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## Isolation and molecular characterization of the *Sinorhizobium meliloti bet* locus encoding glycine betaine biosynthesis

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To cope with osmotic stress, *Sinorhizobium meliloti* accumulates organic compatible solutes such as glutamate, trehalose, N-acetylglutaminylglutamine amide, and the most potent osmoprotectant glycine betaine. In order to study the regulation of the glycine betaine biosynthetic pathway, a genetic and molecular analysis was performed. We have selected a Tn5 mutant of *S. meliloti* which was deficient in choline dehydrogenase activity. The mutation was complemented using a genomic bank of *S. meliloti*. Subcloning and DNA sequencing of a 8.6 kb region from the complemented plasmid showed four open reading frames with an original structural organization of the *bet* locus compared to that described in *E. coli*. (i) The *betB* and the *betA* genes which encode a glycine betaine aldehyde dehydrogenase, and a choline dehydrogenase, respectively, are separated from the *betI* gene (regulatory protein) by an additional gene named *betC*. The BetC protein shares about 30% identity with various sulphatases and is involved in the conversion of choline-O-sulphate into choline. Choline-O-sulphate is used as an osmoprotectant, or as a carbon or sulphur source and this utilization is dependent on a functional *bet* locus. (ii) No sequence homologous to *betT* (encoding a high-affinity choline transport system in *E. coli*) was found in the vicinity of the *bet* locus. (iii) The *betB* and the *betA* genes, as well as the *betI* and the *betC* genes are, respectively, separated by 211 and 167 bp sequences containing inverted repeats. Southern blot analysis indicated that the *bet* locus is located on the chromosome, and not on the megaplasmids.

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### 1. Introduction

The soil bacterium *Sinorhizobium meliloti* has the ability to develop a symbiotic relationship with alfalfa within specific root nodules where it can reduce atmospheric nitrogen into ammonia which is taken up by the plant host. The osmotic environment within the rhizosphere may affect all steps of the plant-microbe interaction, from the root colonization to nodule development and function. Although *S. meliloti* has shown the ability to use different compatible solutes in the presence of an osmotic stress, it appears that glycine betaine (N,N,N-trimethylglycine) is the most potent osmoprotectant and strongly stimulates the growth rate of the bacteria in high salt medium (Le Rudulier and Bernard 1986). Unlike enteric bacteria such as *Escherichia coli*, *S. meliloti* can

catabolize glycine betaine and uses it both as carbon and nitrogen sources for growth. The importance of this compound in osmoregulation is confirmed by the observation that the catabolic pathway is repressed in the presence of high salt concentrations, favouring the intracellular accumulation of the compound over its metabolization (Bernard *et al* 1986; Smith *et al* 1988). Glycine betaine accumulation in *S. meliloti* salt-stressed cells occurs by direct uptake from the environment or by biosynthesis from the oxidation of choline by a two-step pathway with glycine betaine aldehyde as intermediate (Smith *et al* 1988). This pathway has been identified in several bacterial and plant species, where it differs only by the enzymes involved. Gram-positive bacteria such as *Arthrobacter pascens* and *A. globiformis*, and the fungus *Cylindrocarpon didymum* use a soluble choline oxidase

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to catalyse both the steps (Tani *et al* 1979; Rozwadowski *et al* 1991). Higher plants and Gram-negative bacteria catalyse the conversion of choline into glycine betaine with a structurally conserved betaine aldehyde dehydrogenase (BADH); the first step is catalysed by a choline mono-oxygenase in plants and a choline dehydrogenase (CDH) in bacteria like *E. coli*, *Pseudomonas aeruginosa* and *S. meliloti* (Nagasawa *et al* 1976; Rathinasabapathi *et al* 1997; Landfald and Strøm 1986; Smith *et al* 1988).

The glycine betaine biosynthesis pathway has been characterized at the molecular level in *E. coli*, where it consists of four genes, *betTIBA*, encoding respectively, a high-affinity choline transport system, a repressor, the betaine aldehyde dehydrogenase, and the choline dehydrogenase. In *B. subtilis* it consists of two genes, *gbsA* and *gbsB* (Lamark *et al* 1991; Boch *et al* 1996). The *betBA* genes of *S. meliloti* were recently identified and characterized (Pocard *et al* 1997). In order to study the regulation of the glycine betaine biosynthetic pathway, a complete genetic and molecular analysis of the *bet* region was performed which revealed the presence of two additional genes encoding a BetI-like repressor, and a sulphatase that are transcribed together with *betBA*. We present evidence that the sulphatase encoded by *betC* is involved in the conversion of choline-O-sulphate whose uptake is induced by salt stress and choline-O-sulphate or choline.

