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A Plasmid of *Rhizobium meliloti* 41 Encodes Catabolism of Two Compounds from Root Exudate of *Calystegium sepium*

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Our objectives were to identify substances produced by plant roots that might act as nutritional mediators of specific plant-bacterium relationships and to delineate the bacterial genes responsible for catabolizing these substances. We discovered new compounds, which we call calystegins, that have the characteristics of nutritional mediators. They were detected in only 3 of 105 species of higher plants examined: *Calystegia sepium*, *Convolvulus arvensis* (both of the *Convolvulaceae* family), and *Atropa belladonna*. Calystegins are abundant in organs in contact with the rhizosphere and are not found, or are observed only in small quantities, in aerial plant parts. Just as the synthesis of calystegins is infrequent in the plant kingdom, their catabolism is rare among rhizosphere bacteria that associate with plants and influence their growth. Of 42 such bacteria tested, only one (*Rhizobium meliloti* 41) was able to catabolize calystegins and use them as a sole source of carbon and nitrogen. The calystegin catabolism gene(s) (*cac*) in this strain is located on a self-transmissible plasmid (pRme41a), which is not essential to nitrogen-fixing symbiosis with legumes. We suggest that under natural conditions calystegins provide an exclusive carbon and nitrogen source to rhizosphere bacteria which are able to catabolize these compounds. Calystegins (and the corresponding microbial catabolic genes) might be used to analyze and possibly modify rhizosphere ecology.

The factors regulating microbial growth in the rhizosphere are not completely understood but are thought to include substances released by roots (6, 33, 34) that could be species specific and act on microbial populations through negative or positive selection. In the latter case, these secondary metabolites could be responsible for nutritional selection, if they were produced in sufficient quantities and if they were refractory to catabolism by most soil microorganisms. Such substances would then provide an exclusive nutrient source to microorganisms possessing the genetic information necessary for their catabolism (26). The opines encoded by the Ti (tumor-inducing) and Ri (root-inducing) T-DNAs of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* (see reference 40 for a review) are selective nutrients for a variety of soil bacteria under laboratory conditions (5, 32, 41), but their ability to influence bacterial growth under natural conditions has not been reported. The use of these opines as selective agents in the rhizosphere, by inserting opine synthesis genes into the plant host, would run the risk of encouraging the growth of wild opine-utilizing, pathogenic agrobacteria.

We describe here the bacterial catabolism of substances produced by two species of the *Convolvulaceae* family and by *Atropa belladonna*. The synthesis of these compounds, which we call calystegins, is uncommon in the plant kingdom, as is their utilization in a sample of 42 rhizosphere bacteria, most of which were chosen for their ability to interact with plants. Under laboratory conditions these compounds provided selective nutrition to *Rhizobium meliloti* 41. The bacterial catabolic functions were encoded by a self-transmissible plasmid. We discuss the possible ecological significance of calystegin synthesis and catabolism.

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(Some of these results are described in French patent no. 85 08237, May 1985.)

MATERIALS AND METHODS

Calystegin extraction and visualization by high-voltage electrophoresis and silver staining. A minimum of 10 mg of plant tissue was homogenized in 0.02 N HCl (5 μl/10 mg of tissue). The homogenate was heated for 3 min in a boiling-water bath and clarified by centrifugation for 5 min in an Eppendorf microfuge. A 5-μl sample of supernatant was spotted onto Whatman 3MM paper and subjected to 3 kV for 15 min in a buffer consisting of formic acid-acetic acid-water (30:60:910; pH, 1.9) (45). After the samples were dried, the electrophoretogram was stained with silver nitrate (46) by first dipping it in $AgNO_3$ solution (4 g dissolved in 20 ml of H_2O, diluted to 1,000 ml with acetone), followed by drying and developing in a second solution prepared by dissolving 20 g of NaOH in 100 ml of H_2O, and diluting to 1,000 ml with 95% ethanol. After a third drying, the electrophoretogram was fixed in photographic paper fixer and washed. Agropine and mannopine standards were extracted in the same fashion from a tobacco tumor line transformed by *A. tumefaciens* A66 (11).

Root and plant cultures. The methods for establishing transformed root cultures were as described previously (44, 45). Transformed roots were grown in the liquid medium of Monnier (22) with the vitamins of Morel and Wetmore (23). Exudation of calystegins was measured with *Calystegia sepium* plantlets grown in vitro in sterile water containing a mineral nutrient solution (8). The symbiotic properties of *R. meliloti* were determined in vitro by inoculating legumes grown on nitrogen-free agar slants as previously described (47).

