL20</i>	Sambrook <i>et al.</i> (1989)
MLE33	MC4100 <i>recA56</i> Rif ^r (F'2 <i>betA5</i> : : <i>lacZY</i>)	Eshoo (1988)
Plasmids		
pRK290	Broad-host-range cloning vector, IncP1, Tc ^r	Ditta <i>et al.</i> (1980)
pRK415	pRK290 derivative with pUC9 polylinker, Tc ^r	Keen <i>et al.</i> (1988)
pRK2013	ColE1 replicon with RK2 transfer region, Nm-Km ^r	Ditta <i>et al.</i> (1980)
pTJ7.1	Tn5 delivery vector, pBR322 derivative, Ap ^r , Km ^r	S. R. Long†
pBlueScript II SK(-)	Cloning vector, Ap ^r	Stratagene
pCHO34	pRK290 clone selected from gene library, complements the <i>betA</i> : : Tn5 mutation in LTS23-1020	This study
pGBS1020	<i>betA</i> : : Tn5 <i>EcoRI</i> fragment from LTS23-1020 genomic DNA cloned into pBlueScript II SK(-)	This study
pCHO341	8.6 kb <i>Bgl</i> II fragment from pCHO34 cloned into pRK415	This study
pCHO342	7.3 kb <i>Hind</i> III- <i>Bgl</i> II fragment from pCHO34 cloned into pRK415	This study
pCHO343	4.2 kb <i>Pst</i> I fragment from pCHO34 cloned into pRK415	This study
pCHO350	1.5 kb <i>Pst</i> I- <i>Bgl</i> II fragment from pCHO34 cloned into pRK415	This study
pCHO361	3.5 kb <i>Apa</i> I- <i>Bgl</i> II fragment from pCHO34 cloned into pRK415	This study

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a frequency of 1×10^{-4} per cell containing Tn5. These mutants were fed with [*methyl*-¹⁴C]choline and were additionally screened for growth in 20 mM betaine, *N,N*-dimethylglycine, sarcosine and serine, to identify the lesions in the choline pathway (Bernard *et al.*, 1986; Smith *et al.*, 1988). Plasmids were mobilized back from *S. meliloti* into *E. coli* HB101 or DH5 α as previously described (Singh *et al.*, 1990).

DNA manipulations. Plasmid DNA was isolated from *E. coli* strains by the alkaline lysis method and, when necessary, was further purified on a caesium chloride/ethidium bromide gradient (Sambrook *et al.*, 1989), or using the GeneClean kit (Bio101). Due to the paucity of available cloning site in *E. coli*/*Sinorhizobium* shuttle vectors, restriction fragments were first subcloned into pBlueScript II SK(-) prior to subcloning into pRK415 (Keen *et al.*, 1988) and mating into the *Sinorhizobium* host. Chromosomal DNA was purified as described by Ditta (1986). Restriction endonucleases, T4 DNA ligase and other DNA modifying enzymes were obtained from Appligene, New England Biolabs or Stratagene Cloning Systems, and used according to the manufacturers' instructions. Standard procedures were used for agarose gel electro-

phoresis, Southern blotting, ligations and transformation of *E. coli* with plasmid DNA (Sambrook *et al.*, 1989). DNA probes were labelled by using the Prime-a-Gene random priming system (Promega) and [α -³²P]dCTP (Amersham).

DNA sequencing. DNA was sequenced by using the dideoxy chain-termination method of Sanger *et al.* (1977), the Sequenase version 2.0 sequencing kit (United States Biochemical) and [³⁵S]dATP α S (Amersham). Regions of DNA to be sequenced were subcloned into pBlueScript II SK(-). The sequence of the *bet* region was determined on both coding and non-coding strands, using a range of nested deletions prepared by exonuclease III/mung bean nuclease digestion. Bacteriophage T3 and T7 primers (United States Biochemical) were used to prime the sequencing reactions. Sequence analysis was done using the software package from the Wisconsin Genetics Computer Group (Devereux *et al.*, 1984) and BLAST protocols (Altschul *et al.*, 1990).

Plant nodulation assays. The symbiotic proficiency of *S. meliloti* strains was assayed on alfalfa (*Medicago sativa*, cv. Europe) seedlings grown under nitrogen-deficient conditions as described previously (Pocard *et al.*, 1984).

