Microbiology (1997), **143**, 1369–1379 Printed in Great Britain

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 Tn*5* 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*-^{14}C]choline to [*methyl*-^{14}C]betaine, and did not use choline, but still used betaine, as an osmoprotectant. The Tn*5* 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 ressembling 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; Deshnium *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*-^{14}C]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*-^{14}C]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 10^9 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 40000 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		
Sinorhizobium meliloti		
102F34	Wild-type	Ditta *et al.* (1980)
102F34rif	Spontaneous Rif^r derivative of 102F34	This study
LTS23-1020	102F34rif *betA*::Tn5	This study
RCR2011	SU47, wild-type strain	C. Rosenberg*
1021	RCR2011str, Str^r derivative of RCR2011	Meade *et al.* (1982)
Agrobacterium tumefaciens		
GMI9023	C58 cured of pAtC58 and pTiC58	Rosenberg & Huguet (1984)
At125	GMI9023 pRme1021b	Finan *et al.* (1986)
At128	GMI9023 pRme1021a	Finan *et al.* (1986)
Escherichia coli		
DH5α	F$^-$, *supE44* Δ*lac*U169(*ϕ80lacZ*ΔM15) *hsdR*17($r_K^- m_K^+$) *recA1 endA1 gyrA96 thi-1 relA1*	Sambrook *et al.* (1989)
HB101	F$^-$, *hsdS*20($r_B^- m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 leuB6 xyl-5 mtl-1 supE44 rpsL20*	Sambrook *et al.* (1989)
MLE33	MC4100 *recA56* Rif^r (F′2 *betA5*::*lacZY*)	Eshoo (1988)
Plasmids		
pRK290	Broad-host-range cloning vector, IncP1, Tc^r	Ditta *et al.* (1980)
pRK415	pRK290 derivative with pUC9 polylinker, Tc^r	Keen *et al.* (1988)
pRK2013	ColE1 replicon with RK2 transfer region, Nm-Km^r	Ditta *et al.* (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 *betA*::Tn5 mutation in LTS23-1020	This study
pGBS1020	*betA*::Tn5 *Eco*RI fragment from LTS23-1020 genomic DNA cloned into pBlueScript II SK(−)	This study
pCHO341	8·6 kb *Bgl*II fragment from pCHO34 cloned into pRK415	This study
pCHO342	7·3 kb *Hin*dIII–*Bgl*II fragment from pCHO34 cloned into pRK415	This study
pCHO343	4·2 kb *Pst*I fragment from pCHO34 cloned into pRK415	This study
pCHO350	1·5 kb *Pst*I–*Bgl*II fragment from pCHO34 cloned into pRK415	This study
pCHO361	3·5 kb *Apa*I–*Bgl*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*-^{14}C]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 DH5α 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 electrophoresis, 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-^{32}P]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 [^{35}S]dATPαS (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 Tn*5* 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*-^{14}C]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*-^{14}C]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*-^{14}C]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*-^{14}C]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	UD	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)$^{-1}$. UD, 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*-^{14}C]choline for 4 h. The cells were then collected by filtration, extracted in 80% (v/v) ethanol and the ^{14}C-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*-^{14}C]choline or [*methyl*-^{14}C]betaine is expressed as a percentage of the total radioactivity (100000 d.p.m.) recovered from the electrophorograms. The radioactivity of the $^{14}CO_2$- and ethanol-insoluble fractions was negligible, i.e., less than 1% of the supplied [*methyl*-^{14}C]choline.

