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Betaine Use by Rhizosphere Bacteria: Genes Essential for Trigonelline, Stachydrine, and Carnitine Catabolism in *Rhizobium meliloti* Are Located on pSym in the Symbiotic Region

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***Rhizobium meliloti* is known to use betaines synthesized by its host, *Medicago sativa*, as osmoprotectants and sources of energy. It is shown in the present report that the symbiotic megaplasmid (pSym) of *R. meliloti* RCR2011 encodes functions essential to the catabolism of three betaines, trigonelline (nicotinic acid N-methylbetaine), stachydrine (proline betaine or dimethylproline), and carnitine (γ-trimethyl-β-hydroxybutyrobetaine). Preliminary evidence is presented showing that functions on pSym also influence the catabolism of choline and its oxidative product, glycine betaine. Genes implicated in betaine catabolism are found in the symbiotic region of pSym. Trigonelline catabolism functions lie between two clusters of symbiotic genes, *nifKDH* and *nok/fixVI'*. Stachydrine and carnitine functions lie to the right of trigonelline catabolism functions, immediately to the right of *fixVI'*. Information necessary to choline and glycine betaine catabolism is probably encoded to the right of stachydrine catabolism functions.**

Additional keywords: catabolism of secondary metabolites, microbial ecology, nitrogen fixation, nutritional mediators.

The production of secondary metabolites by plants and their selective catabolism by microorganisms are thought to contribute to the regulation of the dynamics of the microbial populations that depend on the plant as a source of energy (Nutman 1965). We have called such metabolites nutritional mediators (Tepfer *et al.* 1988). Studies of the specific catabolism of plant secondary metabolites by soil bacteria originally focused on the opines, substances synthesized in plant tissues genetically transformed by *Agrobacterium* (see review Tempé and Goldmann 1982). Opines are catabolized by *Agrobacterium* (see review Dessaux *et al.* in press) and *Pseudomonas* (Rossignol and Dion 1985; Bouzar and Moore 1987). Members of the genus *Rhizobium* catabolize compounds produced by or in their legume host: homoserine from pea root exudates (Armitage *et al.* 1988) and rhizopine from alfalfa root nodules (Murphy *et al.* 1987). Metabolites characteristic of plants other than legumes can also be catabolized. Tropane derivatives, called calystegins, produced by some morning glories and by *Atropa belladonna* are degraded using genes encoded on pRme41a of *Rhizobium meliloti* Dangeard strain 41 (Tepfer *et al.* 1988; Goldmann *et al.* 1990).

As part of a general effort to identify other nutritional relationships important for the *Rhizobium*-legume association, we examined the betaines, which are fully N-methylated derivatives of amino acids or related compounds. Among the betaines, trigonelline (nicotinic acid N-methylbetaine; Fig. 1) is present in especially high quantities in the seeds of legumes (Tramontano *et al.* 1986). We first determined the spectrum of trigonelline use as a carbon and nitrogen source in a variety of soil bacteria known to interact with plants, and we found that genes essential to the use of trigonelline by *R. meliloti* RCR2011 are located on pSym, adjacent to genes involved in symbiosis. *Medicago sativa* L., one of the plant hosts of *R. meliloti*, synthesizes several other betaines besides trigonelline (Robertson and Marion 1959; Panter and Mudd 1969) (Fig. 1). Among these, choline and stachydrine (proline betaine or dimethylproline) are catabolized by *R. meliloti* (Bernard *et al.* 1986; Gloux and Le Rudulier 1989). Glycine betaine, the oxidative product of choline, is also used by *R. meliloti* (Smith *et al.* 1988). We sought to determine whether their catabolism is also dependent on genes carried by pSym. We comment on the possible importance of these catabolic functions in symbiosis and coevolution.

MATERIALS AND METHODS

Bacterial strains and media. The strains and plasmids used in this study are given in Table 1. One of the strains carrying a deletion in pSym, used for the localization of the trigonelline catabolism (*trc*) genes, GMI5309 (Renalier *et al.* 1987), was auxotrophic for methionine. To test the influence of this deletion on the ability to grow on trigonelline as the sole carbon and nitrogen source, we constructed the prototrophic derivative, GMI5939, by transduction with the phage N3 (Martin and Long 1984).

