The *Sinorhizobium meliloti* Glycine Betaine Biosynthetic Genes (*betICBA*) Are Induced by Choline and Highly Expressed in Bacteroids

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The symbiotic soil bacterium *Sinorhizobium meliloti* has the capacity to synthesize the osmoprotectant glycine betaine from choline-*O*-sulfate and choline. This pathway is encoded by the *betICBA* locus, which comprises a regulatory gene, *betI*, and three structural genes, *betC* (choline sulfatase), *betB* (betaine aldehyde dehydrogenase), and *betA* (choline dehydrogenase). Here, we report that *betICBA* genes constitute a single operon, despite the existence of intergenic regions containing mosaic elements between *betI* and *betC*, and *betB* and *betA*. The regulation of the *bet* operon was investigated by using transcriptional *lacZ* (β -galactosidase) fusions and has revealed a strong induction by choline at concentrations as low as 25 μ M and to a lesser extent by choline-*O*-sulfate and acetylcholine but not by osmotic stress or oxygen. BetI is a repressor of the *bet* transcription in the absence of choline, and a nucleotide sequence of dyad symmetry upstream of *betI* was identified as a putative *betI* box. Measurements of intracellular pools of choline, well correlated with β -galactosidase activities, strongly suggested that BetI senses the endogenous choline pool that modulates the intensity of BetI repression. In contrast to *Escherichia coli*, BetI did not repress choline transport. During symbiosis with *Medicago sativa*, *S. meliloti bet* gene expression was observed within the infection threads, in young and in mature nodules. The existence of free choline in nodule cytosol, peribacteroid space, and bacteroids was demonstrated, and the data suggest that *bet* regulation in planta is mediated by BetI repression, as in free-living cells. Neither Nod nor Fix phenotypes were significantly impaired in a *betI:: Ω* mutant, indicating that glycine betaine biosynthesis from choline is not crucial for nodulation and nitrogen fixation.

Additional keyword: salt stress.

Among compatible solutes accumulated by bacteria to maintain a positive cellular turgor essential for metabolic functions, glycine betaine is one of the most efficient osmolytes (Le Rudulier et al. 1984). Glycine betaine can be either taken up from the environment or synthesized from choline. The molecular details of the choline to glycine betaine syn-

thesis pathway encoding by the *betTIBA* gene cluster have been fully characterized in *Escherichia coli* (Lamark et al. 1991). Import of the precursor choline is mediated by the high-affinity BetT transporter (Styrvoid et al. 1986). The *betA*- and *betB*-encoded proteins are responsible for the enzymatic conversion of choline and glycine betaine aldehyde to glycine betaine. BetA is an oxygen-dependent choline dehydrogenase (CDH) that catalyzes both steps in the oxidation of choline to glycine betaine by the way of glycine betaine aldehyde, whereas BetB is a NAD-dependent betaine aldehyde dehydrogenase (BADH) specific for the last step of the choline oxidation (Andresen et al. 1988; Landfald and Strøm 1986). BetI is a choline-sensing repressor (Landfald and Strøm 1986; Lamark et al. 1991) that can bind, in vitro, the operator sequence (Røkenes et al. 1996). The *betT* and *betIBA* genes are divergently oriented, and the overlapping *betT* and *betI* promoters are negatively regulated by BetI. Two other external stimuli, oxygen and osmotic stress, also regulate the *bet* genes. Oxygen exerts its control via the transacting DNA-binding protein ArcA, whereas the osmotic signal for *bet* induction remains unknown (Lamark et al. 1996).

Sinorhizobium meliloti, the alfalfa symbiotic species, can also synthesize glycine betaine from choline for protection against osmotic stress (Bernard et al. 1986). The *bet* locus that governs this pathway has been characterized at the molecular level, and four genes (*betI*, *betC*, *betB*, and *betA*) have been identified (Østerås et al. 1998; Pocard et al. 1997). The *betI* gene encodes a presumed regulatory protein highly homologous to *E. coli* BetI, which belongs to the TetR/AcrR family with a conserved N-terminal region that contains a helix-turn-helix motif involved in binding to their DNA target sequence. The role of BetI in the regulation of the *S. meliloti bet* genes is yet unclear. The genes encoding the betaine aldehyde dehydrogenase (*betB*) and the choline dehydrogenase (*betA*) were fully analyzed and share many characteristics with their homologs found in *E. coli*. In addition, a new gene (*betC*) has been characterized as encoding a choline sulfatase, which catalyzes the conversion of choline-*O*-sulfate and, at a lower rate, phosphorylcholine into choline. Enterobacteria, which are lacking choline sulfatase, use choline-*O*-sulfate directly as a compatible solute in response to osmotic stress, while in *S. meliloti*, choline-*O*-sulfate is not used as osmoprotectant per se but is first transformed into choline and then glycine betaine to fulfill this role (Østerås et al. 1998). Furthermore, *S. meliloti* is able to catabolize glycine betaine and use it as sole carbon and nitrogen sources for

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growth, a capacity lacking in *E. coli* (Perroud and Le Rudulier 1985). The precursors, choline and choline-*O*-sulfate, can also be used for growth by *S. meliloti*, and that depends on a functional *bet* locus. Moreover, due to the unique presence of the phosphatidylcholine synthase in *S. meliloti* (de Rudder et al. 1999), choline is a direct precursor of phosphatidylcholine, a major lipid membrane component in the *Rhizobiaceae* family, whereas the presence of phosphatidylcholine is rather unusual in other bacterial membranes. These specific physiological aspects may indicate a particular regulation of the choline-glycine betaine pathway in this bacterium as a free-living organism and possibly as symbiotic bacteroids.

The molecular organization of the *bet* locus in *S. meliloti* differs from *E. coli* by two other aspects: i) the absence of *betT* homologues or genes that could potentially encode choline uptake in the region upstream of the sinorhizobial *betI* gene, and ii) the presence of two significantly large noncoding intergenic regions located between *betI*-*betC* (167 bp), and *betB*-*betA* (211 bp) that show characteristics of DNA mosaic elements (Østerås et al. 1998). Thus, although the phenotype of different *bet* mutants suggested that the *betICBA* genes formed a single transcriptional unit, this was not experimentally demonstrated.