RESULTS

Isolation and characterization of a Tn5 mutant impaired in choline oxidation

To isolate mutants of *S. meliloti* impaired in choline utilization, strain 102F34rif was subjected to random Tn5 mutagenesis (Ditta, 1986). Rif^r and Km^r mutants were screened for growth in S medium supplemented as indicated in Methods. Mutants that grew on mannitol/ammonium but failed to grow on choline were further screened for growth on 20 mM betaine, N,N-dimethylglycine, sarcosine and serine, which are intermediates in the betaine catabolic pathway in *S. meliloti* (Smith *et al.*, 1988). One mutant, LTS23-1020, which did not grow on choline, but grew on betaine plates as well as on the other three substrates, was characterized further. Growth tests in liquid medium confirmed that LTS23-1020 failed to grow on choline but grew at the expense of betaine, whereas these two compounds supported growth of the wild-type strain 102F34 (data not shown). Furthermore, transport assays showed that [methyl-¹⁴C]choline was actively taken up through high- and low-affinity systems in LTS23-1020 grown at low or high osmolarity, and that the uptake rates were comparable to those reported previously on 102F34 (Pocard *et al.*, 1989; data not shown). Hence, we assumed that *S. meliloti* LTS23-1020 was impaired in betaine production from choline. Therefore, CDH and BADH activities were measured in cell extracts of 102F34rif and LTS23-1020 grown in LAS medium plus 7 mM choline (Smith *et al.*, 1988). Both strains exhibited high levels of BADH activity (Table 2). Likewise, CDH activity was high in 102F34rif, but was absent in the Tn5 mutant. [methyl-¹⁴C]choline (2 µM) was then fed for 4 h to cultures grown in LAS plus 0.3 M NaCl, a condition which is known to favour the accumulation of betaine as an osmolyte in the wild-type strain 102F34 (Bernard *et al.*,

1986; Smith *et al.*, 1988). As expected, LTS23-1020 was unable to metabolize choline at high osmolarity. Conversely, the parental strain, 102F34rif, actively oxidized choline to betaine which was accumulated (Table 2). When the cells were grown in low-osmolarity LAS medium, [methyl-¹⁴C]choline was not metabolized by the mutant, nor was betaine accumulated in 102F34rif (data not shown), because choline and betaine are both actively catabolized by unstressed cultures of *S. meliloti* 102F34 (Bernard *et al.*, 1986; Smith *et al.*, 1988). To test the osmoprotective effect of exogenous choline, betaine aldehyde and betaine, strain LTS23-1020 was grown in 0.5 M NaCl LAS medium in the presence or the absence of 1 mM of the potential osmoprotectants. Both betaine aldehyde and betaine conferred enhanced salinity tolerance to the CDH-deficient mutant, but choline did not (Fig. 1). Thus, choline itself is not able to restore the growth of *S. meliloti* at high osmolarity.

In all, the results of the nutritional tests, the osmotic stress alleviation assay and those of the radiotracer experiment demonstrate that *S. meliloti* LTS23-1020 is deficient in CDH activity, i.e., shows a BetA phenotype (Styrvold *et al.*, 1986).

Cloning of *S. meliloti* bet genes

To localize the Tn5 insertion in LTS23-1020, genomic DNA was digested with *Eco*RI for which no site exists in Tn5 (Ditta, 1986), ligated into pBlueScript II SK(−), and used to transform *E. coli* DH5α to kanamycin resistance encoded by Tn5. Restriction analysis showed that a Km^r clone contained a plasmid, pGBS1020, with an insert harbouring a Tn5 insertion near one of its ends. Sequencing of the insert ends showed that the insertion mapped within a DNA fragment that showed significant identity to a segment of the *betA* gene from *E. coli*

Table 2. CDH and BADH activities, and fate of [methyl-¹⁴C]choline in three strains of *S. meliloti*

Strain	Enzyme activities*		¹⁴ C-labelled compounds†	
	CDH	BADH	Choline	Betaine
102F34rif	47	53	0.3	98.4
LTS23-1020	ND	53	99.3	0.6
LTS23-1020(pCHO34)	43	84	0.4	96.4

* Choline-induced cultures (Smith *et al.*, 1988) were grown to late exponential phase in LAS medium plus 7 mM choline. Enzyme activities are expressed as nmol substrate oxidized min^{−1} (mg protein)^{−1}. ND, undetectable (less than 0.2 nmol min^{−1} (mg protein)^{−1}). Values are means from three experiments with less than 9% variation.

† Uninduced cultures, grown to late exponential phase in LAS medium plus 0.3 M NaCl, were fed with 2 µM [methyl-¹⁴C]choline for 4 h. The cells were then collected by filtration, extracted in 80% (v/v) ethanol and the ¹⁴C-soluble compounds were identified and quantified following high-voltage electrophoresis of the ethanolic extracts (Bernard *et al.*, 1986; Smith *et al.*, 1988). The radioactivity detected in [methyl-¹⁴C]choline or [methyl-¹⁴C]betaine is expressed as a percentage of the total radioactivity (100 000 d.p.m.) recovered from the electrophorograms. The radioactivity of the ¹⁴CO₂- and ethanol-insoluble fractions was negligible, i.e., less than 1% of the supplied [methyl-¹⁴C]choline.

Fig. 1. Effect of exogenous choline, betaine aldehyde and betaine on the growth rate of *S. meliloti* LTS23-1020 (Tn5 mutant). Cells were grown in high osmolarity medium (LAS plus 0.5 M NaCl) in the absence of osmoprotectant (□), or in the presence of 1 mM choline (■), betaine aldehyde (○) or betaine (△). Control cells were grown in low-osmolarity LAS medium (△).

(Lamark *et al.*, 1991). Thus, the mutation in *S. meliloti* LTS23-1020 is clearly due to the presence of a Tn5 insertion in the gene encoding CDH activity (*betA*).

To obtain a clone containing an intact gene encoding the *betA* function, the CDH deficiency in LTS23-1020 was complemented using a gene bank of *S. meliloti* 102F34 DNA [in *E. coli* HB101(pRK290)] (Ditta *et al.*, 1980).