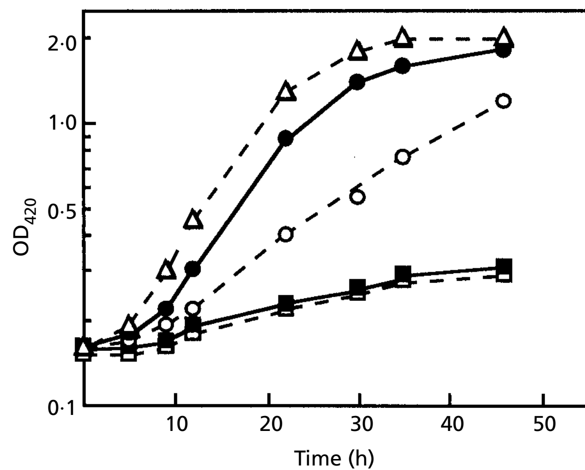

Fig. 1. Effect of exogenous choline, betaine aldehyde and betaine on the growth rate of *S. meliloti* LTS23-1020 (Tn*5* mutant). Cells were grown in high osmolarity medium (LAS plus 0·5 M NaCl) in the absence of osmoprotectant (□), or in the presence of 1 mM choline (■), betaine aldehyde (○) or betaine (●). 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*-^{14}C]choline to [*methyl*-^{14}C]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

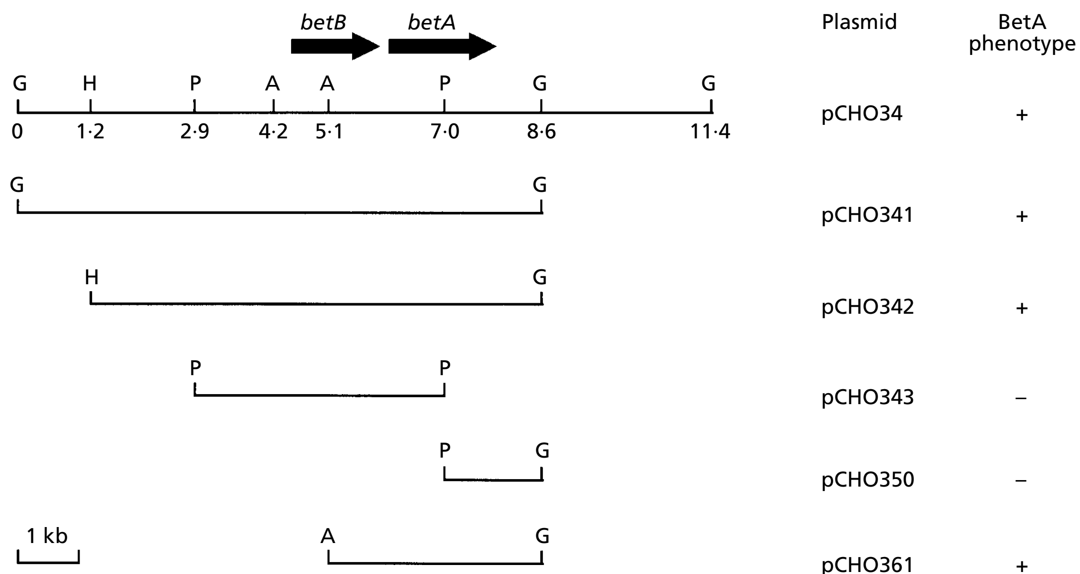

Fig. 2. Subcloning of the *bet* region of *S. meliloti* 102F34. The restriction map of the 11·4 kb DNA insert from pCHO34 and derived subclones is shown. A, *Apa*I; G, *Bgl*II; H, *Hind*III; P, *Pst*I. The approximate locations of the *betA* and *betB* genes, deduced from DNA sequencing, are indicated 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.

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                 *      * * *     *   * *  ** *   *     **    *
S.mel.        ...MRAQPKA SHFIDGEYVE DAAGTVIESI YPATGEIIAR LHAATPGIVE  47
E.coli        ...MSRMAEQ QLYIHGGYTS ATSGRTFETI NPANGNVLAT VQAAGREDVD  47
barley        MAAPPAIPRR GLFIGGGWRE PTLGRHIPVI NPATEDTIGD IPAATAEDVE  50
sugar beet    ..MSMPIPSR QLFIDGEWRE PIKKNRIPII NPSNEEIIGD IPAGSSEDIE  48
spinach       ..MAFPIPAR QLFIDGEWRE PIKKNRIPVI NPSTEEIIGD IPAATAEDVE  48