The present study was initiated to gain an understanding of the regulation of *bet* genes in *S. meliloti* as free-living cells and during the establishment and the maintenance of nitrogen-fixing nodules. We demonstrate that the *bet* genes constitute a single operon transcriptionally induced in free-living cells by choline, while salt stress or reduced oxygen levels had no significant effect on their expression. We also show that BetI mediates the repression of this operon in the absence of choline but is not involved in the regulation of choline transport. Furthermore, our results reveal that significant levels of free choline are present in the nodule cytosol, the peribacteroid space, and the bacteroids in which the *bet* genes are strongly expressed.

RESULTS

The *bet* gene expression is induced by choline.

To study the transcriptional regulation of the *S. meliloti bet* genes, chromosomal *betB-lacZ* and *betA-lacZ* fusions were constructed as described below (Fig. 1), resulting in strains UNA228 and UNA216, respectively (Table 1). Cells were grown overnight in minimal LAS medium (Bernard et al. 1986), and the β -galactosidase activities of both strains were measured after the addition of 7 mM choline in aerobic cultures (21% oxygen). A timecourse analysis showed an increase in β -galactosidase activity starting 3 h after choline addition with maximal expression of the *bet-lacZ* fusions occurring after 5 h (data not shown). Consequently, all subsequent assays were performed 5 h after choline induction (Fig. 2). In the absence of choline, the basal levels of expression of both fusions was less than 300 Miller units, with no inducing effect when salt was added. On the contrary, the addition of choline led to a five- and nine-fold induction of β -galactosidase-specific activity in the UNA228 (*betB-lacZ*) and the UNA216 (*betA-lacZ*) strains, respectively (Fig. 2A and B). Again, the addition of salt did not significantly modify the expressions measured.

Since a very low oxygen tension strongly reduced the expression of *bet* genes in *E. coli* (Eshoo 1988) and considering the fact that *S. meliloti* is subjected to such a condition in planta, we measured the β -galactosidase activity in cells maintained under microaerobiosis (1% oxygen). Compared with the control (21% oxygen), a low oxygen tension did not significantly affect *betB* expression (Fig. 2A) or *betA* expression (data not shown). The ability of *S. meliloti* to metabolize choline (Bernard et al. 1986; Smith et al. 1988) initiated studies on how other carbon sources might affect the induction of the *bet* genes by choline. Cells that carried *betA-lacZ* and *betB-lacZ* fusions on a low copy number plasmid (pCHO341) were

Fig. 1. The *Sinorhizobium meliloti bet* locus. **A**, Physical and genetic organization of the *betICBA* operon (pCHO341). The genes deduced from the nucleotide sequence analysis are represented by boxed arrows. The positions of the *lacZ* fusions are indicated below. Abbreviations for restriction enzymes: Bg = *Bgl*II, E = *Eco*RII, H = *Hind*III, P = *Pst*I, S = *Sal*I, and Sp = *Sph*I. **B**, Physical map of pRK500 and position of the *lacZ* fusion in *betI*. **C**, Alignment of the nucleotide sequences upstream of *betI* gene from *S. meliloti* (Sm) and *Escherichia coli* (Ec). The asterisks indicate conserved nucleotides within the putative *betI* box, and the arrow shows the position of the Ω insertion. Both ATG codons are underlined.

grown in minimal medium with succinate or glucose as the carbon source and with ammonium as the nitrogen source. In the absence of choline, the basal levels of β -galactosidase activity were very similar to that observed in cells grown in LAS medium, and upon addition of choline, a strong induction was observed whether succinate, glucose, or ammonium was present or not (data not shown). Taken together, these results demonstrate choline transcriptional induction of *S. meliloti betBA* genes, while neither salt stress nor low oxygen level have any regulatory effect. Furthermore, *betBA* expression is not subjected to catabolite repression by glucose or by succinate, which plays a major role in the regulation of the general carbon utilization in *S. meliloti* (Arias et al. 1982; Ucker and Signer 1978).

BetI regulates the expression of the *bet* genes.

A *S. meliloti betI::Tn5* mutant displays a leaky phenotype with low BADH and CDH activities (Østerås et al. 1998). Such residual activities are most likely the result of transcription initiated within the transposon Tn5, which reduces the polar effect of the *betI::Tn5* insertion on downstream genes. However, the large intergenic region (167 bp) found between *betI* and *betC* could also contain a weak promoter that would be responsible of the low *betBA* expression. Thus, a double recombinant strain (named UNA294) carrying an Ω -interposon in the *betI* gene, which has a strong polar effect on genes located downstream of the insertion site (Prentki and Krisch 1984), was constructed (discussed below). This mutant strain was totally unable to grow on choline as nitrogen and carbon sources or to use choline as osmoprotectant. It also completely lacked BADH activity and, consequently, was unable to convert choline into glycine betaine (data not shown). A plasmid-located wild-type copy of the *betI* gene (pRK500) could not complement the mutant phenotype of strain UNA294. The wild-type phenotype could only be restored by a plasmid con-

taining the entire *betICBA* locus (pCHO341). Thus, the Bet⁻ phenotype of strain UNA294 is caused by the polar effect of the interposon on downstream genes, as the addition in trans of a functional BetI did not restore BADH (*betB*) and CDH (*betA*) activities. These results demonstrate that the *betICBA* genes form a single operon.

In *E. coli*, Lamark and associates (1996) found that BetI is a repressor that regulates *bet* gene expression in response to the inducer choline. In order to investigate the regulatory function of BetI in *S. meliloti*, a transcriptional *betI-lacZ* fusion was constructed and recombined into the genome of the parental strain 102F34 as well as into the *betI* mutant (strain UNA294) by single recombination. The β -galactosidase activities of the resulting strains UNA338 (*betI-lacZ* in wild-type background) and UNA340 (*betI-lacZ* in UNA294 background) were measured in cells grown in LAS medium after addition of choline, salt, or a combination of both (Fig. 3). As observed for the *betA* and *betB lacZ*-fusion strains, the basal level of β -galactosidase activity in strain UNA338 was not affected by the presence of salt, whereas a three-fold increase in *betI* transcription was observed when 7 mM choline were added. The β -galactosidase activity was slightly higher in cells grown in the presence of both choline and salt compared with choline alone (Fig. 3A). The transcription of *betI* is regulated in a similar way as *betB* and *betA*, as expected for genes in the same operon, but the uninduced basal β -galactosidase activity of the *betI-lacZ* fusion was five- to eight-fold higher than the *betA-* and *betB-lacZ* fusions. This high level is possibly caused by a disequilibrium between the amount of functional BetI and the number of promoter copies. In a BetI⁻ background (strain UNA340), the *betI-lacZ* fusion resulted in a very high level of β -galactosidase activity in all tested conditions, indicating a strong constitutive expression of *betI* (Fig. 3B). The introduction of pRK500 into strain UNA340 restored normal regulation of *betI* expression by choline. These results suggest that BetI mediates the repres-