## 2. Main compatible solutes in salt-stressed *S. meliloti*

It is well established that the main strategy evolved by bacteria to cope with osmotic stress is the accumulation of some solutes, ions or organic compounds of low molecular weight, termed compatible solutes (Le Rudulier *et al* 1984). Such intracellular accumulation relies on transport of osmoprotectants from the surrounding environment and/or synthesis which can be associated with the absence or reduction of their catabolism.

*S. meliloti* cells subjected to osmotic stress are able

to accumulate simultaneously several compatible solutes. The most prevalent cation in the cytoplasm is potassium and its concentration is increased in salt-stressed cells (Le Rudulier and Bernard 1986). Only a few amino acids are frequently accumulated. Glutamate biosynthesis is stimulated when cells are grown in high-osmolarity medium (Smith *et al* 1994). In contrast, proline, which is actively catabolized is not accumulated in response to salt stress. With respect to sugars, the accumulation of trehalose has been detected by <sup>13</sup>C-NMR studies in cells grown at high osmolarity, mainly during the stationary phase (Smith *et al* 1994). In similar growth conditions, *S. meliloti* also accumulates a dipeptide, N-acetylglutaminylglutamine amide (NAGGN), whereas only traces are present at low osmolarity (table 1; Smith and Smith 1989).

Glycine betaine is the most potent osmoprotectant in *S. meliloti*. When added to a growth medium of elevated osmolarity, this betaine strongly stimulates the growth rate. In parallel, its uptake is enhanced while its catabolism is reduced, and thus large accumulation occurs (table 1). *De novo* biosynthesis of glycine betaine has not been reported in *S. meliloti*. However, if choline is available, large amount of glycine betaine can be synthesized (Smith *et al* 1988). The molecular analysis of this pathway is presented in the following sections. Among the betaines, proline betaine (= stachydrine) also acts as a powerful osmoprotectant for *S. meliloti* (Gloux and Le Rudulier 1989).

Besides the betaines, a tetrahydropyrimidine, ectoine, plays a role in the osmotic regulation of *S. meliloti*. Surprisingly, ectoine is not accumulated in high amounts within the cells, its intracellular concentration not being higher than 12 mM even in cells grown in the presence of salt. Thus, ectoine is not directly involved in the osmotic adjustment. Instead, ectoine is catabolized and other compatible solutes such as glutamate, trehalose and NAGGN are accumulated (table 1). The mechanism by which ectoine enhances rhizobial growth at high osmolarity remains unclear (Talibart *et al* 1994).

Table 1. Main compatible solutes in salt-stressed cells of *S. meliloti*, strain 102F34.

Growth conditions	Osmolytes (nmol/mg protein)				
	Glutamate	NAGGN	Trehalose	Glycine betaine	Ectoine
+ 0.5 M NaCl	550	360	200	UD	UD
+ 0.5 NaCl+ 1 mM Glycine betaine	150	40	UD	1150	UD
+ 0.5 NaCl+ 1 mM ectoine	950	420	150	UD	40

In the absence of osmotic stress, none of the osmolytes was detected. UD, undetectable. Results are obtained from Smith *et al* (1994) and Talibart *et al* (1994).

### 3. Characterization of a Tn5 mutant impaired in choline oxidation and cloning of *S. meliloti* bet genes

We have previously isolated Tn5 mutants of *S. meliloti* impaired in choline utilization (Pocard *et al* 1997). One mutant, *LTS23-1020*, which did not grow on choline, but grew on betaine plates was characterized further. Transport assays showed that *LTS23-1020* was not affected in  $^{14}\text{C}$ -choline uptake. However, if *LTS23-1020* exhibited high levels of BADH activity (table 2), CDH activity was absent. As expected, this mutant was unable to metabolize  $^{14}\text{C}$ -choline at high osmolarity, whereas the parental strain, *102F34rif*, actively oxidized choline to glycine betaine which was accumulated in the cells (table 2). Furthermore, choline itself is not able to restore the growth of strain *LTS23-1020* at high osmolarity.