               *   *         *  **  *    * ***  **    *    * **  ****
S.mel.        KAIAAAKRA. ....QPEWAA MSPTARGRIL KRAAELMRQL NRELSELETL  92
E.coli        RAVKSAQQG. ....QKIWAS MTAMERSRIL RRAVDILRER NDELAKLETL  92
barley        LAVAAGGPVL ARRREP.WAR ASGATRAKYL NAIAAKITGK IAYLALLETV  99
sugar beet    VAVAAARRAL KRNKGREWAA TSGAHRARYL RAIAAKVTER KDHFVKLETI  98
spinach       VAVVAARRAF RRN...NWSA TSGAHRATYL RAIAAKITEK KDHFVKLETI  95

              ****   **    *   ***   *   *  *   ** *     * **    *  *
S.mel.        DTGKPIQETI VADPTSGADS FEFFGGVAPA ALNGDY...I PL.GGDF.AY 137
E.coli        DTGKAYSETS TVDIVTGADV LEYYAGLIP. ALEGSQ...I PLRETSF.VY 137
barley        DSGKPKDEA. VADMDDVAAC FEYYAALA.E ALDGKQHAPI SLPMEEFKTY 147
sugar beet    DSGKPFDEA. VLDIDDVATC FEYFAGQA.E AMDAKQKAPV TLPMERFKSH 146
spinach       DSGKPFDEA. VLDIDDVASC FEYFAGQA.E ALDGKQKAPV TLPMERFKSH 143

              * * ****   ******** * ** ** ****  ***** *** ** *** ***
S.mel.        TKRVPLGVCV GIGAWNYPQQ IACWKGAPAL VAGNAMVFKP SENTPLGALK 187
E.coli        TRREPLGVVA GIGAWNYPIQ IALWKSAPAL AAGNAMIFKP SEVTPLTALK 187
barley        VLKEPIGVVG LITPWNYPLL MATWKVAPAL AAGCTAVLKP SELASLTCLE 197
sugar beet    VLRQPIGVVG LITPWNYPLL MATWKIAPAL AAGCTAVLKP SELASITCLE 196
spinach       VLRQPLGVVG LISPWNYPLL MATWKIAPAL AAGCTAVLKP SELASVTCLE 193

               ***  **** * * ***  *   * **  *   **  ****  ** *  ****
S.mel.        IAEILIEAGL PKGLFNVIQG DRA.TGPLLV NHPDVAKVSL TGSVPTGKKV 236
E.coli        LAEIYSEAGL PDGVFNVLPG VGAETGQYLT EHPGIAKVSF TGGVASGKKV 237
barley        LGAICEEIGL PSGVLNIITG LGPDAGAPIA SHPHVDKIAF TGSTATGKTI 247
sugar beet    FGEVCNEVGL PPGVLNIVTG LGPDAGAPLA AHPDVDKVAF TGSSATGSKV 246
spinach       FGEVCNEVGL PPGVLNILTG LGPDAGAPLV SHPDVDKIAF TGSSATGSKV 243

               *  **  **  ********* ******* ** *  *   **   ** * ****
S.mel.        .AGAAAAELK HVTMELGGKS PLIVFDD.AD LESAIGGAML GNFYSTGQVC 284
E.coli        MANSAASSLK EVTMELGGKS PLIVFDD.AD LDLAADIAMM ANFFSSGQVC 286
barley        MT.AAAQMVK PVSLELGGKS PLVTFDDVAD IDKAVEWPML GCFFNGGQVC 296
sugar beet    MA.SAAQLVK PVTLELGGKS PIIVFEDV.D IDQVVEWTMF GCFWTNGQIC 294
spinach       MA.SAAQLVK PVTLELGGKS PIVVFEDV.D IDKVVEWTIF GCFWTNGQIC 291

               *******   * *  *     * * *  * *  *  *  *  * **   **
S.mel.        SNGTRVFVQR KIKEPFLARL KERTEAIVIG DPLDEATQLG PMVSAAQRDK 334
E.coli        TNGTRVFVPA KCKAAFEQKI LARVERIRAG DVFDPQTNFG PLVSFPHRDN 336
barley        SATSRLLLHE KIAEPFLDRL VEWAKNIKIS DPLEEGCRLG SVISKGQYEQ 346
sugar beet    SATSRLLVHE SIAAEFIDRL VKWTKNIKIS DPFEEGCRLG PVISKGQYDK 344
spinach       SATSRLLVHE SIAAEFVDKL VKWTKNIKIS DPFEEGCRLG PVISKGQYDK 341