Table 1. Bacterial stains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Source or reference
Strains		
<i>Escherichia coli</i>		
DH5 α	F <i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>HsdR17</i> (rK ⁻ mK ⁺) <i>recA1</i>	Sambrook et al. 1989
MT616	<i>pro-82 thi-1 hsdR17 supE44 recA56</i>	Finan et al. 1986
S17.1	<i>pro 82 thi-1 hsdR17</i> , RP4 integrated into chromosome, <i>tet::Mu</i> ; <i>kan::Tn7</i> ; <i>TnI</i>	Simon et al. 1983
<i>Sinorhizobium meliloti</i>		
102F34rif	Wild-type derivative, Rif ^r	Pocard et al. 1997
RmUNA294	102F34 derivative, <i>betI::</i> Ω , Sm ^r , Rif ^r	This work
RmUNA216	102F34 derivative, <i>betA-lacZ</i> , Gm ^r , Rif ^r	This work
RmUNA228	102F34 derivative, <i>betB-lacZ</i> , Gm ^r , Rif ^r	This work
RmUNA338	102F34 derivative, <i>betI-lacZ</i> , Nm ^r , Rif ^r	This work
RmUNA340	RmUNA294 derivative, <i>betI::</i> Ω , <i>betI-lacZ</i> , Rif ^r , Sm ^r , Nm ^r	This work
Plasmids		
pUC118	Sequencing vector, ColE1 oriV, Ap ^r	Vieira and Messing 1987
pGEM-T	PCR fragment cloning vector, Ap ^r	Promega
pRK600	ColE1 replicon with RK2 transfer region, Cm ^r	Finan et al. 1986
pSUP202	Replicon ColE1, mob ⁺ , Tc ^r , Ap ^r , Cm ^r	Simon et al. 1983
pKOK5	pSUP202 derivative, source of <i>lacZ-Km^r</i> cartridge, Ap ^r , Km ^r	Kokotek and Lotz 1989
pAB2001	pUC6S derivative, source of <i>lacZ-Gm^r</i> cartridge, Ap ^r , Gm ^r	Becker et al. 1995
pHP45 Ω	Source of Ω -Spc ^r cartridge, Ap ^r , Spc ^r	Prentki and Krisch 1984
pUCSH	1.6- <i>HindIII/SphI</i> fragment containing <i>betI</i> in pUC118, Ap ^r	Osteras et al. 1998
pCHO341	8.6-kb <i>BglIII</i> fragment with the <i>betICBA</i> genes of <i>S. meliloti</i> into pRK415, Tc ^r	Pocard et al. 1997
pCHO34AZ	pCHO341 derivative with <i>betA-lacZ</i> fusion from pAB2001, Tc ^r , Gm ^r	This work
pCHO34BZ	pCHO341 derivative with <i>betB-lacZ</i> fusion from pAB2001, Tc ^r , Gm ^r	This work
pRK500	1.6kb <i>HindIII/SphI</i> fragment from pUCSH into pRK415, Tc ^r	Osteras et al. 1998
pSP402	pSUP202 derivative with <i>betI</i> containing a <i>BamHI</i> restriction site, Ap ^r , Cm ^r	This work
pSP402 Ω	1.6kb <i>HindIII/SphI</i> , with <i>betI::</i> Ω insertion into pSUP202, Spc ^r Ap ^r , Cm ^r	This work
PGEM-T1	PGEM-T derivative with <i>SalI/BamHI</i> 5' region of <i>betI</i> , Ap ^r	This work
pSP211	0.7 kb <i>SalI/BamHI</i> containing <i>betI</i> 5' region into pSUP202, Ap ^r , Cm ^r	This work
pSP211Z	pSP211 derivative with <i>lacZ-Km</i> from pKOK5, Ap ^r , Cm ^r , Km ^r	This work

^a Ap = ampicillin, Cm = chloramphenicol, Gm = gentamicin, Km = kanamycin, Nm = neomycin, Rif = rifampicin, Sm = streptomycin, Spc = spectinomycin, and Tc = tetracycline.

sion of the *bet* gene transcription in the absence of choline. Since a binding site for BetI, named the *betI* box, has been located in the promoter region of the *betBA* and *betT* genes of *E. coli* by DNA binding experiments (Røkenes et al. 1996), homologous sequence was searched out upstream of the *S. meliloti betI* gene. Indeed, a sequence of dyad symmetry, ATTGATTGA*TTGGATCAAT (the asterisk shows the center of symmetry), identified 32 bp upstream of the putative ATG start codon of *betI* showed high homology to the *betI* box characterized in *E. coli* (Fig. 1C).

Substrate specificity of BetI repressor.

It has been shown previously that choline-*O*-sulfate and, to a lesser extent, phosphorylcholine can be converted into choline by the choline sulfatase encoded by the *S. meliloti betC* gene (Østerås et al. 1998). Thus, the specificity of BetI-mediated *bet*

genes repression was assayed by monitoring the β -galactosidase activities in strains UNA338 (*betI-lacZ*) and UNA216 (*betA-lacZ*) grown at low osmolarity in the presence of various choline-derivative compounds added at a final concentration of 1 mM (Table 2). In strain UNA338, choline-*O*-sulfate, acetylcholine, and to a lesser degree, phosphorylcholine were inducing the *bet* gene transcription as well as choline, while glycine betaine was totally ineffective. Similar results were obtained with strain UNA216. It should be noted that, in both strains, a strong induction was already obtained at a choline concentration as low as 25 μ M, and maximal β -galactosidase activity required less than 100 μ M choline (data not shown). From these results, it seems that BetI displays a narrow specificity towards choline and acetylcholine, although the latter compound is not metabolized via the pathway encoded by the *bet* operon. Choline-*O*-sulfate and phosphorylcholine might act

Fig. 2. β -Galactosidase activities in various *Sinorhizobium meliloti* 102F34 strains carrying *bet-lacZ* fusions integrated into the chromosome. **A**, *betB-lacZ* fusion, strain UNA228. **B**, *betA-lacZ* fusion, strain UNA216. Cells were grown overnight in LAS minimal medium (Bernard et al. 1986) and either choline (7 mM), NaCl (0.3 M), or both were added 5 h before β -galactosidase activities were measured. They were maintained at 21% oxygen or under microaerobiosis (1% oxygen) during the same period of time. Values are means of duplicates from three independent cultures, and standard deviations are shown.