Rhizobium, *Pseudomonas*, *Azospirillum*, and *Klebsiella pneumoniae* Schroeter were grown in TY medium (0.5%

tryptone, 0.3% yeast extract, 2 mM $CaCl_2$). *Agrobacterium tumefaciens* (Smith and Townsend) Conn and *A. rhizogenes* (Riker *et al.*) Conn were grown as described in Petit and Tempé (1978). *Escherichia coli* (Migula) Castellani and Chalmers was cultured in LB medium (see Maniatis *et al.* 1982). Minimal medium was SM for *Rhizobium* (Klapwijk *et al.* 1977) and M9 for *E. coli* (Sambrook *et al.* 1989). Media for growth tests were solidified with agar (Touzart, France) at 8 g/L and supplemented with a betaine as the sole carbon and nitrogen source. Antibiotics were used at the following concentrations: tetracycline, 10 μg/ml; spectinomycin, 200 μg/ml; and neomycin, 100 μg/ml.

Chemicals. Trigonelline, nicotinic acid, carnitine, choline, and glycine betaine were purchased from Sigma (St. Louis, MO). Stachydrine and homarine were from Extrasynthèse (Genay, France).

Microbiological techniques. Methods for conjugal crosses were as described (Truchet *et al.* 1985). Derivatives of pLAFR1 were mobilized with the helper plasmid pRK2013, and *R. meliloti* transconjugants were selected on TY medium supplemented with the appropriate antibiotics.

Growth curves in liquid medium were determined after two types of preculture: in minimal SM medium, containing a betaine as the sole carbon and nitrogen source for 130 hr; and overnight in complete TY medium, followed by 3 hr in fresh TY medium. Growth of the cultures was determined by monitoring increases in turbidity at 650 nm. Experiments were repeated at least once.

Choline incorporation was determined in 5-ml cultures containing 2 μCi of methyl-[^{14}C] choline (Amersham Corp., Arlington Heights, IL). Bacteria were cultured overnight in TY medium, before being exposed to labeled choline. At 10-min intervals after addition of labeled choline, bacteria were collected from 0.5-ml samples using Millipore filters. They were washed, and incorporated radioactivity was determined by liquid scintillation spectrometry. In one sample, cells were disrupted by sonication and the presence of choline and glycine betaine was assessed by high voltage paper electrophoresis followed by autoradiography.

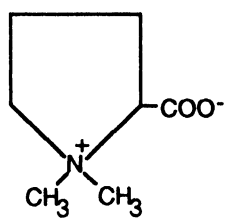
Betaine degradation. Bacteria (OD_{650} = 0.5) were incubated at 28° C for 48 hr, with agitation, in 200 μl of SM minimal medium containing 50 μg of either trigonelline, choline, glycine betaine, stachydrine, or carnitine as the sole carbon and nitrogen source (Tepfer *et al.* 1988). After 40 hr, the bacterial supernatant was concentrated to dryness, resuspended in 10 μl of H_2O and assayed by high voltage electrophoresis at pH 1.9 and staining with Dragendorff's reagent (Dawson *et al.* 1969). The limit of detection of betaines was 10 μg after electrophoresis. Experiments were repeated with 100 μg of substrate, and betaines were no longer detected, except in the case of stachydrine, when faint spots were sometimes observed.

choline $(CH_3)_3N^+CH_2CH_2OH$

carnitine $(CH_3)_3N^+CH_2CH(OH)CH_2COO^-$

glycine betaine $(CH_3)_3N^+CH_2COO^-$

stachydrine



trigonelline

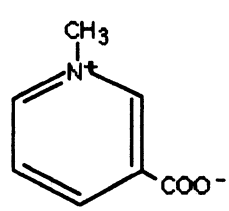


Fig. 1. Structures of the betaines. A characteristic trait is the quaternary amine shown as N^+.

RESULTS

Catabolism of trigonelline by *R. meliloti* strains 41 and RCR2011. Both strains grew on plates of minimal medium with trigonelline as the sole carbon and nitrogen source. We designated the trigonelline catabolism phenotype as Trc^+ and determined the minimal and maximal concentrations for colony formation for the two strains. No colonies formed on SM medium containing trigonelline at a concentration below 0.1% (5.8 mM). Cells were killed at trigonelline concentrations above 0.34%, because subsequent culture in complete TY liquid medium with a conventional carbon source produced no viable bacteria. The optimal trigonelline concentration for growth was 0.2%, and colonies appeared in 3–4 days. Disappearance of trigonelline was verified in liquid medium, thus confirming that growth was correlated with the catabolism of the substrate. Chemically related molecules such as nicotinic acid, which is the immediate precursor of trigonelline in *Pisum sativum* (Tramontano *et al.* 1983), or homarine (2-picolinic acid N-methylbetaine) were not utilized by strains 41 and RCR2011, indicating that trigonelline catabolism is chemically specific.