This bank was mated into LTS23-1020 using HB101(pRK2013) as the mobilizing strain. None of the strains used in the triparental mating could grow on choline plates since LTS23-1020 contains a *betA*::Tn5 insertion and *E. coli* strains do not catabolize choline further than betaine (Le Rudulier *et al.*, 1984; Landfald & Strøm, 1986). Exconjugants harbouring plasmids complementing the *betA* mutation were selected on S medium supplemented with 20 mM choline as the sole carbon and nitrogen source, and tetracycline for pRK290 selection. Two independent complementation experiments yielded about 60 tetracycline-resistant and choline-proficient clones after 6–7 d. Plasmid DNA was prepared from four clones from each mating experiment and subjected to *Bgl*II digestion. All recombinant plasmids carried the same 11.4 kb DNA insert. One of these plasmids was named pCHO34 and was further characterized by restriction analysis (Fig. 2).

To ascertain that pCHO34 effectively complements the *betA*::Tn5 mutation in *S. meliloti* LTS23-1020, it was used to transform *E. coli* HB101 and mated back into LTS23-1020. One exconjugant, LTS23-1020(pCHO34), was selected and subjected to a series of physiological and biochemical tests to verify its BetA⁺ phenotype. As expected, the exconjugant was able to grow in liquid S medium plus 20 mM choline, and displayed salinity tolerance comparable to the wild-type when grown in 0.5 M NaCl LAS medium plus 1 mM choline (data not shown). Furthermore, the exconjugant actively converted [*methyl*-¹⁴C]choline to [*methyl*-¹⁴C]betaine which accumulated in salt-stressed cells as it also did in the salt-stressed parental strain 102F34rif (Table 2). Subcloning DNA fragments from pCHO34 into pRK415

Fig. 2. Subcloning of the *bet* region of *S. meliloti* 102F34. The restriction map of the 11.4 kb DNA insert from pCHO34 and derived subclones is shown. A, *Apal*; G, *Bgl*II; H, *Hind*III; P, *Pst*I. The approximate locations of the *betA* and *betB* genes, deduced from DNA sequencing, are indicated above the restriction map. The BetA phenotype, as defined in the text, of *S. meliloti* LTS23-1020 exconjugants harbouring subclones of pCHO34 is shown on the right.