Fig. 3. β -Galactosidase activities in *Sinorhizobium meliloti* 102F34 carrying *betI-lacZ* fusion integrated into the chromosome. **A**, *betI-lacZ* fusion in wild-type background, strain UNA338. **B**, *betI-lacZ* fusion in *betI::* Ω mutant background, strain UNA340. Cells were grown overnight in LAS minimal medium and either choline (7 mM), NaCl (0.3 M), or both were added 5 h before β -galactosidase activities were measured. Cells were maintained at 21% oxygen.

directly on BetI with less efficiency or indirectly through hydrolysis into choline via the BetC protein.

Evaluation of the importance of the endogenous choline pool in BetI regulation.

The data presented above show significant variations in the increase of expression measured after choline induction in strains UNA228 (five-fold increase), UNA216 (nine-fold), UNA338 (three-fold), and UNA340 complemented with pRK500 (7.5-fold). Such disparity could simply be caused by different *lacZ* fusion constructions (*betA*, *betB*, and *betI*) having different expression levels. It is also possible that the level of induction depends on the intracellular choline concentration in strains with various genetic backgrounds. To answer this hypothesis, the strains were grown in LAS medium and were maintained 5 h in the presence of 1 mM choline before the intracellular free choline concentration was determined as described below. The two strains able to convert choline into glycine betaine (UNA338 [*betI-lacZ*] and UNA228 [*betB-lacZ*]), which allows transcription of *betA* from the gentamicin cartridge promoter, contained choline at an intracellular concentration of 10 nmol per mg of protein (Fig. 4). In contrast, strains UNA340, UNA340 (pRK500), and UNA216, which are unable to oxidize choline into glycine betaine, showed an accumulation of endogenous choline at concentrations six- to seven-fold higher than in the previous strains. A direct correlation can thus be observed between a high intracellular choline level and high β -galactosidase activity. This strongly suggests that BetI senses the intracellular pool of choline and modulates its level of regulation accordingly.

The status of the endogenous choline pool depends on fluctuations in the rates of choline transport and utilization. Consequently, besides its role in the regulation of the *bet* gene expression, a hypothetical role of BetI in the regulation of choline uptake had to be addressed. Therefore, choline uptake activity at 10 μ M of substrate was compared in the wild-type strain 102F34, the *betI:: Ω* mutant (UNA294), and the same strain complemented with a wild-type copy of *betI* (pRK500) or *betICBA* (pCHO341). In the wild-type strain, choline uptake was stimulated three-fold when the cells were grown with 1 mM choline. While addition of salt had no effect on choline transport in the absence of choline, it increased by 50% the uptake activity in choline-induced cells to a level 4.5-fold higher than in the control cells (Table 3). These results are consistent with a previous report on choline uptake in *S. meliloti* (Pocard et al. 1989). By contrast, choline uptake in strains UNA294 and UNA294(pRK500) was not stimulated by choline at low or high osmolarity. Only when the whole *bet* operon carried on

pCHO341 was transferred into the *betI* mutant was wild-type regulation of choline transport restored. These results suggest that BetI is not involved per se in the regulation of choline uptake and that its induction by extracellular choline occurs via another mechanism. The absence of transport stimulation by choline in the mutant strain UNA294 is probably due to the accumulation of intracellular choline when oxidation into glycine betaine can no longer occur, resulting in feedback inhibition that is BetI independent. The intracellular choline pool is thus finely regulated by two different mechanisms for transport and metabolism.

Regulation of the *bet* genes expression during symbiosis.

S. meliloti is able to develop a symbiotic relationship with the host plant alfalfa within specific root nodules. The expression of the *bet* genes was monitored in situ during the nodulation and nitrogen fixation process. Seven days after infection, the indigo coloration resulting from the expression of the *betB-lacZ* (UNA228), *betA-lacZ* (UNA216), *betI-lacZ* (UNA338), *betI:: Ω /betI-lacZ* (UNA340) fusions was observed inside root hair infection threads (Fig. 5A and B) and the infection zone of young developing nodules (Fig. 5C and D). No staining was observed in infection threads or in young nodules induced by the wild-type 102F34 strain. The expression of the *bet-lacZ* fusions was also followed in mature two-week-old nitrogen fixing nodules and referred to the specific histological zones of alfalfa nodules (Vasse et al. 1990). The apical meristematic zone formed of uninfected cells never showed any β -galactosidase activity (Fig. 5E, F, and G). In contrast, significant staining was detected in pre-fixing zone II, in which bacteria are released from growing infection threads. Within the nitrogen-fixing zone III containing fully differentiated bacteroids, β -galactosidase activity was always strongly expressed (Fig. 5E and F). However, in nodules induced by strain UNA228 (*betB-lacZ*), the intensity of the indigo coloration was always lower than in other strains. Quantitative measurements of β -galactosidase activities within bacteroids isolated from five-week-old nodules confirmed that the expression of the *betI-lacZ* fusion was about five times higher in the *betI* mutant strain UNA340 than in the wild-type strain. These results suggest that *bet*

Table 2. β -Galactosidase activity of *Sinorhizobium meliloti* 102F34^a

Culture conditions ^b	β -Galactosidase activity (Miller units/mg protein)	
	Strain UNA338	Strain UNA216
Control	1,844 $\pm$ 300	474 $\pm$ 120
+Choline	7,077 $\pm$ 970	2,719 $\pm$ 470
+Choline-O-sulfate	4,704 $\pm$ 970	2,807 $\pm$ 580
+Acetylcholine	5,943 $\pm$ 1090	2,700 $\pm$ 510
+Phosphorylcholine	3,563 $\pm$ 960	2,455 $\pm$ 530
+Phosphatidylcholine	1,732 $\pm$ 190	500 $\pm$ 50
+Glycine betaine	1,485 $\pm$ 290	430 $\pm$ 110

^a Carrying either a *betI-lacZ* fusion (strain UNA338) or a *betA-lacZ* fusion (strain UNA216) integrated into the chromosome and grown under various physiological conditions

^b Cells were grown in low-osmolarity LAS minimal medium (control). Choline and derivatives were added at a final concentration of 1 mM, and β -galactosidase activity was measured 5 h later. Values are means of duplicates from three independent cultures $\pm$ standard deviation.