R. meliloti GMI13, a derivative of strain 41, cured of pRme41a, which was previously identified as carrying the calystegin catabolic genes (Tepfer *et al.* 1988), grew on trigonelline as the sole carbon and nitrogen source. This plasmid therefore does not carry genes necessary for trigonelline catabolism.

Spontaneous Trc^- derivatives of strains 41 and GMI13 were isolated after storage in the cold. Because loss of symbiotic functions after cold storage has already been described (Banfalvi *et al.* 1981), we thought it possible that genetic rearrangements in the vicinity of the symbiotic genes

carried by the Sym plasmid of strain 41 had affected trigonelline catabolism. In a preliminary effort to determine whether pSym carries genes involved in trigonelline catabolism, the Trc$^+$ 2011 derivative GMI245, (RP4, pSym::Tn*5*::Tn*7*) was mated with a spontaneous Trc$^-$ mutant of *R. meliloti* GMI13. Transconjugants were selected carrying RP4, previously shown to mobilize pSym or pSym fragments (Truchet *et al.* 1984). They were then screened for trigonelline catabolism by substrate breakdown and growth tests. Among 10 GMI13 × GMI245 transconjugants studied, one was isolated that had acquired the Trc$^+$ phenotype, suggesting that in *R. meliloti* strain 41 *trc* genes are located on pSym.

pSym-encoded catabolism of trigonelline in *R. meliloti* RCR2011. Further efforts to localize *trc* genes were made using *R. meliloti* strain RCR2011 because of the availability of derivatives carrying deletions in and subclones from the Sym plasmid (Renalier *et al.* 1987). Strain GMI766, a spontaneous mutant of RCR2011 with a large deletion in pSym was Trc$^-$, as was GMI5939, a RCR2011 derivative carrying an approximately 65-kb deletion to the right of the *nifAB* genes (Fig. 2). In an attempt to complement the Trc$^-$ phenotype of GMI766, three recombinant plasmids carrying pSym inserts were introduced into GMI766: pGMI467, pGMI469, and pGMI471 (Fig. 2). Transconjugants were selected for pLAFR1 with tetracycline and

Table 1. Bacterial strains, plasmids, and phages used in this study

Bacterium, plasmid, or phage	Relevant characteristics[a]	Catabolic phenotype[b]	Source or reference[c]
Bacteria			
Rhizobium meliloti (wild-types)			
RCR 2011	Isolate from alfalfa	Trc$^+$, Stc$^+$ Chc$^+$, Gbc$^+$ Crc$^+$	Rosenberg *et al.* 1981
41, 1322, CC169, V7, Ls2a, A145, S26, L530	Various isolates from alfalfa	Trc$^+$	Rosenberg *et al.* 1981
R. meliloti 41 derivatives			
AK631	Non-mucoid derivatives of 41	Trc$^+$	Kondorosi *et al.* 1984
GMI13	Derivative of AK631, cured of pRme41a	Trc$^+$	Tepfer *et al.* 1988
R. meliloti RCR2011 derivatives			
GMI766	Nod$^-$Δ(*nod nifA*)766, Spc^r	Trc$^-$, Stc$^-$ Chc$^-$, Gbc$^-$ Crc$^-$	Renalier *et al.* 1987
GMI5309	Δ(*fixABC nifA nifB*)53, (Tn*5*) auxotroph		Truchet *et al.* 1985
GMI5939	Δ(*fixABC nifA nifB*)53, (Tn*5*) prototroph	Trc$^-$, Stc$^-$ Crc$^+$	This work
GMI255	Δ(*fix-1074 nod nifKDH*)7125, (Tn*5*) Nal^r	Trc$^+$, Stc$^+$ Chc$^+$, Gbc$^+$ Crc$^+$	Renalier *et al.* 1987
GMI245	pSym2011::Tn*5*::Tn*7*, RP4	Trc$^+$	J. Dénarié[c]
LBR46	GMI766 (pGMI467)	Trc$^-$, Stc$^+$ Chc$^-$, Gbc$^-$ Crc$^+$	This work
LBR47	GMI766 (pGMI469)	Trc$^-$, Stc$^-$ Chc$^-$, Gbc$^-$ Crc$^-$	This work
LBR48	GMI766 (pGMI471)	Trc$^+$, Stc$^-$ Chc$^-$, Gbc$^-$ Crc$^-$	This work
GMI708	RCR2011 Rif^r	Trc$^+$, Stc$^+$	Renalier *et al.* 1987
R. meliloti GMI708 derivatives			
GMI5576	GMI708 ΔJB8 Nm^r, Bl^r, Rif^r,	Stc$^+$	Renalier *et al.* 1987
GMI5577	GMI708 ΔJB10 Nm^r, Bl^r, Rif^r	Stc$^+$	Renalier *et al.* 1987
GMI5579	GMI708 ΔJB109 Nm^r, Bl^r, Rif^r	Stc$^+$	Renalier *et al.* 1987
GMI5590	GMI708 ΔJB32 (R751pMG2) Nm^r, Bl^r, Rif^r, Gm^r	Stc$^+$	Renalier *et al.* 1987
GMI5593	GMI708 ΔMD36 (R751pMG2) Nm^r, Bl^r, Rif^r, Gm^r	Stc$^-$	Renalier *et al.* 1987
GMI5595	GMI708 ΔJB16 Nm^r, Bl^r, Rif^r	Stc$^-$	Renalier *et al.* 1987