<i>S. mel.</i>	...MRAQPKA	SHFIDGEYVE	DAAGTVIESI	YPATGEIAR	LHAATPGIVE	47
<i>E. coli</i>	...MSRMAEQ	QLYIHGGYTS	ATSGRTFETI	NPANGNVLAT	VQAAGREDVD	47
barley	MAAPPAIPRR	GLFIFGGWRE	PTLGRHIPVI	NPATEDTIGD	IPAAATAEDVE	50
sugar beet	..MSMPIPSR	QLFIDGEWRE	PIKKNRIPVI	NPSNEEIIIGD	IPAGSSIEDIE	48
spinach	..MAFFIPAR	QLFIDGEWRE	PIKKNRIPVI	NPSTEEIIIGD	IPAAATAEDVE	48
<i>S. mel.</i>	*AIAAAKRA.	...QPEWAA	MSPTARGRIL	KRAAELMRQL	NRELSELETL	92
<i>E. coli</i>	RAVKSAAQGG.	...QKIWAS	MTAMERSRIL	RRAVDILRER	NDELAKLETL	92
barley	LAVAAGGPVL	ARRREP.WAR	ASGATRAKYL	NAIAAKITGK	IAYLALLETV	99
sugar beet	VAVAAARRAL	KRNKGREWAA	TSGAHRARYL	RAIAAKVTER	KDHFVKLETI	98
spinach	VAVAAARRAF	RRN...NWSA	TSGAHRATYL	RAIAAKITEK	KDHFVKLETI	95
<i>S. mel.</i>	****PIQETI	VADPTSGADS	FEFFGGVAPA	ALNGDY...I	PL.GGDF.AY	137
<i>E. coli</i>	DTGKAYSETS	TVDIVTGADV	LEYVAGLIP.	ALEGSQ...I	PLRETSF.VY	137
barley	DSGKPKDEA.	VADMDVAAC	FEYVAAAL.E	ALDGKQHAPI	SLPMEEFKTY	147
sugar beet	DSGKPFDEA.	VLDIDDVATC	FEYFAGQA.E	AMDAKQKAPV	TLPMERFKSH	146
spinach	DSGKPFDEA.	VLDIDDVASC	FEYFAGQA.E	ALDGKQKAPV	TLPMERFKSH	143
<i>S. mel.</i>	TKRVPLGVCV	GIGAWNPQQ	IACWKGAAPAL	VAGNAMVFKF	SENTPLGALK	187
<i>E. coli</i>	TRREPLGVVA	GIGAWNPPIQ	IALWKSAPAL	AAGNAMIFKP	SEVTLPLTALK	187
barley	VLKEPIGVVG	LITPWNYPPL	MATWKVAPAL	AAGCTAVLKP	SELASLTCL	197
sugar beet	VLRFPIGVVG	LITPWNYPPL	MATWKIAPAL	AAGCTAVLKP	SELASITCL	196
spinach	VLRFPLGVVG	LITPWNYPPL	MATWKIAPAL	AAGCTAVLKP	SELASVTCLE	193
<i>S. mel.</i>	IAEILLIEAGL	PKGLFNVIQG	DRA.TGPLLV	NHPDVAKVSL	TGSVPTGKKV	236
<i>E. coli</i>	LAEIYSEAGL	PDGVFNVLPG	VGAETGQVLT	EHPIAKVSF	TGGVASGKKV	237
barley	LGAICEEIGL	PSGVLNIITG	LGPDAGAPIA	SHPHVDKIAF	TGSTATGKTI	247
sugar beet	FGEVNEVGL	PPGVNLIVTG	LGPDAGAPLA	AHPDVKVAF	TGSSATGSKV	246
spinach	FGEVNEVGL	PPGVNLITG	LGPDAGAPLV	SHPDVKIAF	TGSSATGSKV	243
<i>S. mel.</i>	*AGAAAELK	HVTMELGGKS	PLIVFDD.AD	LESAGGAML	GNFYSTGGVC	284
<i>E. coli</i>	MANSAASSLK	HVTMELGGKS	PLIVFDD.AD	LDLAADIAMM	ANFFSSGGVC	286
barley	MT.AAAQMVK	PVSLLEGGKS	PLVTFDDVAD	IDKAVEWPM	GCFWNGGVC	296
sugar beet	MA.SAAQLVK	PVTLLEGGKS	PLIVFEDV.D	IDQVVEWTF	GCFWNGGVC	294
spinach	MA.SAAQLVK	PVTLLEGGKS	PLVVFEDV.D	IDQVVEWTF	GCFWNGGVC	291
<i>S. mel.</i>	SNGTRVVFQR	KIKEPFLARL	KERTEAIVIG	DPLDEATQLG	PMVSAARQDK	334
<i>E. coli</i>	TNGTRVVFPA	KCKAAFEQKI	LARVERIRAG	DVDFPQTNFG	PLVSPFPHRDN	336
barley	SATSRLLLHE	KIAEPFLDRL	VEWAKNIKIS	DPLEEGCRLG	SVISKGQYEQ	346
sugar beet	SATSRLLVHE	SIAAEFIDRL	VKWTKNIKIS	DPFEEGCRLG	PVISKGQYDK	344
spinach	SATSRLLVHE	SIAAEFVDKL	VKWTKNIKIS	DPFEEGCRLG	PVISKGQYDK	341
<i>S. mel.</i>	VFSYIGKGA	EGARLVTTGG	I.PNNVSGEG	TYIQPTVFAD	DTDGMTIARE	383
<i>E. coli</i>	VLRYIAKGE	EGARVLCGGD	VLKGDGPDNG	AWVAPTTFD	CSDDMTIVRE	386
barley	IKKFISTARS	EGATILHGGD	..RPKHLGKG	FFIEPTINTG	VSTSMQIWRE	394
sugar beet	IMKFISTAKS	EGATILCGGS	..RPEHLKKG	YFIEPTIISD	ISTSMQIWRE	392
spinach	IMKFISTAKS	EGATILYGGG	..RPEHLKKG	YFIEPTIVTD	ISTSMQIWKE	389
<i>S. mel.</i>	****EIPFGVMCVL	DFDDEVEVIA	****RANATEFGLS	AGVFTADLTR	****AHRVADRLEA	433
<i>E. coli</i>	****EIFGFMVMSIL	TYESEDEVIR	****RANDTDYGLA	AGIVTADLNR	****AHRVIHQLEA	436
barley	****EVFGFVICVK	VFKTESEAVE	****LANDTHYGLA	GGVISDDLER	****CERIAKVIHS	444
sugar beet	****EVFGFVLCVK	TFSSSEDEALD	****LANDTEYGLA	SAVFSKDLE	****CERVSKLLES	442
spinach	****EVFGFVLCVK	TFSSSEDEAIA	****LANDTEYGLA	AAVFSNDLER	****CERITKALEV	439
<i>S. mel.</i>	****GTLWINTYNL	CPVEIFPGGS	****KQSGFGRENS	VAAALNHYTEL	****KITVYGMGPV	483
<i>E. coli</i>	****GICWINTWGE	SPAEMPVGGY	****KHSIGIGRENG	VMTLQSYTQV	****KSIQVEMAKF	486
barley	****GIVWKNCSQP	TLVQAPWGGN	****KRSFGFREL	EWGLENYLSV	****KQVTRYCKDE	494
sugar beet	****GAVWVNCSP	CFVHAPWGGI	****KRSFGFREL	EWGIENYLN	****KQVTSDISNE	492
spinach	****GAVWVNCSP	CFVQAPWGGI	****KRSFGFREL	EWGIQNYLN	****KQVTDISDE	489
<i>S. mel.</i>	EAPY.....					487
<i>E. coli</i>	QSIF.....					490
barley	LYGWYQRPK	L				505
sugar beet	PWGMYKSP..					500
spinach	PWGMYKSP..					497