The Tn5 insertion in *LTS23-1020* was localized in the gene encoding CDH activity (*betA*). The CDH deficiency was complemented using a gene bank of *S. meliloti* 102F34 DNA (Ditta *et al* 1980). Exconjugants harboring plasmids complementing the *betA* mutation were selected, plasmid DNA was prepared, and subjected to BglIII digestion. All recombinant plasmids carried the same 11.4 kb DNA insert. One of these plasmids, *pCHO34*, was selected for further characterization. Exconjugant *LTS23-1020(pCHO34)* was able to grow on minimal medium plus 20 mM choline, and displayed salinity tolerance comparable to the wild-type when grown in high salt medium plus 1 mM choline. Furthermore, the exconjugant actively converted  $^{14}\text{C}$ -choline to  $^{14}\text{C}$ -glycine betaine as in the parental strain *102F34rif* (table 2). Subcloning from *pCHO34* was undertaken, and *pCHO361* (figure 1A) contains the smallest insert (a 3.5 kb *ApaI*-*BglIII* fragment) that complements the *betA* mutation (Pocard *et al* 1997).

### 4. Molecular characterization of the bet locus

We have determined the nucleotide sequence of both strands of the *BglIII*-fragment (8.6 kb) from plasmid

*pCHO341* (figure 1A). Analysis of this sequence revealed four non-overlapping open reading frames encoding proteins designated BetI, BetC, BetB, and BetA, and being transcribed in the same direction (figure 1B).

The *S. meliloti betA* gene encodes a 548-residue polypeptide which shows 50% identity to the enteric CDH (Pocard *et al* 1997). By contrast, these two dehydrogenases share only 21% identical residues with the choline oxidase of *A. globiformis* (Deshmum *et al* 1995), essentially in the C- and N-terminal regions. However, the two dehydrogenases as well as 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 (Lamark *et al* 1991).

The *S. meliloti betB* gene encodes a 487-amino acid protein (Pocard *et al* 1997) which shares 54 and 40% identical residues with the BetB protein of *E. coli* (Lamark *et al* 1991) and the plant BADHs, respectively (McCue and Hanson 1992; Ishitani *et al* 1995). All BADHs are similar in length (487 to 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] is highly conserved in the five BADHs.

The *betI* gene encodes a 203 amino acid protein which shows homology to regulatory proteins of the TetR/AcrR family (Pan and Spratt 1994). These regulators are characterized by a conserved N-terminal region which contains a helix-turn-helix motif involved in binding to their DNA target sequence (Wissmann *et al* 1991). BetI from *S. meliloti* shows 33.5% identity with the BetI repressor of *E. coli*, which has been shown to regulate the expression of the *betIBA* operon depending on the choline concentration (Eshoo 1988; Lamark *et al* 1991; Røkenes *et al* 1996). The role of BetI in the regulation of the *bet* genes expression in *S. meliloti* is under investigation.

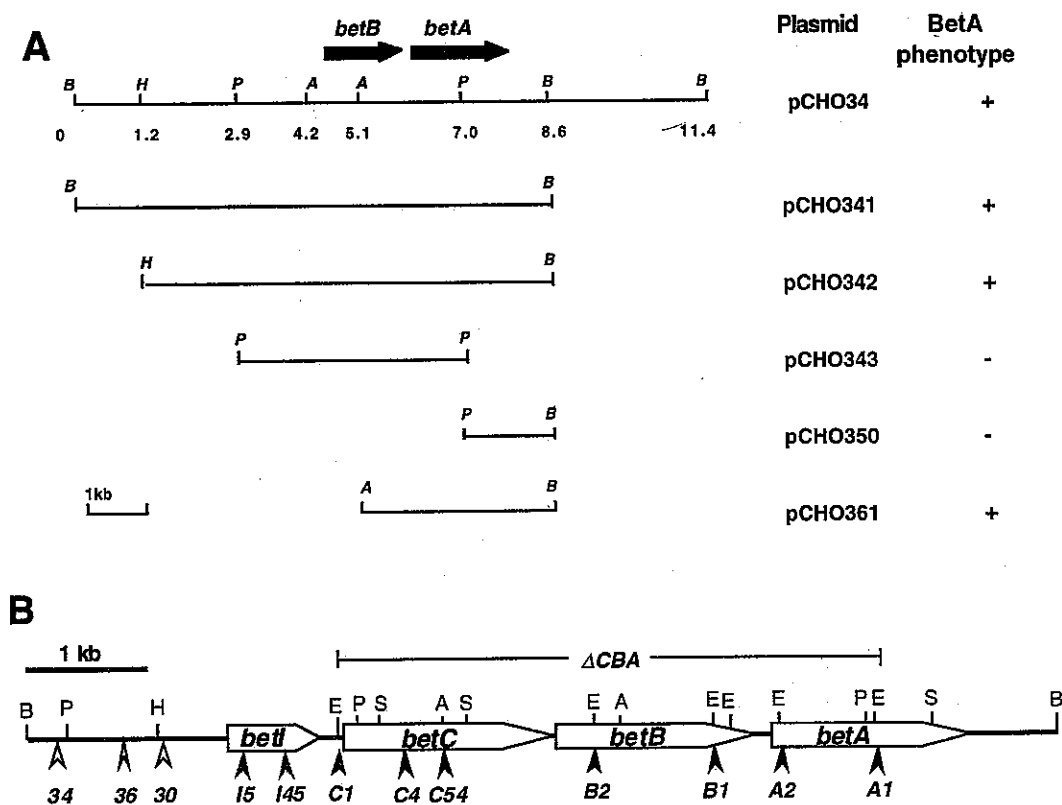
The *betC* gene, which is not present in the *E. coli*