(**Table 1.** *continued on next page.*)

[a] Spc^r, Nal^r, Nm^r, Bl^r, Rif^r, Gm^r. Tc^r, and Km^r indicate resistance to spectinomycin, nalidixic acid, neomycin, bleomycin, rifampicin, gentamicin, tetracycline, and kanamycin, respectively.

[b] Trc, Stc, Chc, Gbc, Crc indicate catabolic phenotypes for trigonelline, stachydrine, choline, glycine betaine, and carnitine, respectively.

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tested for the Trc$^+$ phenotype. Only GMI766 (pGMI471), designated LBR48 (Fig. 3A), acquired the Trc$^+$ phenotype, indicating that *trc* genes are located on the pSym in a region of about 30 kb between the *nifKDH* genes and the *nok/fixVI'* cluster previously described by Renalier *et al.* 1987.

Search for other soil bacteria that degrade trigonelline. Strains of *Rhizobium*, *Agrobacterium*, *Azospirillum*, and *Pseudomonas* were tested for growth on solid minimal medium containing 0.2 or 0.3% trigonelline as the sole carbon and nitrogen source. *E. coli* and *K. pneumoniae* were also tested (Table 1). All of the *Rhizobium* and *Agrobacterium* species formed colonies in three days, except for two *Rhizobium leguminosarum* strains, RCR0403 (bv. *trifolii* Jordan) and 248R (bv. *viceae* Jordan), which grew more slowly. Thus, trigonelline catabolism appears to be a general trait in the Rhizobiaceae. An *A. tumefaciens* derivative (GMI9023), cured of all detectable plasmids, was Trc$^+$, indicating that the *trc* genes in this *Agrobacterium* are chromosomal. Several of the *Pseudomonas* strains that catabolize opines also grew on trigonelline. In contrast, *E. coli* and two free-living, nitrogen-fixing bacteria, *Azospirillum* and *K. pneumoniae* were unable to grow with trigonelline as the sole carbon and nitrogen source.

Catabolism of betaines by *R. meliloti*. Betaines previously identified in *M. sativa* are stachydrine, carnitine, choline, and glycine betaine (Robertson and Marion 1959; Mears and Mabry 1971). We thus examined catabolism of these substances in *Rhizobium*. Growth tests in liquid minimal medium confirmed that *R. meliloti* strain RCR2011 uses stachydrine, carnitine, choline, and glycine betaine as carbon and nitrogen sources. Strains carrying deletions in pSym (Fig. 2) were tested for their capacity to catabolize stachydrine, carnitine, choline, and glycine

Table 1. (*continued*)