Fig. 3. Sequence alignment of the *S. meliloti* BetB gene product and homologous proteins from *E. coli* and higher plants. The deduced amino acid sequences were aligned with the PILEUP program of the GCG software package. The numbers indicate positions in the amino acid sequences. Dots within the sequences indicate gaps introduced to give optimal alignment. *S. mel.*, *S. meliloti* BetB, (GenBank accession number U39940, this study); *E. coli*, *E. coli* BetB (X52905, Lamark, et al., 1991); barley, *Hordeum vulgare* BADH (D26448, Ishitani et al., 1995); sugar beet, *Beta vulgaris* BADH (X58462, McCue & Hanson, 1992); spinach, *Spinacia oleracea* BADH (M31480, Weretilnyk & Hanson, 1989). Identical amino acid residues in all five sequences are shown in bold print, and those identical in the two bacterial proteins are marked by asterisks. Boxes indicate a decapeptide sequence and a cysteine residue which are highly conserved in betaine alcohol dehydrogenases and in general aldehyde dehydrogenases. Bold dots denote amino acid residues potentially involved in NAD-binding.

(Keen et al., 1988) was undertaken to precisely map the sequences which restore choline oxidation in *S. meliloti* LTS23-1020. The subclones were tested for growth in liquid S medium plus 20 mM choline, osmoprotection with 1 mM choline in 0.5 M NaCl LAS medium, and oxidation of [methyl-¹⁴C]choline at high osmolarity. pCHO361 (Fig. 2) contains the smallest insert (a 3.5 kb *ApaI*-*BglII* fragment) that complements the *betA* mutation, but neither pCHO343 nor pCHO350 restored the BetA⁺ phenotype in LTS23-1020. Hence, the *PstI* site

common to both of these plasmids most likely maps within *betA*.

Nucleotide sequence of the *betBA* genes

The sequence determined from the *PstI* site in pCHO350 (Fig. 2) overlapped with that determined from the T7 primer in pGBS1020. Thus, the two recombinant plasmids, pGBS1020 and pCHO350, share identical *Sinorhizobial* DNA sequences which are significantly hom-

<i>S.mel.</i>	MQP DFVIIG SGSAGS ALAY RLSE DGRNSV IVLE FGGSD. 38
<i>E.coli</i>	MQP DYIIIG AGSAGN VLAT RLTE DPNTSV LLLE AGGPDY 39
<i>A.glob.</i>	MHIDNIENLS DREF DYIVVG GGSAGA AVAA RLSE DPAVSV ALVE AGPDDR 50
<i>S.mel.</i>	.VGPF IQMPA ALAW PM SMNR YNW GYLSE PE PNLNNRR ITA PRG KVIGGSS 87
<i>E.coli</i>	RDFRT QMPA ALAF PL QGR YNW AYETE PE PFMNNRR MEC GRG KGLGGSS 89
<i>A.glob.</i>	GVPEVLQ LD R WMEL LE SG.. YDWD YPIEPQ EN.GNSFMRH ARAK VMGGCS 97
<i>S.mel.</i>	***** SING VMVVRG HSE DFNRW.E ELG AQGWAYA DVL PYYK ME HSHGG E EG.. 134
<i>E.coli</i>	LINGMCYIRG NALDL DN WAG EPGL EN WSYL DCL PYYR KA E TRDMG EN .. 137
<i>A.glob.</i>	SHNSCIAFWA PRED LDEWEA KYG ATGWNAE AAW PL YKR LE TNEDAG PD AP 147
<i>S.mel.</i>	WRG TD GL HL V QRG. P VKN PL FHA PIEAGKE AG FEVTE.DY NGS KQ EG FG L 182
<i>E.coli</i>	YHG GD GPVSV TTSK PG VN PL FEA MI EAG VQ AG YPR TD .DL NGY Q Q EG FG FP 186
<i>A.glob.</i>	HHG DS GPVHL .MNV P PKD PT GVA LLDACEQ AG IPRA KFN T GTTVV NG ANF 196
<i>S.mel.</i>	*MEQT.TWRGR RWS AA SAY LR PAL KRP N VEL .IRCFARKIV IE.NGR AT GV 229
<i>E.coli</i>	MDRTVT PG GR RAS TARGYLD QAK SR PN LTI RTHAMTDHII PD .GKR AV GV 235
<i>A.glob.</i>	FQIN RR ADGT RSS SVSYIH PIV EQENFTL LTGLR AR QLV FD ADR CT GV 246
<i>S.mel.</i>	*IERGGRIEV VK.. AN REVI VS ASSFNS PK LLML SGIG PR AHL K EM GIDV 277
<i>E.coli</i>	EWLEGDSTIP TRAT ANKEVL LC AGAIAS PQ ILQ RS GV NA ELL AE FD IP L 285
<i>A.glob.</i>	DIVDSAFGR HLRT ARNE EV LST GAID TP K LLML SGIG PA AHL AEHGIEV 296
<i>S.mel.</i>	KVDR PG VQ ON LQD HMEFYQ QVST KPVSLY SWL PWF WQ GV AGA QW LF FKR 327
<i>E.coli</i>	VHEL PG VQ EN LQD HLEMYLQ YECK EPVSLY PAL QW WN QPK IGA EW LF GGT 335
<i>A.glob.</i>	LGGL PR GRRA PAG PPG.... ..RR GA VR GQ AAH GRRV HAV VGD RHL HP HR 340
<i>S.mel.</i>	*GLGISNQFES CAFL RSAPGV KQP DIQYHFL PVA ISYDGKA AAK SHG FQ VH 377
<i>E.coli</i>	GVGASNHFEA GGF IRSREEF AWP NIQYHFL PVA INYN GSN AVK EHG FQ CH 385
<i>A.glob.</i>	G RP GP PRP DDALRLR AVR HEHPA.A RLP H GE RAS 374
<i>S.mel.</i>	**VGYNL S SRG NVSL RS SD PK ADP VIRFNYM S HP ED WEK F.DLRAV 421
<i>E.coli</i>	VGS MR SPSRG HVRI KSRDPH QHP AILFNYM S HE QD WQ EFRDAIRI 430
<i>A.glob.</i>	AS PT S RT PA PAAL SGCAA TSA IS PW STR ATS PTQ KGH D MRV MVAGIRK 424
<i>S.mel.</i>	TGE IF GE KAF DLY R GP E IQ P GEK VQ TD E IE DGFL REHLES AYH PC GT CKM 471
<i>E.coli</i>	TRE IM HQ PAL DQY R GE IS P GVE CQ TD E QL DEF VNRHAET AFH PC GT CKM 480
<i>A.glob.</i>	ARE IA AQ PAM AEWT GRE LS P GVE AQ TD E EL QDY IRK THNT VYH VG TV RM 474
<i>S.mel.</i>	*GAKDD P MAVV DP ETRVIGVD GLR VADSSIF PHI TYGNLNA PSI MT GE KA 521
<i>E.coli</i>	GY.. DEM SVV DGE GRVHGLE GLR VVDASIM PQI ITGNLNA TTI MI GE KIA 528
<i>A.glob.</i>	GAVE DE MSPL DP ELRVKGVT GLR VGDASVM PEH VTVP NP I TVM MI GE RCA 524
<i>S.mel.</i>	DHIL GRQ PLA RSN QEPWINP RWAV SDR. 548
<i>E.coli</i>	DMIR GQ EAL P RST AGYFVAN GMP VRAKK 556
<i>A.glob.</i>	DLIR SARAGE TTT ADAELSA ALA 547