Fig. 4. Intracellular pool of free choline in various *Sinorhizobium meliloti* strains. Cells were grown in LAS minimal medium (control) and maintained 5 h in the presence of 1 mM choline. Lanes 1 and 2, *betI-lacZ* fusion in wild-type background, strain UNA338; lane 3, *betI-lacZ* fusion in *betI:: Ω* mutant background, strain UNA340; lane 4, strain UNA340(pRK500); lane 5, *betB-lacZ* fusion, strain 228; lane 6, *betA-lacZ* fusion, strain 216. Values are means from three independent cultures, and standard deviations are shown.

gene expression in planta is regulated by BetI in a similar way as in free-living cells. Furthermore, the β -galactosidase activity obtained with the *betA*- and *betB-lacZ* fusions well corroborates the BADH and CDH activities previously determined in bacteroids isolated from alfalfa nodules (Fougère and Le Rudulier 1990a).

Evidence for endogenous free choline in alfalfa nodules.

The natural occurrence of free choline is widespread in higher plants, including legumes. By example, alfalfa roots can exude choline, which is then available for *S. meliloti* in the rhizosphere (de Rudder et al. 1999), but the distribution of choline within nodules, if any, has not yet been investigated. Since choline is the main inducer for the expression of the *bet* genes, the expression observed in planta during all stages of the establishment of symbiosis would suggest that choline is available to the microsymbiont. This hypothesis was addressed by investigating whether this compound is present within the cytosol, the peribacteroid space, and the bacteroids. In three- and five-week-old nodules, choline could be detected and quantified in the three compartments (Table 4). The highest concentration was measured in the cytosol and remained almost constant over time. The significant levels found in the peribacteroid space strongly suggest that choline is transported through the peribacteroid membrane, and the amount found in bacteroids is consistent with the existence of choline uptake activity already described in alfalfa bacteroids (Fougère and Le Rudulier 1990b). On the basis of intracellular aqueous volumes of bacteroids measured previously (Fougère and Le Rudulier 1990b; discussed below), the free choline concentration was estimated at 0.8 to 1 mM, which is largely sufficient to induce the *bet* operon and is in full agreement with the results observed in planta.

Symbiotic phenotype of a *betI* mutant on alfalfa plants.

The symbiotic capacity of the *betI* mutant (strain UNA294) was tested on alfalfa. Seedlings were inoculated with the mutant and the wild-type strain as a control, and the weight of nodules was determined after 4 and 6 weeks. Both strains were similarly efficient in inducing nodulation. The acetylene reduction activity was assayed 4 weeks after inoculation and 2 weeks later with plants subjected or not to salt stress (0.2 M NaCl) during this period. With 2-week-salt-stressed plants from two separate experiments and measurements on six replicates of five plants each, acetylene reduction activities observed for *betI*-nodulated plants (18 ± 3 nmol of ethylene produced per mg of nodules [fresh weight] per h) was always slightly lower (approximately 20%) from that measured in wild type-nodulated plants (23 ± 4). However, because of non-negligible standard deviations, we cannot conclude that nitrogen fixation in plants inoculated with the *betI* mutant was significantly altered in plants subjected to salt stress. Thus, glycine betaine biosynthesis from choline is not crucial for an efficient nitrogen fixation, even in plants submitted to 0.2 M NaCl, as

observed previously (Pocard et al. 1997), and the absence of the BetI repressor does not alter the regulation of another pathway of symbiotic importance. Other osmoprotective molecules, such as proline betaine produced by the plant host, might be used by the bacteroids to deal with either such levels of stress, higher salt concentrations, or both might be needed for significant protection by the choline-glycine betaine pathway. This hypothesis is currently being tested.

DISCUSSION

We have previously identified a *S. meliloti betICBA* locus involved in glycine betaine biosynthesis from choline-*O*-sulfate or choline (Østerås et al. 1998). In this study, we have demonstrated that this locus constitutes a single operon, despite the existence of intergenic regions containing mosaic elements between *betIC* and *betBA*. We also report the regulation of this operon in free-living cells and have addressed the question of its expression during symbiosis.

Ex planta expression studies of various *bet-lacZ* transcriptional fusions have shown that the *S. meliloti bet* operon is regulated at the transcriptional level, with choline, acetylcholine, choline-*O*-sulfate, and, to a lesser extent, phosphorylcholine acting as inducers but not glycine betaine. In contrast to *E. coli* (Lamark et al. 1996), neither oxygen nor salt stress is involved in the regulation of the choline-to-betaine pathway that produces the major osmoprotectant glycine betaine. We believe that the differences between the two bacteria are due to the fact that, in *E. coli*, glycine betaine functions only as an osmoprotectant and cannot be further catabolized (Perroud and Le Rudulier 1985) whereas *S. meliloti* uses choline and glycine betaine either as nutritional sources or as osmoprotectants (Bernard et al. 1986; Smith et al. 1988). In *E. coli*, the *betIBA* gene regulation by choline is mediated by the choline-sensing repressor BetI (Lamark et al. 1996). It has been demonstrated that a purified BetI*, which is a genetically modified BetI protein with seven extra N-terminal amino acids, can specifically bind to the promoter region at its axis of symmetry (Røkenes et al. 1996). In vitro studies have also shown that the inducer choline caused a somewhat enhanced binding of BetI* to the promoter, and it was proposed that BetI is an unusual negative regulatory protein because it is bound to its operator site even during induction. Our results show that, in *S. meliloti*, BetI mediates the repression of the *bet* gene transcription in the absence of choline. The similar regulatory behavior of the BetI proteins of *E. coli* and *S. meliloti* and the identification of a putative *betI* box in the promoter region of the *S. meliloti betICBA* locus suggest a similar repression mechanism by BetI in the two species. The analysis of the annotated sequence of the *S. meliloti* genome (Galibert et al. 2001) has shown the presence of a gene named Sma1726 on the megaplasmid pSymA, encoding a putative regulator that shows 34% homology to BetI. However, this protein is not involved in the regulation of the choline-to-betaine pathway, as the *betI-lacZ* fusion is expressed constitu-