Bacterium, plasmid, or phage	Relevant characteristics[a]	Catabolic phenotype[b]	Source or reference[c]
R. leguminosarum bv. *viceae*			
PX31, PBI1	Wild isolate from pea nodules	Trc$^+$	N. Amarger[c]
248R	Wild isolate from pea nodules	Trc$^+$ (slow)	Van Brussel *et al.* 1982
R. leguminosarum bv. *phaseoli*			
8002, 84001	Wild isolates from bean nodules	Trc$^+$	Lamb *et al.* 1982
R. leguminosarum bv. *trifolii*			
32R	Wild isolates from clover nodules	Trc$^+$	N. Amarger
RCR0402, RCR0403	Wild isolate from clover nodules	Trc$^+$ (slow)	Rothamsted, U.K.
Agrobacterium rhizogenes			
8196	Wild type mannopine strain	Trc$^+$	J. Lippincott[c]
A4	Wild type agropine strain	Trc$^+$	L. Moore[c]
A. tumefaciens			
T37	Wild type nopaline strain	Trc$^+$	G. Morel[c]
B6-806	B6 derivative cured of phage omega	Trc$^+$	J. Tourneur[c]
Bo-542	Wild type agropine strain	Trc$^+$	J. Tempé[c]
C58	Wild type nopaline strain	Trc$^+$	G. Morel
C58C1	C58 derivative cured of pTi	Trc$^+$	J. Tempé
GMI9023	C58C1 pAt$^-$ (no detectable plasmids)	Trc$^+$	Rosenberg *et al* 1984
Pseudomonas sp.			
CH173, CH39, 211	Octopine-utilizing strains	Trc$^+$	P. Dion[c]
CH412, CH418,203	Octopine-utilizing strains	Trc$^-$	P. Dion
Azospirillum brasilense			
CD1, R07, Sp7	From rhizospheres of tropical grasses	Trc$^-$	C. Elmerich
Azospirillum lipoferum			
Br17	Wild type, motile	Trc$^-$	C. Elmerich
Klebsiella pneumoniae			
UNF933		Trc$^-$	J. Dénarié
Escherichia coli			
GMI3355	K12 derivative Nal^r Rif^r	Trc$^-$	Julliot and Boistard 1979
Plasmids			
pLAFR1	Inc-P1 Tc^2 cos$^+$ rlx$^+$		Friedman *et al.* 1982
pGMI467	pLAFR1-prime; about 35-kb insert of pSym RCR2011, Tc^r		Van Brussel *et al.* 1982
pGMI469	pLAFR1-prime; 27-kb insert of pSym RCR2011, Tc^r		M.-H. Renalier
pGMI471	pLAFR1-prime; 30-kb insert of pSym RCR2011, Tc^r		M.-H. Renalier
pRK2013	Helper plasmid for mobilization of pLAFR1 derivatives, *tra* (RK2), *ori* ColE1, Km^r		Friedman *et al.* 1982
pGMI53	RP4-prime; 70-kb insert from pSym RCR2011 with Tn*5* insertion in the *nif* region, Tra$^+$, Tc^r, Ap^r, Km^r, Bl^r		Van Brussel *et al.* 1982
Phage			
N3	Transducing phage of *R. meliloti*		Maniatis *et al.* 1982

betaine (Fig. 3). One strain, GMI255, carrying a deletion of 280 kb to the left of *nifKDH*, catabolized the four betaines. On the other hand, as in the case of trigonelline, strain GMI766, carrying a deletion of more than 100 kb to the right of *nifKDH*, could not catabolize stachydrine, carnitine, choline, and glycine betaine, suggesting that genes essential to the use of these compounds are located on pSym to the right of *nifKDH*. The recombinant pLAFR1′ plasmids used to localize the *trc* region (pGMI467, pGMI471, and pGMI469) were thus tested for their ability to restore to GMI766 the catabolism of the other four betaines (Fig. 3). Plasmid pGMI467, introduced into GMI766 (designated strain LBR46), restored catabolism of stachydrine (Fig. 3B). Catabolism of choline and glycine betaine could neither be restored by these plasmids (Fig. 3C,D), nor by pGMI53, which contains an insert of 70 kb from the region of pSym deleted in GMI5939. In the case of carnitine, LBR46 grew slowly compared with the wild strain (Fig. 3E), and whereas carnitine was no longer detected in the medium, a more slowly migrating, Dragendorff-positive compound appeared (results not shown), indicating that pGMI467 encodes one or more (but not all) steps in carnitine use. Strain GMI5939, which is Trc^- and Stc^-, can catabolize carnitine, thus the genes implicated in its catabolism (*crc*) must lie on the right-hand end of pGMI467.

These experiments were repeated under different conditions of preculture, in TY medium instead of minimal medium. For trigonelline, glycine betaine, and choline, results (not shown) were similar to those obtained using minimal medium, except that longer lag periods were observed. Plasmid pGMI471 (strain LBR48) again completely restored the Trc phenotype. However, in the case of stachydrine, a particularly long lag occurred with strain LBR46 (Fig. 3F), indicating that pGMI467 did not completely restore the wild phenotype.

To more precisely locate the strachydrine catabolism (*stc*) genes, strains with deletions in the region of pSym corresponding to the insert of pGMI467 (i.e., containing *nok*, *fixVI′*, and *nodD*$_2$) (Renalier *et al.* 1987; Honma and Ausubel 1987) were tested for their ability to catabolize stachydrine. Strains GMI5576 and GMI5577 (carrying deletions in *fixVI′*) strain GMI5579 (with a deletion in *nok*) and strain GMI5590 (with a deletion to the left of *nok*) had a Stc^+ phenotype (results not shown). GMI5595, carrying a deletion extending to the left and right of *fixVI′*, was unable to use stachydrine. The Stc^- phenotype was also observed (results not shown) for GMI5593, which carries a 7.6-kb deletion immediately to the right of the *fixVI′* genes (Fig. 2).