Fig. 4. Sequence alignment of three bacterial choline-oxidizing enzymes. *S. meli.*, *S. meliloti* CDH (BetA, GenBank accession number U39940, this study), *E. coli*, *E. coli* CDH (BetA, X52905, Lamark *et al.*, 1991), and *A. glob.*, *A. globiformis* choline oxidase (CodA, X84895, Deshniun *et al.*, 1995). Identical amino acid residues in all three sequences are shown in bold print, and those identical in the two dehydrogenases from *S. meliloti* and *E. coli* are marked by asterisks. The putative ADP-binding site of FAD near the N-terminus of the three proteins is boxed. The inverted triangle between positions 358 and 359 indicates the site of the Tn5 insertion in strain LTS23-1020.

ologous to a segment of the *betA* gene from *E. coli* (Lamark *et al.*, 1991). In addition, sequencing of pCHO361 from the *Ap*I site showed significant homology to the *E. coli betB* gene. Hence, sequencing of the *bet* region from *S. meliloti* 102F34 was pursued on both DNA strands. The complete nucleotide sequence of the sinorhizobial *bet* region (GenBank accession number U39940) revealed two ORFs which have the same orientation and displayed 60% and 57% identical nucleotides to the *betB* and *betA* genes of *E. coli*, respectively (Lamark *et al.*, 1991).

The *S. meliloti betB* gene encodes a 487 amino acid protein (Fig. 3) which shares 54% identical and 71% similar residues with the BetB protein of *E. coli* (Lamark *et al.*, 1991), and only 40% identical and 62% similar residues with the plant BADHs (Weretilnyk & Hanson, 1990; McCue & Hanson, 1992; Ishitani *et al.*, 1995). All BADHs are similar in length (487–505 amino acids). The most conserved regions in both the bacterial and plant BADHs are confined to the central three-tenths and, to

a lesser extent, to the C-terminal region of the protein. A decapeptide motif [VT(L/M)ELGGKSP] and an array of amino acid residues highly conserved in the five BADHs (Weretilnyk & Hanson, 1990; Lamark *et al.*, 1991) are highlighted in Fig. 3. The decapeptide motif in the two bacteria displays only one deviation (a Met residue instead of a Leu residue) from the consensus sequence occurring in plant BADHs. The *S. meliloti betA* gene encodes a 548 residue polypeptide which shows 50% identity and 68% similarity to the enteric choline dehydrogenase throughout the polypeptide sequence (Fig. 4). By contrast, these two dehydrogenases share only 21% identical residues with the choline oxidase from *A. globiformis* (Deshniun *et al.*, 1995), essentially in the C- and N-terminal regions. However, both the two dehydrogenases and the oxidase possess, at their N-terminus, a 'glycine box' containing a conserved motif (GXGXXG) and a series of amino acids which are characteristic features of flavoproteins (Wierenga *et al.*, 1986; Hanukoglu & Gutfinger, 1989; Lamark *et al.*, 1991).