Preparation and biological enrichment of extracts prior to catabolic tests. Treatment with Dowex 50, followed by elec-

trophoretic analysis of the eluate, showed that calystegins copurified with the amino acid fraction. This fraction was evaporated to dryness, dissolved in water, filter sterilized, and added to a log-phase culture of *A. tumefaciens* T37. After 40 h a sample was tested for the presence of calystegins and amino acids by high-voltage paper electrophoresis and silver nitrate and ninhydrin staining. Strain T37 removed all detectable ninhydrin-positive compounds except calystegins and mannopine (data not shown). Little or no neutral sugars remained after this biological purification. After bacteria were removed by centrifugation and filtration, the root and rhizome extracts were re-run on Dowex 50 to remove medium salts and the purity of these extracts was assessed by electrophoresis and staining. These methods were essentially as described previously for the extraction and biological enrichment of opines (13).

Catabolic tests. Reaction mixtures (200 μl) included a biologically enriched (see above) extract from transformed roots, containing approximately 20 μg of calystegins A and B, 20 μg of mannopine, inorganic salts (minus N) of SM medium (19), and bacteria at an initial concentration with an optical density at 650 nm of 0.5. After 40 h at 28°C, the bacteria were removed by centrifugation and the supernatant was evaporated to dryness. The supernatant was redissolved in 20 μl of water, and a 5-μl portion of this solution was subjected to high-voltage paper electrophoresis, followed by silver staining to assay calystegin degradation.

Microbiological techniques. The bacterial strains, phages, and plasmids discussed here are listed in Table 1. The conditions used for bacterial growth and conjugation experiments (47) and the details of the genetic manipulations (31) were as described previously. Tagging of pRme41a with Tn*5* was performed by first mutagenizing pGMI4104, an RP4-prime carrying a pRme41a insert, in *Escherichia coli* with Tn*5*. Tn*5* insertions were introduced into pRme41a in *R. meliloti* by modifications (16) of the marker exchange method (35). Elimination of the vector was achieved by selecting clones resistant to phages pRR1 and GU5, which are specific for bacteria carrying IncP1 plasmids. A pRme41a thus tagged with Tn*5* (pGMI59) was mobilized into an *A. tumefaciens* strain cured of its Ti plasmid by using another RP4-prime (pGMI4142) carrying a region of pRme41a which does not overlap the insert of pGMI4104. *A. tumefaciens* transconjugants were selected in the presence of kanamycin (30 μg/ml), while donors were counter-selected with rifampin (100 μg/ml) and streptomycin (100 μg/ml). *R. meliloti* 41 was cured of pGMI59 with a pGMI4142::Mu-1 plasmid. Insertion of Mu-1 into RP4 derivatives was as already described (4). The cointegrate pGMI59-GMI4142::Mu-1 was unstable, and kanamycin-sensitive derivatives were obtained which were cured of both pGMI59 and pGMI4142::Mu-1. Plasmids were visualized by published procedures (30).

RESULTS

Detection of calystegins. Our first objective was to isolate plant secondary metabolites which might serve as selective agents for specific rhizosphere bacteria. We reasoned that these compounds might be carbon or nitrogen sources and would be liberated into the rhizosphere in quantities sufficient to selectively nourish bacteria capable of catabolizing these compounds. We therefore screened 105 species of plants, representing 26 families (Table 2), by subjecting crude extracts of subterranean and aerial plant organs to high-voltage electrophoresis and silver staining. Since we were looking for abundance and specificity, we were attracted to a pair of substances (designated calystegins A and B) which were first observed in *C. sepium* (morning glory). They are distinguishable as two spots when the sample concentration is low (see Fig. 4, lane 1), while at high concentrations they migrate as an elongated spot (Fig. 1). Calystegins A and B are abundant in roots and rhizomes, but they are absent (or present in only low concentrations) in stems and leaves (Fig. 1). *C. sepium* plants grown in vitro liberated calystegins into the medium (data not shown). We found substances with identical electrophoretic properties in *Convolvulus arvensis* and *Atropa belladonna*. Thus along with the abundance (see below) and the specific accumulation of calystegins in underground plant organs, the rarity of these compounds among the 105 plants species tested suggested that calystegins could play a role in plant-microorganism relationships in the rhizosphere.