In an effort to determine the nature of the defect in strain GMI766, which is Trc^-, Stc^-, Chc^- (choline), Glc^- (glycine betaine), and Crc^-, we monitored the incorporation and degradation of labeled choline, relative to the wild type strain. In the time period examined, choline was taken up slower than in the wild type (results not shown). Using high voltage paper electrophoresis, we found that after 40 min the majority of the radioactivity was found in glycine

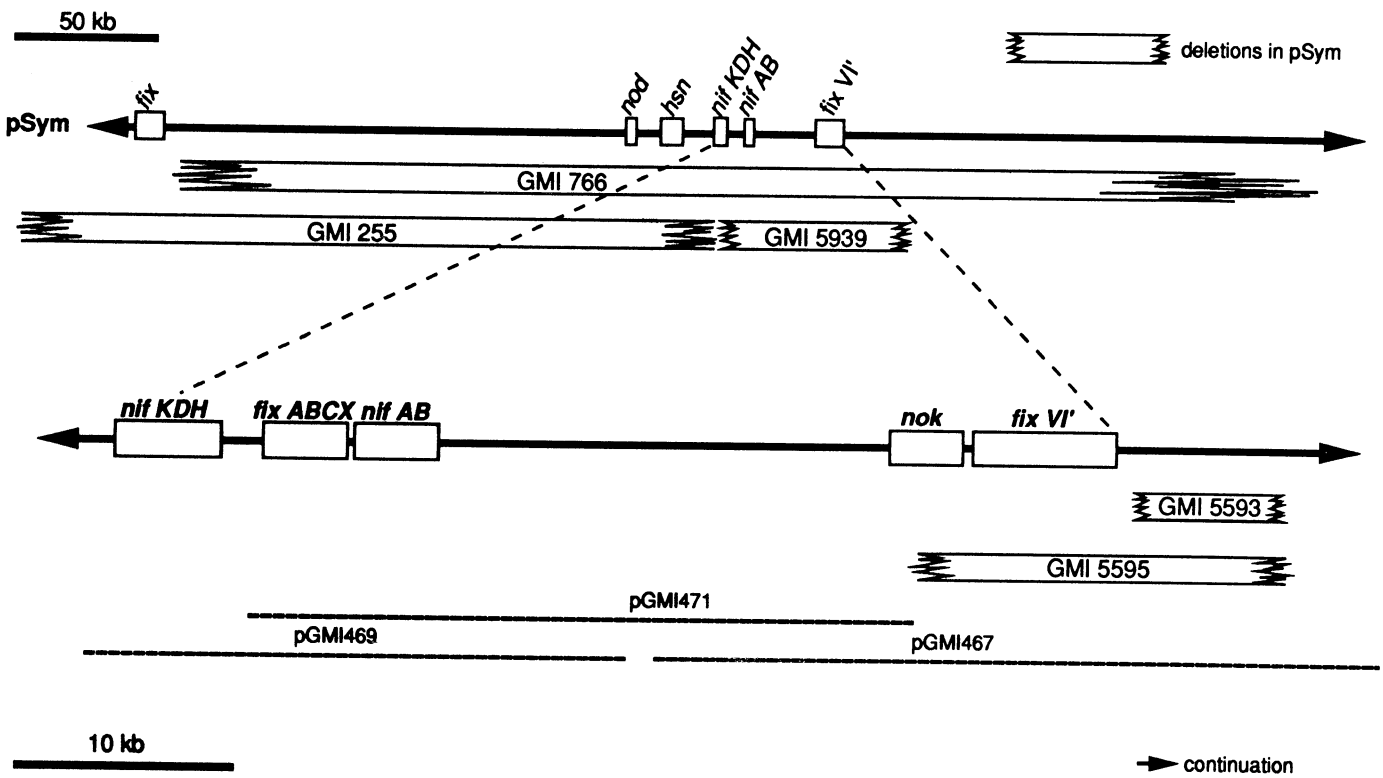

Fig. 2. Partial genetic map of pSym from *Rhizobium meliloti* strain 2011, showing regions containing known genetic loci in boxes with unbroken borders. Deletions are shown below by boxes with broken borders indicating uncertainty in the boundaries. Plasmids used in this study are indicated by dashed lines. *nok* (nodulation kinetics) refers to the *nod* locus defined by Renalier *et al.* (1987). *fixVI′* is a functional fix region repeat (Renalier *et al.* 1987).

betaine, as in the wild type (results not shown). We did not, thus, observe a defect in the first step of choline catabolism. Because the slow transport observed would seem insufficient to explain growth inhibition, the inability of GMI766 to grow on choline may be due to slow transport plus inhibition of catabolic functions, which occur after the conversion of choline to glycine betaine.