Fig. 5. Autoradiograph of Southern blots of *Xho*I-restricted DNA from strains of *S. meliloti*, *Ag. tumefaciens* and *E. coli* hybridized with 32 P-labelled *betA* (a) or *betB* (b) gene probes from *S. meliloti* 102F34. Each lane was loaded with 4 μ g genomic DNA. The hybridization was performed at 65 °C in 6 $\times$ SSC buffer and the membrane was washed twice in 1 $\times$ SSC at 65 °C. Lanes: 1, *S. meliloti* 102F34; 2, *S. meliloti* 1021; 3, *S. meliloti* LTS23-1020; 4, *S. meliloti* RCR2011; 5, *Ag. tumefaciens* GM19023; 6, *Ag. tumefaciens* At128; 7, *Ag. tumefaciens* At125; 8, *E. coli* HB101. Molecular mass markers are indicated in base pairs.

Symbiotic proficiency of LTS23-1020 and genomic location of the *betBA* genes

Besides the chromosome, *S. meliloti* contains two symbiotic megaplasms called pSyma and pSymb (Burkhardt *et al.*, 1987; Sobral *et al.*, 1991). Genes essential for the catabolism of at least three betaines, carnitine, trigonelline and stachydrine (proline betaine), map near the *nod* region of pSyma in *S. meliloti* RCR2011 (Goldman *et al.*, 1991). *stc* (stachydrine catabolism) mutants show delayed and reduced nodulation patterns (Goldman *et al.*, 1994), and genes encoding the catabolism of trigonelline are expressed throughout the *Sinorhizobium*/alfalfa symbiosis (Boivin *et al.*, 1990). Moreover, it was suggested that genes encoding the catabolism of choline and/or glycine betaine may also map to the *nod* region of pSyma (Goldman *et al.*, 1991). Therefore, we sought to determine both the symbiotic phenotype of *S. meliloti* LTS23-1020, and the replicon location of the *betBA* genes.

Nodulation tests conducted on alfalfa seedlings over 6 weeks with strains LTS23-1020 and 102F34rif showed that both strains were similarly efficient in inducing nodulation, although nitrogen fixation was slightly reduced by the *betA* mutation (data not shown). Thus,

strain LTS23-1020 is both Nod⁺ and Fix⁺. *S. meliloti* 102F34 DNA probes used in hybridization experiments were two *Eco*RI restriction fragments of 799 bp and 885 bp for the *betA* and *betB* genes, respectively. The *betA* probe (Fig. 5a) strongly hybridized (3.4 kb band) to DNA from *S. meliloti* 102F34, 1021 and 2011. The *betB* probe (Fig. 5b) also gave a strong signal (1.8 kb band) with the same strains. The 3.4 kb band hybridizing to the *betA* probe was shifted downward to 1.7 kb in strain LTS23-1020 (Fig. 5a, lane 3), due to the insertion of the Tn5 transposon, which introduced additional *Xho*I sites in the inactivated *betA* gene. No hybridization was detected between the two sinorhizobial probes and DNA from the Bet⁺ strain *E. coli* HB101 under the high stringency conditions used in the experiment. To permit the rapid determination of the replicon location of the *bet* genes in *S. meliloti*, derivatives of *Ag. tumefaciens* GM19023 (Rosenberg & Huguet, 1984), containing either the pSyma (At128) or the pSymb (At125) megaplasms from *S. meliloti* 1021 (Finan *et al.*, 1986), were also used. Both probes strongly hybridized to a 7 kb *Xho*I fragment present in all three strains (Fig. 5a, b, lanes 5–7), probably representing the *bet* genes of *Ag. tumefaciens*. No extra band corresponding to the *bet* gene fragments from strain 102F34 was detected with At128 and At125 DNA, indicating that the *S. meliloti* *betBA* genes are not located on the megaplasms.

DISCUSSION

Physiological and biochemical data obtained with a mutant deficient in choline dehydrogenase activity demonstrate that choline *per se* is not an osmoprotectant in *S. meliloti*, as previously reported for *E. coli* (Styrvold *et al.*, 1986). Indeed, choline is osmoprotective only after subsequent oxidation to betaine which can be accumulated to high concentrations in osmotically stressed cells (Le Rudulier *et al.*, 1984; Styrvold *et al.*, 1986; Csonka & Hanson, 1991). The two genes encoding the enzymes responsible for betaine production from choline were cloned and sequenced. They show 60% and 57% identity to the *betB* and *betA* genes of *E. coli* (Lamark *et al.*, 1991). Despite this, functional complementation of a *betA* mutation in *E. coli* MLE33 (Eshoo, 1988) with the *sinorhizobial* genes was unsuccessful (data not shown).