              *  ** *** ****   **            *     **** *   * *** **
S.mel.        VFSYIGKGKA EGARLVTGGG I.PNNVSGEG TYIQPTVFAD DTDGMTIARE 383
E.coli        VLRYIAKGKE EGARVLCGGD VLKGDGFDNG AWVAPTVFTD CSDDMTIVRE 386
barley        IKKFISTARS EGATILHGGD ..RPKHLGKG FFIEPTINTG VSTSMQIWRE 394
sugar beet    IMKFISTAKS EGATILCGGS ..RPEHLKKG YFIEPTIISD ISTSMQIWRE 392
spinach       IMKFISTAKS EGATILYGGS ..RPEHLKKG YYIEPTIVTD ISTSMQIWKE 389

              *******  *     * ***  *** *  **  **  **** * ****   ***
S.mel.        EIFGPVMCVL DFDDEVEVIA RANATEFGLS AGVFTADLTR AHRVADRLEA 433
E.coli        EIFGPVMSIL TYESEDEVIR RANDTDYGLA AGIVTADLNR AHRVIHQLEA 436
barley        EVFGPVICVK VFKTESEAVE LANDTHYGLA GGVISDDLER CERIAKVIHS 444
sugar beet    EVFGPVLCVK TFSSEDEALD LANDTEYGLA SAVFSKDLER CERVSKLLES 442
spinach       EVFGPVLCVK TFSSEDEAIA LANDTEYGLA AAVFSNDLER CERITKALEV 439

              *  ****     * * * **  * ** ****  *  *  **   *   * *
S.mel.        GTLWINTYNL CPVEIPFGGS KQSGFGRENS VAALNHYTEL KTVYVGMGPV 483
E.coli        GICWINTWGE SPAEMPVGGY KHSGIGRENG VMTLQSYTQV KSIQVEMAKF 486
barley        GIVWKNCSQP TLVQAPWGGN KRSGFGRELG EWGLENYLSV KQVTRYCKDE 494
sugar beet    GAVWVNCSQP CFVHAPWGGI KRSGFGRELG EWGIENYLNI KQVTSDISNE 492
spinach       GAVWVNCSQP CFVQAPWGGI KRSGFGRELG EWGIQNYLNI KQVTQDISDE 489

S.mel.        EAPY...... . 487
E.coli        QSIF...... . 490
barley        LYGWYQRPSK L 505
sugar beet    PWGWYKSP.. . 500
spinach       PWGWYKSP.. . 497
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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*-^{14}C]choline at high osmolarity. pCHO361 (Fig. 2) contains the smallest insert (a 3·5 kb *Apa*I–*Bgl*II fragment) that complements the *betA* mutation, but neither pCHO343 nor pCHO350 restored the BetA$^+$ phenotype in LTS23-1020. Hence, the *Pst*I site common to both of these plasmids most likely maps within *betA*.