DISCUSSION

We set out to examine the possibility that betaines from *M. sativa* are nutritional mediators. We first concentrated on trigonelline, which, although generally detected in plants, is present in high quantities in the seeds and seedlings of legumes. We found that it can serve as the sole carbon and nitrogen source for diverse soil bacteria known to interact with plants and, more interestingly, for *Rhizobium*. In *R. meliloti* strain RCR2011, genes essential for trigonelline catabolism (*trc*) are carried by the symbiotic plasmid (pSym) between two clusters of symbiotic genes *nifKDH* and *nok/fixVI'*. Genes important in the catabolism of stachydrine and carnitine, other betaines present in alfalfa, are also carried by the pSym of *R. meliloti* RCR2011, but to the right of the *trc* genes in the region containing *fixVI'* and $nodD_2$ (Renalier *et al.* 1987; Honma and Ausubel 1987). Complete carnitine catabolism was not restored by pGMI467, thus this plasmid encodes functions governing only part of the degradative pathway required for efficient catabolism. For choline and glycine betaine at least some information essential to their utilization is

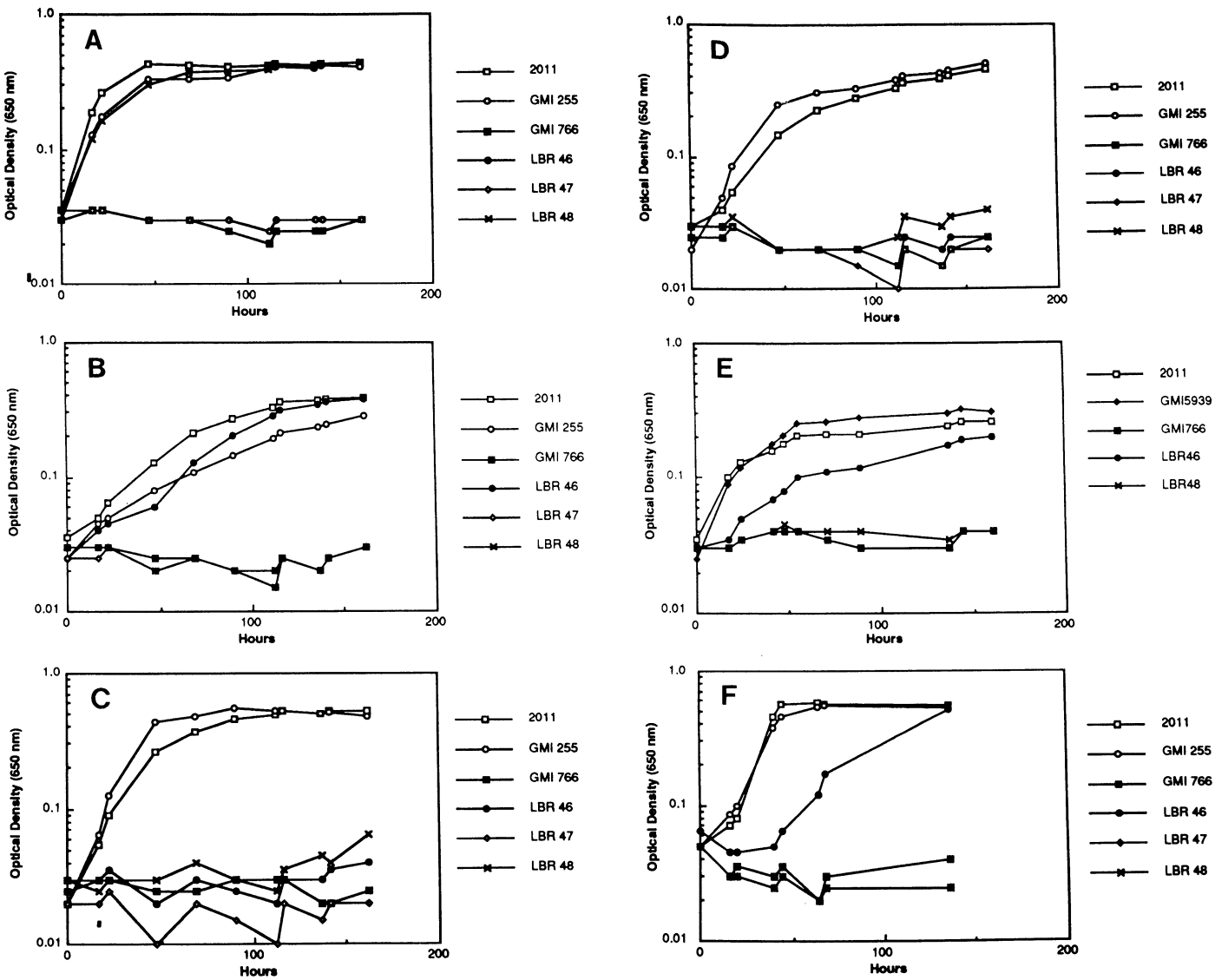

Fig. 3. Growth curves on five different betaines as sole carbon and nitrogen sources: **A,** trigonelline; **B,** stachydrine; **C,** choline; **D,** glycine betaine; **E,** carnitine; **F,** stachydrine. In A–E, growth was monitored after preculture on the betaine being tested and in F after culture in TY medium. 2011, *Rhizobium meliloti* wild type 2011; GMI255, strain 2011 containing pSym with a large deletion that does not interfere with growth on betaines; GMI766, strain 2011 containing pSym with a large deletion that interferes with growth on betaines; LBR46, GMI766 containing pGMI467; LBR47, GMI766 containing pGMI469; LBR48, GMI766 containing pGMI471. In some instances of no growth, data points are not visible due to their superposition.

situated on pSym, but less closely linked to the symbiotic genes, located more than 70 kb to the right of *nifKDH*.

Catabolic functions have already been assigned to *Rhizobium* plasmids. Interestingly, the symbiotic plasmids carry genes controlling the catabolism of two secondary metabolites produced in legume hosts, either by the plant or the symbiotic *Rhizobium* (Johnston *et al.* 1988; Murphy *et al.* 1987). On the other hand, genes involved in the catabolism of a family of nonlegume secondary metabolites, the calystegins, are encoded on a different replicon that is not essential for symbiosis (Tepfer *et al.* 1988). In contrast to the calystegins, trigonelline, stachydrine, carnitine, choline and glycine betaine are produced by numerous plants, indicating that if they were nutritional mediators, specificity would have to depend on differences in the degree and timing of their production by host and nonhost plants. The finding that trigonelline appears in the host only during the first stages of seed germination (Tramontano *et al.* 1986) suggests that it could play a role in the timing of plant-microorganism interactions, perhaps as a signal in the early stages of seedling colonization.