Table 2. CDH and BADH activities, and fate of  $^{14}\text{C}$ -choline in *S. meliloti*.

Strain	Enzyme activities <sup>a</sup>		$^{14}\text{C}$ -labelled compounds <sup>b</sup>	
	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

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

<sup>b</sup>Uninduced cultures, grown to late exponential phase in minimal medium plus 0.3 M NaCl, were fed with 2  $\mu\text{M}$   $^{14}\text{C}$ -choline for 4 h. The cells were extracted in 80% ethanol and the  $^{14}\text{C}$ -soluble compounds were quantified (Bernard *et al* 1986). The radioactivity detected in choline or betaine is expressed as a percentage of the total radioactivity recovered.



**Figure 1.** Subcloning of the chromosomal insert of the plasmid pCHO34 (A), and physical and genetic map of the *bet* region of *S. meliloti* 102F34 (B). The BetA phenotype of LTS23-1020 exconjugants harboring subclones of pCHO34 indicates (i) utilization of choline as sole carbon source, (ii) osmoprotection by choline or glycine betaine aldehyde, (iii) production of  $^{14}\text{C}$ -glycine betaine from  $^{14}\text{C}$ -choline and (iv) presence of CDH or BADH activities. Restriction sites shown are *Apa*I (A), *Bgl*III (B), *Eco*RI (E), *Hind*III (H), *Pst*I (P), and *Sma*I (S). Various Tn5 and  $\Omega$  insertions which have been generated are shown by arrowheads under the map; the arrowhead filling indicates a minus (filled), leaky (gray) or wild-type (open) choline growth phenotype.

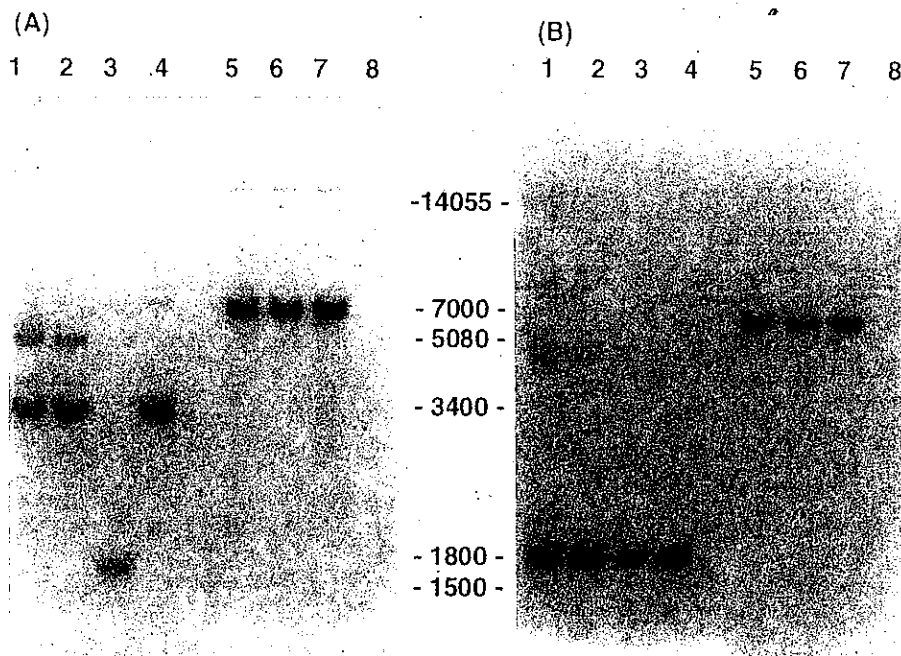
*bet* operon, encodes a 512 amino acid protein which reveals significant homology to a family of hydrolases, mostly sulphatases. The highest homology was found with a phosphonate monoester hydrolase from *Burkholderia caryophylli* (27% identity, GenBank accession number U44852), human iduronate sulphatase and arylsulphatase (26% identity, SwissProt accession numbers P22304 and P51689), a hypothetical sulphatase-like protein from *E. coli* (24% identity, SwissProt accession number P31447) and arylsulphatases from *Pseudomonas aeruginosa*, *E. coli* and *Klebsiella pneumoniae* (23% identity, SwissProt accession numbers P51691, P25549 and P20713). Indeed, the *betC* product encodes a sulphatase involved in the conversion of choline-O-sulphate into choline. This choline sulphatase is induced by its substrate and/or its product, and is absent in a mutant strain which carries a Tn5 insertion in *betC*.