Nucleotide sequence of the *betBA* genes

The sequence determined from the *Pst*I 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-

```
S.mel.    .......... .MQADFVIIG SGSAGSALAY RLSEDGRNSV IVLEFGGSD.  38
E.coli    .......... .MQFDYIIIG AGSAGNVLAT RLTEDPNTSV LLLEAGGPDY  39
A.glob.   MHIDNIENLS DREFDYIVVG GGSAGAAVAA RLSEDPAVSV ALVEAGPDDR  50

S.mel.    .VGPFIQMPA ALAWPMSMNR YNWGYLSEPE PNLNNRRITA PRGKVIGGSS  87
E.coli    RFDFRTQMPA ALAFPLQGKR YNWAYETEPE PFMNNRRMEC GRGKGLGGSS  89
A.glob.   GVPEVLQLDR WMELLESG.. YDWDYPIEPQ EN.GNSFMRH ARAKVMGGCS  97

S.mel.    SINGMVYVRG HSEDFNRW.E ELGAQGWAYA DVLPYYKRME HSHGGEEG.. 134
E.coli    LINGMCYIRG NALDLDNWAQ EPGLENWSYL DCLPYYRKAE TRDMGEND.. 137
A.glob.   SHNSCIAFWA PREDLDEWEA KYGATGWNAE AAWPLYKRLE TNEDAGPDAP 147

S.mel.    WRGTDGPLHV QRG.PVKNPL FHAFIEAGKE AGFEVTE.DY NGSKQEGFGL 182
E.coli    YHGGDGPVSV TTSKPGVNPL FEAMIEAGVQ AGYPRTD.DL NGYQQEGFGP 186
A.glob.   HHGDSGPVHL .MNVPPKDPT GVALLDACEQ AGIPRAKFNT GTTVVNGANF 196

S.mel.    MEQT.TWRGR RWSAASAYLR PALKRPNVEL .IRCFARKIV IE.NGRATGV 229
E.coli    MDRTVTPQGR RASTARGYLD QAKSRPNLTI RTHAMTDHII FD.GKRAVGV 235
A.glob.   FQINRRADGT RSSSSVSYIH PIVEQENFTL LTGLRARQLV FDADRRCTGV 246

S.mel.    EIERGGRIEV VK..ANREVI VSASSFNSPK LLMLSGIGPR AHLKEMGIDV 277
E.coli    EWLEGDSTIP TRATANKEVL LCAGAIASPQ ILQRSGVGNA ELLAEFDIPL 285
A.glob.   DIVDSAFGRT HRLTARNEVV LSTGAIDTPK LLMLSGIGPA AHLAEHGIEV 296

S.mel.    KVDRPGVGQN LQDHMEFYFQ QVSTKPVSLY SWLPWFWQGV AGAQWLFFKR 327
E.coli    VHELPGVGEN LQDHLEMYLQ YECKEPVSLY PALQWWNQPK IGAEWLFGGT 335
A.glob.   LGGLPRRGRA PAGPPG.... ..RRGAVRGQ AAHGRRVHAV VGDRHLHPHR 340

S.mel.    GLGISNQFES CAFLRSAPGV KQPDIQYHFL PVAISYDGKA AAKSHGFQVH 377
E.coli    GVGASNHFEA GGFIRSREEF AWPNIQYHFL PVAINYNGSN AVKEHGFQCH 385
A.glob.   G......... ......RPGP PRPDDALRLR AVRHEHPA.A RLPHHGERAS 374

S.mel.    VGYNLSKSRG NVSLRSSDPK ADPVIRFNYM S.....HPED WEKF.DLRAV 421
E.coli    VGSMRSPSRG HVRIKSRDPH QHPAILFNYM S.....HEQD WQEFRDAIRI 430
A.glob.   ASPRTSRTPA PAALSGCAAA TSAISPWSTR ATSPTQKGHD MRVMVAGIRK 424

S.mel.    TGEIFGEKAF DLYRGPEIQP GEKVQTDEEI DGFLREHLES AYHPCGTCKM 471
E.coli    TREIMHQPAL DQYRGREISP GVECQTDEQL DEFVRNHAET AFHPCGTCKM 480
A.glob.   AREIAAQPAM AEWTGRELSP GVEAQTDEEL QDYIRKTHNT VYHPVGTVRM 474

S.mel.    GAKDDPMAVV DPETRVIGVD GLRVADSSIF PHITYGNLNA PSIMTGEKSA 521
E.coli    GY..DEMSVV DGEGRVHGLE GLRVVDASIM PQIITGNLNA TTIMIGEKIA 528
A.glob.   GAVEDEMSPL DPELRVKGVT GLRVGDASVM PEHVTVNPNI TVMMIGERCA 524

S.mel.    DHILGRQPLA RSNQEPWINP RWAVSDR. 548