Since calystegins were detected by methods used to assay agropine and mannopine, substances initially discovered in a tobacco tumor induced by *A. tumefaciens* (11), an extract of this tobacco line was used as an external standard in rough estimates of calystegin content. On a fresh-weight basis, agropine and calystegins are present in similar quantities (Fig. 1). Agropine was previously estimated to comprise 5 to 7% of the dry weight in this tumor line (37). By comparison with mannopine content we conclude that calystegin synthesis represents a major anabolic commitment on the part of the plant. This fact and their presence in root exudates again suggested that calystegins could play a role in rhizosphere nutritional relationships.

Calystegin production in transformed root cultures. Before embarking on a search for soil bacteria that degrade calystegins, we established axenic root organ cultures of *C. sepium* in order to provide a continuous, uniform, and copious supply of these substances. Since attempts to culture roots excised from seeds germinated in vitro gave poor results, we used genetic transformation by *A. rhizogenes* 8196 to induce

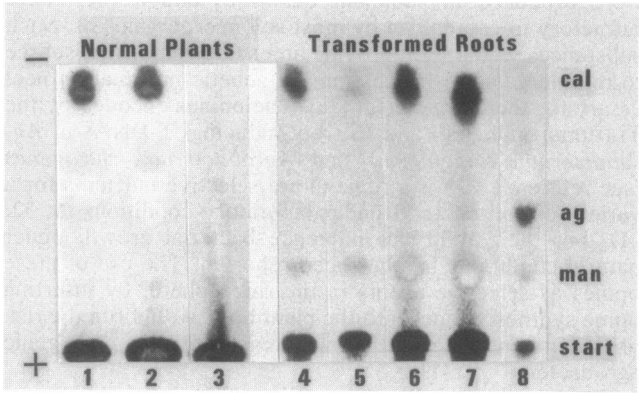

FIG. 1. (Left) Calystegins A and B extracted from roots, rhizomes, and leaves of normal *C. sepium* plants grown in pots. Lanes: 1, 5 mg (fresh weight) of roots of a normal, pot-grown plant; 2, 5 mg (fresh weight) of rhizomes; 3, 5 mg (fresh weight) of leaves. (Right) Calystegins A and B extracted from transformed roots cultured in vitro or accumulated in growth medium after 21 days of culture. Lanes: 4, extract representing 5 mg of transformed roots; 5, 5 μl of spent medium after 21 days of culture; 6, concentrate of 62 μl of spent medium; 7, concentrate of 125 μl of medium; 8, migration standard (extract representing 5 mg of A66 tumor line) (11). Abbreviations: start, point of deposit of samples; man, mannopine; ag, agropine; cal, calystegins A and B. (Roots of normal plants grown in vitro excreted detectable, but less, calystegin than transformed roots in culture [data not shown].)