The organization of the *bet* region in *S. meliloti* differs from that in *E. coli* (Lamark *et al.*, 1991) by the absence of *betI* and *betT* homologues in the 500 bp region upstream of the *sinorhizobial betB* gene. No coding sequence was identified downstream of *betA*. In addition, *betB* and *betA* are separated by a 210 bp non-coding sequence in *S. meliloti* while homologous genes are tandemly linked in *E. coli* (*betBA*; Lamark *et al.*, 1991), and *A. globiformis* (*betB* and *codA*; Deshnum *et al.*, 1995). It is also noteworthy that the intergenic sequence between *betB* and *betA* in *S. meliloti* contains a region of inverted repeats whose transcript could form a stem-loop structure which may function as a terminator of transcription (Platt, 1986). Interestingly, a computer search of the GenBank database also reveals that this region of dyad symmetry shares high homology with a similar structure located downstream from the *ftsZ2* (cell division) gene of *S. meliloti* 1021 (Margolin & Long, 1994) and from the *nolC* (cultivar-specific nodulation) gene from *S. fredii* USDA257 (Krishnan & Pueppke, 1991). The significance of these sequence similarities, and the function of the intergenic sequence between *betB* and *betA*, are unclear. Experiments are currently under way to determine whether the *sinorhizobial betB* and *betA* genes belong to the same operon, or are transcribed from distinct promoters.

The alignment of the deduced BADH sequences from *S. meliloti*, *E. coli*, barley, sugar beet and spinach (Fig. 3) shows that the *sinorhizobial* BADH presents higher homology to that from *E. coli*, which is highly specific for betaine aldehyde (Lamark *et al.*, 1991), than to the plant BADHs which can oxidize a variety of aldehydes (Weretilnyk & Hanson, 1990). However, despite these significant differences in substrate specificity, the BADH from *S. meliloti* contains a conserved decapeptide motif and a series of highly conserved amino acids (Fig. 3), which are postulated to be catalytically essential, possibly by forming the ADP-binding domain of the NAD coenzyme (Weretilnyk & Hanson, 1990; Lamark *et al.*, 1991; Ishitani *et al.*, 1995).

The three known bacterial choline-oxidizing enzymes (Fig. 4) are similar in length (547–556 residues) and contain a so-called 'glycine box' which most likely

functions as a binding site for the ADP moiety of the FAD coenzyme (Wierenga *et al.*, 1986). The algorithm of Kyte & Doolittle (1982) was used to determine the hydrophobic character of the predicted BetA protein from *S. meliloti*. This protein displays a profile which can readily be superimposed on that obtained for the membrane-linked BetA protein from *E. coli* (Lamark *et al.*, 1991; Landfald & Strøm, 1986). These data agree with our unpublished observations showing that CDH activity is also membrane-bound in *S. meliloti*. Conversely, choline oxidase is a flavoprotein which is a cytosolic, hydrogen-peroxide-forming oxidase in *A. globiformis* and *A. pascens* (Rozwadowski *et al.*, 1991; Deshnum *et al.*, 1995). In *E. coli* and *A. pascens*, CDH and choline oxidase, respectively, catalyse both steps of betaine biosynthesis from choline and we predict that a similar situation occurs in *S. meliloti*. The differences in their cellular location, and in the specific catalytic mechanisms which differentiate general dehydrogenases from oxidases (Brouquisse *et al.*, 1989), most likely account for the rather low sequence homology displayed by the two classes of bacterial choline-oxidizing enzymes (Fig. 4). The structural relationship of these enzymes with the chloroplastic, ferredoxin-dependent, choline monooxygenase from higher plants (Brouquisse *et al.*, 1989) cannot yet be established.

At low osmolarity, *S. meliloti* can use choline and betaine as carbon and nitrogen sources. Both betaine biosynthesis (CDH and BADH activities) and catabolism are strongly stimulated by growth on choline (Bernard *et al.*, 1986; Pocard, 1987; Smith *et al.*, 1988). At high osmolarity, CDH activity remains the same and BADH activity is slightly increased, while the activities of enzymes involved in betaine degradation strongly decrease, thus favouring betaine accumulation. Nevertheless, significant catabolism of betaine still occurs in osmotically stressed cells if a sufficient amount of exogenous choline or betaine is supplied to the culture (Pocard, 1987; Smith *et al.*, 1988). In contrast, betaine functions only as a compatible solute in *E. coli* (Le Rudulier *et al.*, 1984; Landfald & Strøm, 1986), whereas it is only used as a growth substrate in *A. pascens* (Rozwadowski *et al.*, 1991). These physiological and biochemical specificities in *S. meliloti* are indicative of an original regulation of the choline–betaine pathway in this bacterium. The regulation of the expression of the *sinorhizobial betBA* genes is currently under study in free-living cells. It will also be analysed in bacteroids, either isolated or *in planta*, at different symbiotic stages. Henceforward, the metabolic engineering of the biosynthetic and catabolic pathways for betaine into other agronomically important strains of *Rhizobium* and *Sinorhizobium* is a novel approach to enhance salinity tolerance in those strains. Two recent studies showing that the expression of the *bet* genes from *E. coli* and the *codA* gene from *A. globiformis* confers betaine accumulation and osmoprotection in the salt-sensitive cyanobacterium *Synechococcus* sp. PCC7942 (Deshnum *et al.*, 1995; Nomura *et al.*, 1995) support this goal. Indeed, increased osmotic tolerance in rhizobia

might favour their propagation in the soil and, hence, their symbiotic efficiency. Such bacteria might also gain a selective advantage in the rhizosphere where choline is present as a byproduct of the degradation of plant phospholipids and plant sap exudates in which phosphorylcholine is an abundant component (Maizel *et al.*, 1956).

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