Interestingly, the *bet* genes of *S. meliloti* are not closely linked to each other like in *E. coli*, but separated by large intergenic regions of 167 and 211 bp between

*betIC* and *betBA*, respectively. A highly conserved domain within these two regions has also been found downstream of the *S. meliloti* *ftsZ* gene (control of cell division; accession number L25440), and downstream of the *Rhizobium leguminosarum* bv *trifolii* *frk* gene (fructokinase; accession number U08434). No coding sequence was identified downstream of *betA*, and the upstream region (500 bp) of *betI* did not contain any ORF homologous to *betT* which encodes the high-affinity choline transporter in *E. coli*.

### 5. Symbiotic phenotype of *bet* mutants and genomic location of the *bet* locus

Besides the chromosome, *S. meliloti* contains two symbiotic megaplasmids called *pSymA* and *pSymB*. Genes essential for the catabolism of at least three betaines (i.e., carnitine, trigonelline, and stachydrine) map near the *nod* region of *pSymA* in *S. meliloti*, and stachydrine



**Figure 2.** Autoradiograph of Southern blots of *Xho*I-restricted DNA from strains of *S. meliloti*, *A. tumefaciens* and *E. coli* hybridized with [<sup>32</sup>P]-labelled *betA* (A) or *betB* (B) gene probes from *S. meliloti* 102F34. Each lane was loaded with 4 µg genomic DNA. Lanes: 1, *S. meliloti* 102F34; 2, *S. meliloti* 1021; 3, *S. meliloti* LTS23-1020; 4, *S. meliloti* RCR2011; 5, *A. tumefaciens* GMI9023; 6, *A. tumefaciens*, At128; 7, *A. tumefaciens* At125; 8, *E. coli* HB101. Molecular mass markers are indicated in base pairs.

catabolism mutants show delayed and reduced nodulation patterns (Goldman *et al* 1994). It was suggested that genes encoding the catabolism of glycine betaine may also map to the *nod* region of *pSymA* (Goldman *et al* 1994). Therefore, we sought to determine both the replicon location of the *bet* locus and the symbiotic phenotype of *S. meliloti* LTS23-1020.

Two *Eco*RI restriction fragments of 799 bp and 885 bp for the *betA* and *betB* genes, respectively, were used as DNA probes in hybridization experiments. Both probes strongly hybridized to DNA from *S. meliloti* 102F34, 1021, and 2011 (figure 2). The band hybridizing to the *betA* probe was shifted downward in strain LTS23-1020 (figure 2A, 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 rhizobial probes and DNA from *E. coli* HB101. Derivatives of *A. tumefaciens* GMI9023 (Rosenberg and Huguet 1984), containing either the *pSymA* (At128) or the *pSymB* (At125) megaplasmid from *S. meliloti* 1021 (Finan *et al* 1986), were used for determination of the replicon location of the *bet* genes. Both probes strongly hybridized to a *Xho*I fragment present in all three strains (figure 2A and 2B, lanes 5 to 7), representing probably the *bet* genes of *A. 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.

Nodulation tests conducted on alfalfa seedlings during 6 weeks with strains LTS23-1020 and 102F34rif showed that both strains were equally efficient in inducing nodulation. Although nitrogen fixation was slightly reduced by the *betA* mutation, strain LTS23-1020 is both Nod<sup>+</sup> and Fix<sup>+</sup>.

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