Table 3. Choline uptake activity in *S. meliloti* 102F34 wild-type and *betI* : Ω mutant (strain UNA294) cells

Strains	Choline uptake (nmol/5 min/mg protein) ^a			
	MCAA	MCAA + cho	MCAA + NaCl	MCAA + cho + NaCl
102F34	6.5 $\pm$ 0.9	19.2 $\pm$ 1.1	4.7 $\pm$ 0.1	29.1 $\pm$ 4.3
<i>betI</i> (UNA294)	5.1 $\pm$ 0.1	5.1 $\pm$ 0.1	3.1 $\pm$ 0.2	3.2 $\pm$ 0.8
<i>betI</i> (pRK500)	5.4 $\pm$ 0.2	5.1 $\pm$ 0.1	3.4 $\pm$ 0.2	3.6 $\pm$ 0.2
<i>betI</i> (pCHO341)	nd ^b	nd	nd	29.3 $\pm$ 0.5

^a Cells were grown in MCAA medium, were harvested at an OD₆₀₀ of 0.8, and were assayed for uptake of [methyl-¹⁴C] choline at a final concentration of 10 μ M. Choline and NaCl were added at 1 mM and 0.3 M, respectively. Values are the mean from duplicates of two independent cultures $\pm$ standard deviation.

^b nd = not determined.

tively in a *betI* mutant background and only the addition of the wild-type copy of *betI* can restore full repression of the *bet* operon in the absence of the inducer (Fig. 3B).

In *E. coli*, the *betT* gene (choline transport) is transcribed divergently from the *betIBA* operon, but both transcripts are regulated simultaneously by BetI in response to the inducer choline (Lamark et al. 1996). In *S. meliloti*, the location of one or more genes encoding one or several choline transporters is currently not known, but it is obvious from uptake measurements that choline uptake in this species is not regulated by BetI (Table 3). Previous work has demonstrated the existence of three kinetically distinct choline uptake activities, in particular, one high-affinity activity that is inducible by choline itself. Interestingly enough, glycine betaine is an equally potent in-

ducer of this activity (Pocard et al. 1989), which is not the case in *E. coli*. It is thus understandable that the transport of choline in *S. meliloti* is regulated by another pathway than BetI, which simply monitors the intracellular concentration of the substrate.

Besides its dual role in osmoprotection, as precursor of glycine betaine, and in nutrition, as carbon and nitrogen sources, choline plays a major role in the biosynthesis of phosphatidylcholine (PC), an essential membrane component in a limited number of bacterial species, including rhizobia (Goldfine 1982). In *S. meliloti*, PC, which is required for normal growth (de Rudder et al. 2000), can be produced either by the methylation pathway, in which phosphatidylethanolamine is N-methylated three times with S-adenosylmethionine as a methyl donor in order to yield PC, or by the novel PC

Fig. 5. Histochemical localization of *betIBA* gene expression during *Sinorhizobium meliloti*-*Medicago sativa* nodule development. β -Galactosidase activity was detected using X-gal as substrate. **A** and **B**, *betA-lacZ* (strain UNA216) and *betI-lacZ* (strain UNA338) fusions, respectively, in root hairs 7 days after infection. **C** and **D**, *betI-lacZ* and *betA-lacZ* fusions in young 7-day-old nodules. **E** and **F**, *betB-lacZ* (strain UNA228) and *betI-lacZ* fusions, respectively, in mature 2-week-old nodules. **G**, β -Galactosidase staining in a 2-week-old nodule obtained with the control parental strain 102F34. Specific histological zones of mature nodules (I, II, and III) are indicated on cross sections. Scale bars: **A** and **B**, 25 μ m; **C** and **D**, 100 μ m; and **E**, **F** and **G**, 400 μ m.

synthase pathway, in which PC is formed in one step from choline and CDP-diacylglycerol (de Rudder et al. 1999; Sohlenkamp et al. 2000). A *S. meliloti* mutant deficient in the methylation pathway and grown in minimal medium can form PC only when choline is added. Although the regulation of PC synthase expression is yet unknown, choline is a potential inducer, and it will be of interest to determine whether BetI is involved.

Since *S. meliloti* requires choline for several essential pathways, it depends on high-affinity choline uptake systems to take up the choline present in its natural habitat at a rate sufficient for growth, especially in environments of high osmotic strength due to elevated concentrations of salts or drought, when a large amount of osmoprotectants is required. The presence of free choline and choline derivatives in plants and plant saps is well established (Rhodes and Hanson 1993; Wyn Jones and Storey 1981) and, in the case of alfalfa, the host plant of *S. meliloti*, choline exudation into the rhizosphere has been demonstrated. It has been shown that addition of alfalfa root exudates in minimal medium without choline enables *S. meliloti* mutants defective in phospholipid N-methyltransferase to produce wild-type amounts of PC (de Rudder et al. 1999). The *bet-lacZ* fusion data presented in this report revealed that the *bet* operon is expressed in planta at all stages of symbiosis. The induction of the *bet* genes probably occurs at a very early stage in the rhizosphere and continues throughout the successive symbiotic steps from the infection thread formation to the differentiated bacteroids (Fig. 5). The substrate-driven regulation observed in free-living cells suggests that free choline is present in root hairs and nodules. Direct quantification of choline in the different nodule compartments confirm the presence of a significant amount of choline in the cytosol, the peribacteroid space, and the bacteroids (Table 4), indicating that choline is transported into symbiosomes and bacteroids. This fact is supported by preliminary experiments conducted with isolated alfalfa symbiosomes showing choline uptake through the peribacteroid membrane (J. C. Trinchant, unpublished data) and previous uptake studies with isolated alfalfa bacteroids indicating the existence of high- and low-affinity choline transport activities in bacteroids (Fougère and Le Rudulier 1990b). Choline, via glycine betaine catabolism, could be used as an energy source by *S. meliloti*, not only in the colonization of the rhizosphere but also during root infection and bacteroid differentiation, as we have previously shown that isolated bacteroids actively catabolize glycine betaine (Fougère and Le Rudulier 1990a). However, since a *betI* mutant is not significantly impaired in nitrogen-fixing capacity, this energy source is not essential to support the nitrogen-fixation process in alfalfa nodules, at least under our experimental conditions. Finally, in view of the multiple roles of choline in *S. meliloti*, an in-depth study of the regulation of both its uptake and its conversion, either as osmoprotectant or as phosphatidylcholine, should contribute to a better understanding of the complexity of choline utilization in this agronomically important bacterium that can benefit, in the rhizosphere and during all steps of the symbiosis, from the choline produced by the plant host.