TABLE 1. Bacteria, plasmids, and phages

Bacterium, plasmid, or phage[a]	Relevant characteristics[b]	Source or reference
Bacteria		
Rhizobium meliloti		
41*, 1322*, 102F51*, A145*, S26*, CC169*, 2011*, V7*, Ls2a*, L5-30*	Various wild isolates from alfalfa nodules	29
AK 631	Nonmucoid derivative of 41	20
GMI13	Derivative of AK631 cured of pRme41a	This work
Rhizobium leguminosarum		
PX31*	Wild isolate from pea nodules	N. Amarger
PBL1*, 248*	Wild isolate from pea nodules	48
Rhizobium trifolii		
RCR0402*, RCR0403*	Wild isolate from clover nodules	Rothamsted, United Kingdom
32R*	Wild isolate from clover nodules	N. Amarger
Rhizobium phaseoli 8002*	Wild isolate from bean nodule	21
Agrobacterium tumefaciens		
T37*	Wild-type nopaline strain	G. Morel
B6-806*	Wild-type octopine strain	J. Tourneur
Bo-542*	Wild-type agropine strain	J. Tempé
C58*	Wild-type nopaline strain	G. Morel
C58C1	C58 derivative cured of pTi	J. Tempé
GMI9050	Str^r Rif^r derivative of C58C1	49
GMI9019	GMI9050(pGMI4142), -(pRme41a::Tn*5*), c-(pGMI59)	16 This work
Agrobacterium rhizogenes		
A4*	Wild-type agropine strain	L. Moore
8196*	Wild-type mannopine strain	J. Lippincott
Azospirillum lipoferum		
4ATr*	Not mobile	J. Balandreau
4B*	Mobile	J. Balandreau
Br17*	Mobile	C. Elmerich
Azospirillum brasiliense CD1*, R.07*, Sp7*, K77*, and KR77*	Rhizospheres of tropical grasses	C. Elmerich
Klebsiella oxytoca 1ABI*	Rhizosphere of rice	J. Balandreau
Pseudomonas paucimobilis 29AJ* and 5AJ*	Rhizosphere of rice	J. Balandreau
Pseudomonas sp. strains CH183*, CH142*, CH418*, CH39*, 203*, and 211*	Octopine-utilizing strains	P. Dion
Beijerinckia sp. strain 5*	Rice rhizosphere	J. Balandreau
Enterobacter cloacae C*	Rice rhizosphere	J. Balandreau
Plasmids		
RP4	IncP1, Tc^r Km^r Ap^r Tra^+	9
pRme41a	Cryptic plasmid from *R. meliloti* 41, Cac^+ Tra^+ 225 kilobases	31
pGMI59	pRme41a derivative, carrying a Tn*5* insertion, Cac^+ Tra^+	31
pGMI35	pRme41a derivative, carrying a Tn*5* insertion which interferes with calystegin catabolism, Tra^+	This work
pGMI4142	RP4 derivative, carrying a fragment of pRme41a cloned in the *Hin*dIII site, Tc^r Ap^r Tra^+	31
pGMI4104	RP4 derivative, carrying a different fragment of pRme41a cloned in the *Hin*dIII site, Tc^r Ap^r Tra^+	P. Boistard (16)
Phages		
pRR1	Specific to bacteria carrying a P-1 plasmid	39
GU5	Specific to bacteria carrying a P-1 plasmid	49
Mu-1	Lysogenic for *E. coli*	4

[a] An asterisk (*) indicates wild isolates tested for calystegin metabolism.
[b] Resistance phenotypes: Str, streptomycin; Rif, rifampin; Tc, tetracycline; Km, kanamycin; Ap, ampicillin; Tra^+, transfer positive.

adventitious root formation on *C. sepium* stems. Such bacterially induced roots contain a portion (T-DNA) of the Ri (root-inducing) plasmid, which confers upon roots the ability to grow rapidly in culture (42, 44, 45). Thus a continuous culture of genetically transformed *C. sepium* roots was established which exhibited reduced apical dominance (frequent branching) and tip elongation rates of 1 to 2 cm per day. Kilogram quantities were produced in liquid medium. Detailed studies of the growth of these cultures are published elsewhere (18). Transformed *C. sepium* roots abundantly liberated calystegins into the medium (Fig. 1). After 3 weeks of culture, a 5-μl portion of culture medium was sufficient to detect these compounds, and on a fresh-weight basis their concentration in the medium was lower, but of the same order of magnitude, as in roots from the same culture (Fig. 1). Thus, a significant portion of the calystegins produced by these roots is released into the medium through excretion or cell death.

TABLE 2. Survey of 105 plant species for presence of calystegins in roots and leaves