Because trigonelline is present in particularly high concentrations in the seeds of legumes, attempts have been made to link it to the symbiotic interaction (Tramontano *et al.* 1986). Trigonelline has been found in root exudates (Ikegami *et al.* 1982), and it is a weak inducer of *nod* gene expression (Schmidt *et al.* 1986). It is also known to regulate the cell cycle in the roots of legumes (Evans and Tramontano 1984). It thus appears that trigonelline, like other plant secondary metabolites (e.g., phenolic compounds), may have multiple functions, both in the plant and in associated microorganisms.

Stachydrine, carnitine, choline, and glycine betaine are osmoprotectants that are essential to the survival of diverse organisms under osmotic stress (Wyn Jones and Storey 1981; Csonka 1989). Soil bacteria (e.g., *Rhizobium*) rely on plants as a source of betaines. Their osmoprotective roles in *R. meliloti* have been extensively studied (Bernard *et al.* 1986), and it has been shown that catabolism of choline and glycine betaine is osmoregulated (Smith *et al.* 1988). Nevertheless, the question of the osmoprotective activities of these substances under natural conditions has not been answered.

Although the betaines cannot at present be clearly identified as nutritional mediators, the localization of genes encoding their catabolism in the proximity of genes directly involved in nodulation and nitrogen fixation again raises the hypothesis that betaine catabolism genes have a role in nitrogen-fixing symbiosis or were important in its evolution.

Given the competition among soil bacteria for plant carbon sources, we would suggest that a preliminary step in plant-bacterium coevolution would likely be the acquisition of catabolic functions that permit the bacterium to adapt to a specific host's rhizosphere. Thus, in *Rhizobium*, the evolution of endosymbiosis may have followed and may have at least been partially based on the acquisition of catabolic functions permitting adaptation to a legume host. At the present time, *Rhizobium* interacts with plants both as an ectocommensal and an endosymbiont. The presence of genes encoding the catabolism of betaines on pSym and calystegins on another replicon (pRme41a), not known to be involved in nitrogen-fixing symbiosis, may be a reflection of these dual identities. Thus, aside from the question of the direct role of betaines in symbiosis, the structural linkage between symbiotic and catabolic genes might be a reflection of the sequence in which they evolved. Interestingly, trigonelline induces both *nod* and *trc* functions (Schmidt *et al.* 1986; Boivin *et al.* 1990), suggesting that catabolic and symbiotic functions may still retain common modes of regulation.

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