Table 4. Concentration of free choline (nmol/g fresh weight of nodules)^a

	Cytosol	Peribacteroid space	Bacteroids
3-week-old nodules	146 ± 6	10 ± 1	25 ± 5
5-week-old nodules	124 ± 7	9 ± 2	23 ± 6

^a In cytosol, peribacteroid space, and bacteroids from alfalfa nodules. Values are means of three measurements ± standard deviation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions.

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were routinely grown at 37°C in Luria-Bertani (LB) medium (Sambrook et al. 1989), and *S. meliloti* strains were grown in LB supplemented with 2.5 mM MgSO₄ and 2.5 mM CaCl₂ (LBmc) at 30°C. When required, antibiotics were added at the final concentration described previously (Finan et al. 1984; Pocard et al. 1997). Defined minimal medium used for *S. meliloti* was carbon- and nitrogen-free S medium supplemented with 20 mM choline (Smith et al. 1988). For physiological experiments, the *S. meliloti* strains were grown overnight in LBmc medium and transferred in LAS medium containing 10 mM sodium lactate and 10 mM sodium aspartate (Bernard et al. 1986), prior to transfer under inducing conditions. For experiments under microaerobiosis, air was replaced by a 1% oxygen and 99% argon atmosphere. When indicated, the osmotic strength of the media was increased by the addition of 0.3 M NaCl.

DNA manipulations, PCR amplifications, and DNA sequencing.

Restriction analysis, ligation, transformation, plasmid DNA extraction, Southern blotting, and DNA hybridization were performed by standard methods (Sambrook et al. 1989). DNA probe were labeled, using the Prime-a-Gene random priming system (Promega, Charbonnières, France) and [α -³²P] dCTP (Amersham Corp., Little Chalfont, U.K.). Total DNA from *S. meliloti* was isolated by the CTAB (cetyl-trimethylammonium-bromide) method, as described previously (Meade et al. 1982). PCR mixtures contained 50 pmol of each primer synthesized by MWG Biotech (Edersberg, Germany), 500 ng of plasmid template, a 200- μ M concentration each of dATP, dTTP, dGTP, and dCTP, 1× Taq polymerase buffer, and 1 U of Taq DNA polymerase (Appligene, Illkirch, France) in a final volume of 50 μ l. The reaction mixtures were cycled in a Biometra DNA thermocycler (T gradient model; Biometra GmbH, Göttingen, Germany), as previously described (Boscari et al. 2002), with an annealing temperature varying from 50 to 68°C, depending on primers used. PCR-amplified fragments were purified from agarose gel prior to enzymatic restriction and cloning. The nucleotide sequences of both DNA strands were established using the fluorescent ABI dye-labeled deoxy terminator method (MWG Biotech, Edersberg, Germany).

Plasmid constructions and mutagenesis of *S. meliloti* 102F34.

To construct a *betI*:: Ω mutant allele, a *Bam*HI restriction site was first created by PCR at the position 21 bp after the putative ATG of the *betI* gene, using the plasmid pUCSH as DNA template. The 5' upstream region of *betI* was amplified with primer *betI5* (5'-GCA CCG GAT CCA TTC C-3') and universal primer from bacteriophage M13, while the 3' end of *betI* was amplified using *betI4*, the reverse primers of *betI5*, and the universal reverse M13 primer. The resulting *Hind*III/*Bam*HI 737-bp fragment (corresponding to the 5' region of *betI*) and the *Bam*HI/*Sph*I 852-bp fragment (corresponding to the 3' end of *betI*) were subcloned into pUC118 before verification by sequencing to ensure that PCR amplification had not introduced any error. The pSP402 plasmid was subsequently constructed by sequential ligation of both purified inserts into the suicide vector pSUP202. The Ω -interposon (Sp^r/Sm^r) purified from the plasmid pHP45 Ω was finally inserted at the unique *Bam*HI restriction site of pSP402 to yield pSP402 Ω .

For the construction of the *betI::Ω* mutant strain, the plasmid pSP402Ω was transformed into *E. coli* S17-1 and was transferred into *S. meliloti* 102F34 by biparental mating, as previously described (Simon et al. 1983). *S. meliloti* double genomic recombinant clones were isolated as Rif^r, Sm^r, or Cm^r and were verified for their inability to use choline as unique carbon and nitrogen sources. The correct recombination of the interposon in the genomic *betI* gene was verified by hybridizations, and a selected clone was designated UNA294.

The *betI-lacZ* fusion was created by inserting a polar *lacZ-Km* cartridge in the *betI* gene. The 5' upstream region of *betI* was first amplified by PCR using pUCSH as DNA template and the primers *pbetI.1* (5'- GCG ATC GAG GCC AGC CCC TGC AT-3') and *pbetI.2* (5'-GAC GGA TCC GCG CGA TCT CCG ACA TGG TCA- 3'). The 780-bp DNA fragment was cloned into the vector pGEM-T, resulting in plasmid pGEM-TII, before DNA sequence verification. The *SalI/BamHI* fragment purified from pGEM-TII was then cloned and ligated into pSUP202, and the 4.7-kb *lacZ-Km* cartridge from pKOK5 was inserted at the unique *BamHI* restriction site of the resulting construct. The orientation of the cartridge was verified by restriction analysis, and the new plasmid containing the *betI-lacZ* fusion was transferred into the *S. meliloti* 102F34 and UNA294 strains by triparental mating, using *E. coli* MT616 (pRK600) as helper. A simple genomic recombinant of strain 102F34, selected as Rif^r and Nm^r, was named UNA338, and a derivative of UNA294, selected as Sm^r and Nm^r, was designated UNA340.