Plant sp.	Presence of calystegins[a]
Bryophyta (Sphagnum sp.)	No
Pteridophyta (Polypodium vulgare)	No
Spermaphyta	
Araceae (Arum maculatum)	No
Borraginaceae	
Heliotropium peruvianum	No
Myosotis silvatica	No
Symphytum officinale	No
Buxaceae (Buxus sempervirens)	No
Caryophyllaceae	
Dianthus caryophyllus	No
Silene vulgaris	No
Stellaria media	No
Chenopodiaceae (Beta vulgaris)	No
Compositae	
Bellis perennis	No
Calendula officinalis	No
Chrysanthemum indicum hybridum	No
Chrysanthemum leucanthemum	No
Cichorium intybus	No
Lactuca sativa	No
Matricaria chamomilla	No
Taraxacum officinale	No
Tragopogon porrifolius	No
Convolvulaceae	
Calystegia sepium	Yes
Convolvulus arvensis	Yes
Ipomea tricolor	No
Cruciferae	
Alliaria petiolata	No
Brassica napus var. *oleifera*	No
Brassica oleracea	No
Cheiranthus cheiri	No
Nasturtium officinale	No
Raphanus sativus	No
Cucurbitaceae	
Cucumis melo	No
Cucurbita maxima	No
Cucumis sativus	No
Cyperaceae	
Carex flacca	No
Cyperus papyrus	No
Gramineae	
Agropyrum repens	No
Alopecurus myosuroïdes	No
Avena sativa	No
Dactylis glomerata	No
Festuca arundinacea	No
Hordeum distichum	No
Secale cereale	No
Triticum aestivum	No
Labiatae	
Ajuga reptans	No
Mentha viridis	No
Origanum vulgare	No
Rosmarinus officinalis	No
Salvia pratensis	No
Leguminosae	
Cytisus scoparius	No
Medicago sativa	No
Pisum sativum	No
Onobrychis viciifolia	No
Phaseolus vulgaris	No
Trifolium pratense	No
Ulex nanus	No
Vicia faba	No
Vicia sativa	No
Liliaceae	
Allium sativum	No
Asparagus officinalis	No
Convallaria majalis	No
Hyacinthus orientalis	No
Lilium candidum	No
Tulipa gesneriana	No
Orchidaceae	
Dactylorhiza maculata	No
Listera ovata	No
Ophrys apifera	No
Polygonaceae	
Polygonum lapathifolium	No
Reynoutria japonica	No
Rumex obtusifolius	No
Primulaceae	
Hottonia palustris	No
Primula veris subsp. *veris*	No
Renonculaceae	
Adonis vernalis	No
Anemone coronaria	No
Delphinium ajacis	No
Ranunculus repens	No
Rosaceae	
Crataegus laevigata	No
Fragaria vesca	No
Malus domestica	No
Prunus domestica	No
Pyrus communis	No
Rosa sp.	No
Rubus idaeus	No
Rubus sp.	No
Rubiaceae	
Galium cruciata	No
Rubia tinctorum	No
Scrophulariaceae	
Digitalis purpurea	No
Linaria vulgaris	No
Veronica persica	No
Solanaceae	
Atropa belladonna	Yes
Capsicum annuum	No
Datura innoxia	No
Datura stamonium	No
Lycopersicum esculentum	No
Nicotiana tabacum	No
Petunia hybrida	No
Solanum capsicastrum	No
Solanum melongena	No
Solanum tuberosum	No
Umbelliferae	
Apium dulce	No
Archangelica officinalis	No
Daucus carota	No
Foeniculum vulgare	No
Petroselinum segetum	No
Valerianaceae	
Valeriana officinalis	No
Valerianella olitoria	No

[a] Calystegins, when present, were found primarily in roots.

Transformed *C. sepium* roots also produce mannopine (Fig. 1), an opine characteristic of roots containing Ri T-DNA (28, 43). Mannopine catabolism is characteristic of certain *A. tumefaciens* and *A. rhizogenes* strains (see reference 25 for a discussion of opine specificity). Mannopine coextracted with calystegins A and B (see below and Fig. 1) and served as an internal standard in catabolic tests used to search for soil bacteria able to catabolize calystegins.

Search for soil bacteria which degrade calystegins. Extracts of transformed roots (containing calystegins and mannopine) or extracts of rhizomes excised from normal plants (containing only calystegins) were partially purified prior to catabolic tests. Strains of the genera *Agrobacterium*, *Azospirillum*, *Pseudomonas*, and *Rhizobium* were chosen in the search for soil bacteria able to degrade calystegins because these bacteria are known to associate with plants and because in many cases they have been described genetically (10, 15). A total of 42 strains of rhizosphere bacteria (marked with asterisks in Table 1) were tested for calystegin catabolism. These bacteria were isolated by various researchers from a broad spectrum of higher plant organs and rhizospheres. Examples of the results are given in Fig. 2.

Catabolism of calystegins A and B was positive for only one of the strains examined, *R. meliloti* 41 (Fig. 2, lane 11). We designated this calystegin catabolism phenotype Cac$^+$. An extract of lysed strain 41 from the postincubation culture did not contain calystegins, showing that degradation, not just assimilation, had taken place (data not shown). Strain 41 also degraded the compounds found in underground organs of *Convolvulus arvensis* and *Atropa belladonna*, which have the same electrophoretic properties as calystegins. As expected, mannopine catabolism was positive for *A. tumefaciens* strains carrying octopine-type Ti plasmids and for *A. rhizogenes* strains. It was negative for strains harboring nopaline-type Ti plasmids, thus confirming the reliability of the catabolic tests. Unexpectedly, *R. meliloti* V7 and L5-30 catabolized mannopine (data not shown).