E.coli    DMIRGQEALP RSTAGYFVAN GMPVRAKK 556
A.glob.   DLIRSARAGE TTTADAELSA ALA....  547
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Fig. 4. Sequence alignment of three bacterial choline-oxidizing enzymes. *S. mel., 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, Deshnium *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 Tn*5* 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 *Apa*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* (Deshnium *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).

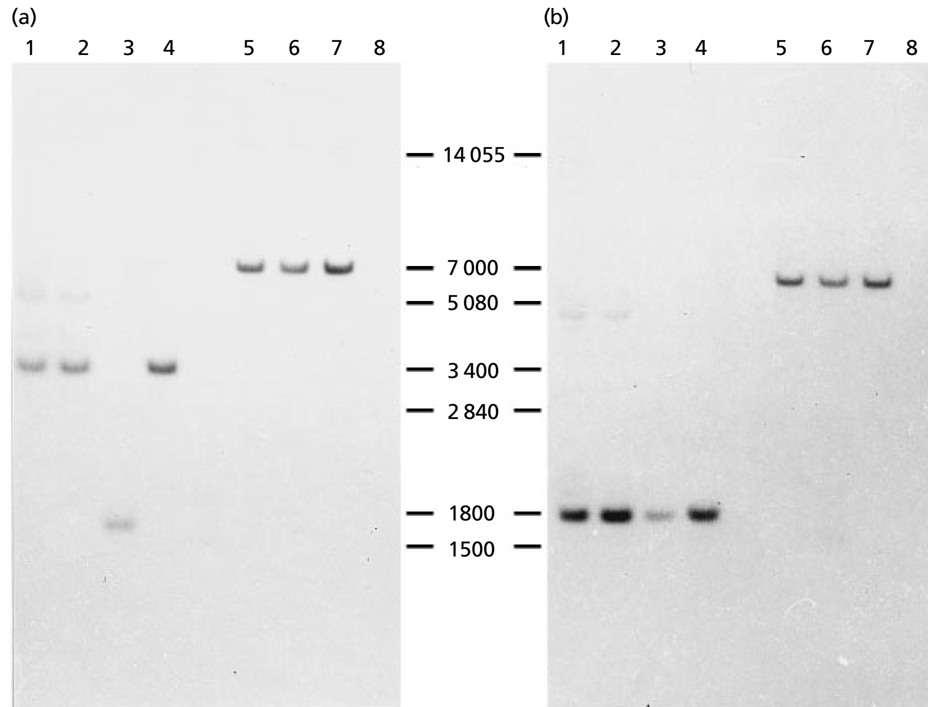

Fig. 5. Autoradiograph of Southern blots of *Xho*I-restricted DNA from strains of *S. meliloti*, *Ag. tumefaciens* and *E. coli* hybridized with ^{32}P-labelled *betA* (a) or *betB* (b) gene probes from *S. meliloti* 102F34. Each lane was loaded with 4 µg genomic DNA. The hybridization was performed at 65 °C in 6 × SSC buffer and the membrane was washed twice in 1 × 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* GMI9023; 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 megaplasmids 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 Tn*5* 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* GMI9023 (Rosenberg & Huguet, 1984), containing either the pSyma (At128) or the pSymb (At125) megaplasmid 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 megaplasmids.

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*; Deshnium *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 hydropathic 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; Deshnium *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 (Deshnium *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).

ACKNOWLEDGEMENTS

This work was funded by the European Communities BIOTECH Programme, as part of the Project of Technological Priority 1993–1996 (BIO2CT930400, D.L.R.), the Ministère de l'Education Nationale, DREIF (D.L.R.) and the National Science Foundation (grant DCB-8903923, L.T.S.). E.B. received a doctoral fellowship from the Ministère de l'Enseignement Supérieur et de la Recherche. We acknowledge S. Uratsu for her expert technical assistance. We are grateful to G. Ditta for providing the genomic bank of *S. meliloti*, and to M. Eshoo, T. Finan, N. T. Keen, S. R. Long and C. Rosenberg for providing bacterial strains and plasmids. M. Østerås and D. Hérouart are thanked for helpful discussions.

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Received 12 July 1996; revised 21 October 1996; accepted 8 November 1996.