To construct the *betA-lacZ* and *betB-lacZ* fusions, derivatives of pCHO341, containing the whole *bet* cluster (Østerås et al. 1998), were obtained after partial digestion with *EcoRI* and ligation with the 4.5-kb *EcoRI* fragment carrying a nonpolar *lacZ-Gm^r* cartridge from pAB2001. The location and orientation of the cartridge at the correct *EcoRI* site in the resulting plasmids pCHO34AZ (*betA-lacZ*) and pCHO34BZ (*betB-lacZ*) were verified by restriction analysis. The broad host range plasmids were transferred into *S. meliloti* 102F34 by conjugation, and the *betA-lacZ* and *betB-lacZ* fusions were recombined into the 102F34 genome using the plasmid incompatibility technique (Ruvkun and Ausubel 1981). The correct locations of the *lacZ-Km* cartridge in the *betI-lacZ* strains UNA338 and UNA340 and of the *lacZ-Gm* cartridge in the *betA-lacZ* and *betB-lacZ* double recombinant strains named UNA216 and UNA228, respectively, were verified by hybridization.

Transport assays.

The radioactive [methyl-¹⁴C] choline (2.04 GBq/mmol) was purchased from Amersham Corp. Cells were grown in MCAA medium containing 0.1% sodium malate and 0.1% casamino acids (Smith et al. 1988), were harvested at an optical density at 600 nanometers (OD₆₀₀) of 0.8 to 1.0, were washed twice in the medium used for the culture, and were resuspended at the same OD. All assays were carried out at 30°C with 1 ml of cell suspension and radioactive choline (100,000 dpm) at 10 μM for 1 to 5 min. Reactions were terminated by rapid filtration through GF/F glass microfiber filters (Whatman, Maidstone, U.K.), as described previously (Pocard et al. 1989). The radioactivity remaining on the filters was determined with a liquid scintillation spectrometer (model LS6000SC, Beckman Instruments, Villepinte, France).

Chemicals, enzymatic assays, and intracellular choline determination.

Choline chloride and choline esters were purchased from Sigma Aldrich (Saint Quentin Flavier, France). Choline-*O*-sulfate was prepared from choline chloride and sulfuric acid

as previously described (Østerås et al. 1998; Stevens and Vohral 1955). β-Galactosidase activity was determined by Miller's method using *o*-nitrophenyl-β-D-galactoside as the substrate (Miller 1972). Protein concentration was measured according to Bradford (1976) using bovine serum albumine as standard.

Intracellular choline pools were determined on 100 ml of free-living cells grown in LAS medium with the addition of 1 mM choline 5 h before the analysis. The cells were harvested at an OD₆₀₀ of 0.8, were washed three times in 50 mM potassium phosphate buffer, pH 7.8, were resuspended in 1 ml of the same buffer, and were sonicated on ice (6 × 30 s, one pulse per s). The extract was clarified by centrifugation at 10,000 × *g* (10 min.) at 4°C. Choline was measured by a method adapted from Nie and associates (1993). The reaction was conducted in 50 mM Tris-HCl buffer, pH 7.8, containing 100 μl of bacterial crude extract, 200 μg of phenol, 590 μg of 4-aminoantipyrine, 4 units of horseradish peroxidase, and 2.5 units of choline oxidase in a total volume assay of 1 ml. The reaction mixture was incubated 30 min. at 37°C, and the OD₅₀₀ was measured. Choline chloride solution was used as standard at concentrations ranging from 0 to 50 μg per ml in the assay. The same method was used for choline determination in three- and five-week-old nodules. Freshly harvested nodules (2 to 3 g, fresh weight) were used to prepare extracts from cytosol, bacteroids, and peribacteroid spaces. They were gently crushed in Mes-Tris buffer (25 mM, pH 7.2) adjusted with mannitol to 450 mosmol (Trinchant et al. 1994), and the homogenate was used to obtain the cytosol fraction and the symbiosomes, according to Trinchant and associates (1998). Symbiosomes were purified, and the corresponding free bacteroids were liberated by vigorously vortexing purified symbiosome suspensions for 5 min. A centrifugation (5000 × *g*, 15 min) allowed us to separate the bacteroid pellets from the supernatant that constitutes the peribacteroid space fraction. The bacteroid pellets were extracted with 70% ethanol (Fougère et al. 1991), and all extracts were kept at -20°C until used for choline determinations. The choline concentration inside the bacteroids was calculated using the following relationships: 1 g of fresh weight nodules contains 15.3 ± 0.7 mg dry weight in bacteroids (Fougère et al. 1991), which corresponds to 4.0 ± 0.3 mg of protein, and the intracellular aqueous volume of bacteroids is 6.9 ± 0.7 μl per mg protein (Fougère and Le Rudulier 1990b).

Plant growth and nitrogen fixation assays.

Alfalfa (*Medicago sativa* cv. Europe) seedlings were grown on a sterilized mixture of vermiculite and sand (10:1, vol/vol) and were inoculated nine days after sowing. The plantlets were maintained in a growth chamber with 16 h light (24°C) and 8 h dark (20°C). They were watered with a nitrogen-free nutrient medium (Trinchant et al. 1994). Osmotic stress was applied four weeks after inoculation by adding NaCl to the growth medium (final concentration 0.2 M) and was maintained for two weeks. Nitrogen fixation capacity was determined by C₂H₂ reduction using a gas chromatograph (ATI-Unicam model 610) equipped with a Porapak T column (Trinchant et al. 1994).

Histochemical detection of β-galactosidase activity in nodules.

M. sativa plantlets were grown in sterile test tubes (3 plantlets per tube) with nitrogen-free medium and were inoculated with the appropriate strain, as previously described (Pocard et al. 1997). One or two weeks after inoculation, histochemical detection of β-galactosidase activity was

performed according to Boivin and associates (1990) and Jiménez-Zurdo and associates (1997). Roots and mature nodules were fixed with 1.25% glutaraldehyde in 0.2 M cacodylate, pH 7.2. Sections of two-week-old nodules (100 µm) were obtained with a vibratome (VT 1000 Plus, Labo Nord, France) and were fixed again with 1.25% glutaraldehyde. Samples were transferred to the staining solution, were cleared (30 s) with sodium hypochlorite, were rinsed with distilled water, and were mounted on slides. They were observed and photographed using an Olympus microscope. For the quantification of β -galactosidase activity, five-week-old nodules from 40 plants grown on the vermiculite/sand mixture were collected. Bacteroids were isolated as described above, and enzymatic determination was performed as for free-living cells.

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