Plasmid-encoded calystegin catabolism. Three plasmids have been detected in *R. meliloti* 41: a cryptic 225-kilobase plasmid (pRme41a) and two megaplasmids of more than 1,200 kilobases (2, 3, 29). For practical reasons a compact-colony, nonmucous variant (AK631) of *R. meliloti* 41 was used in the following experiments (20). After tagging the cryptic pRme41a plasmid by marker exchange with transposon Tn*5*, we isolated an *R. meliloti* AK631 derivative cured of pRme41a (Fig. 3). This strain (GMI13) was Cac$^-$, indicating that pRme41a carries the *cac* gene(s) (Fig. 3). pRme41a::Tn*5* (referred to as pGMI59) was observed to be self-transmissible at a low frequency (about 10^{-7}) to *R. meliloti* L5-30 and GMI13 (16). Reintroduction of the plasmid into the cured strain GMI13 restored the Cac$^+$ phenotype (data not shown). Self-transfer into *A. tumefaciens* was not detected. We therefore used a derivative (pGMI4142) of the broad-host-range plasmid RP4 to mobilize pGMI59 into GMI9050, a Str^r Rif^r derivative of *A. tumefaciens* C58C1, a recipient strain cured of its Ti plasmid (Fig. 3). Whereas the control GMI9050(pGMI4142) was Cac$^-$, the GMI9050 (pGMI59, pGMI4142) transconjugants catabolized calystegins A and B, showing that the gene(s) responsible for calystegin catabolism (*cac*) is located on pRme41a (Fig. 3). pRme41a is incompatible with pAtC58, a 410-kb cryptic plasmid of GMI9050. GMI9050(pGMI59) transconjugants are thus cured of pAtC58 (31). The curing of pAtC58 does not render GMI9050(pGMI59) Cac$^+$, since GMI9050 cured of pAtC58 is Cac$^-$ (data not shown).

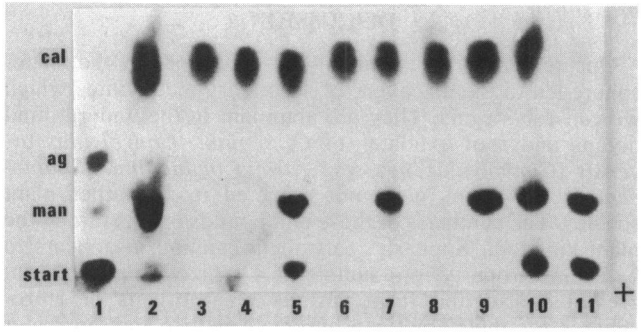

FIG. 2. Catabolic specificity of rhizosphere bacteria toward calystegins. Lanes: 1, standard extract from A66 tumor line (11); 2, control without bacteria; 3, *A. rhizogenes* A4; 4, *A. rhizogenes* 8196; 5, *A. tumefaciens* C58; 6, *A. tumefaciens* Bo542; 7, *A. tumefaciens* C58C1; 8, *A. tumefaciens* B6-806; 9, *A. tumefaciens* T37; 10, *R. meliloti* L5-30; 11, *R. meliloti* 41. Abbreviations: start, point of deposit of samples; man, mannopine; ag, agropine; cal, calystegins A and B.

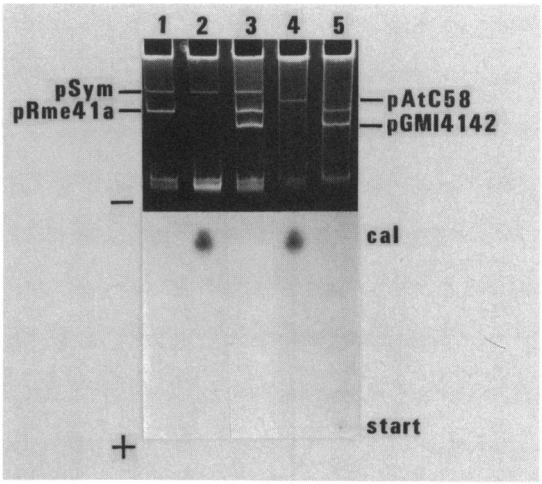

FIG. 3. (Top) Plasmid profiles of bacterial strains used to show that the Cac$^+$ phenotype correlates with the presence of pRme41a. Lanes: 1, *R. meliloti* AK631 carrying pRme41a, as well as a megaplasmid; 2, GMI13, a derivative of strain AK631, cured of pRme41a; 3, GMI17, strain AK631(pGMI59) containing pGMI4142, an RP4 derivative used to mobilize pRme41a; 4, GMI9050, an *A. tumefaciens* cured of the Ti plasmid, but still harboring pAtC58, a cryptic plasmid; 5, GMI9019, GMI9050 into which pGMI4142 and pGMI59 have been introduced, causing the loss of pAtC58. (Bottom) Catabolic tests using calystegin extracted from rhizomes of normal plants, showing that Cac functions are encoded in pRme41a, using the same strains as in the top panel (se Fig. 2 for methods). Catabolic tests using calystegin from transformed roots gave identical results (e.g., only strains containing pRme41a were Cac$^+$ [data not shown]). Abbreviations: start, point of deposit of samples; cal, calystegins A and B.

Differential growth. Cac$^-$ and Cac$^+$ *R. meliloti* 41 derivatives (GMI13 and AK631) were tested for their ability to grow in a medium containing calystegin extracts and no other source of carbon and nitrogen. Extracts used in growth experiments were prepared as described in Materials and Methods and then further purified by gel filtration and preparative paper electrophoresis. Growth of Cac$^+$ bacteria was stimulated (relative to the Cac$^-$ controls) as a function of the calystegin concentration in the medium (Fig. 4A). This

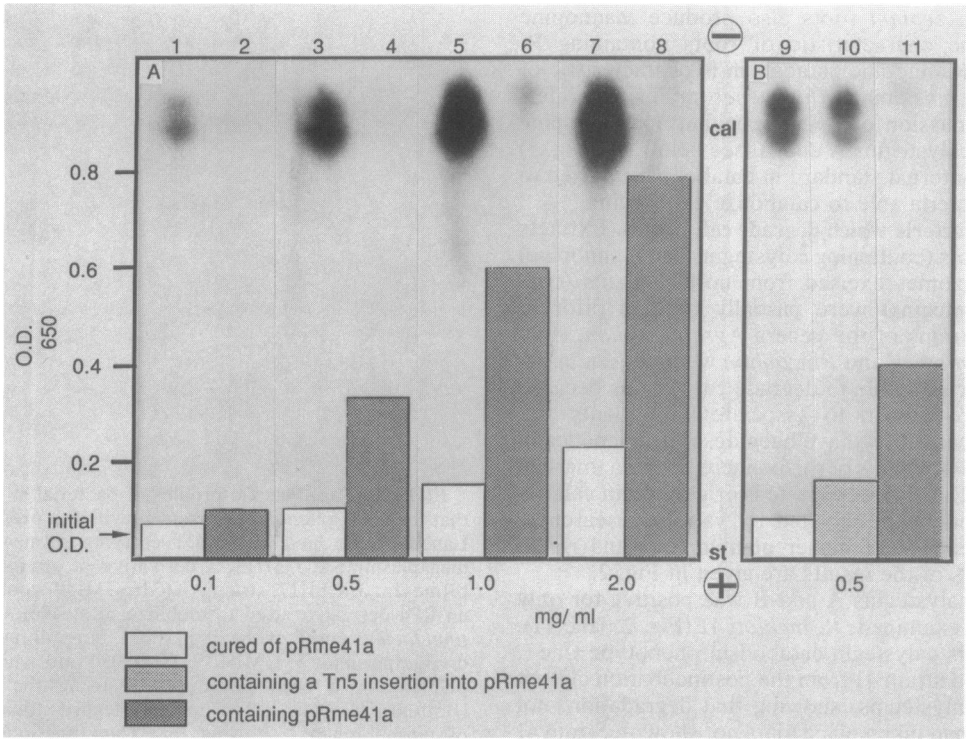

FIG. 4. (A) Calystegin-dependent growth. *R. meliloti* wild-type and the same bacterium cured of pRme41a (strain GMI13) were grown in SM medium with various amounts of calystegins A and B as the sole carbon and nitrogen source. Growth was determined by monitoring the optical density at 650 nm, and calystegin degradation was assayed by high-voltage paper electrophoresis and silver staining of the supernatant after 3 days of culture (see Fig. 2 for methods). The growth histogram is superimposed on the electrophoretogram. (Calystegins A and B were partially purified as described in Materials and Methods, followed by further purification using gel filtration on Trisacryl GF05 and preparative high-voltage paper electrophoresis.) Abbreviations: st, point of deposit of samples; cal, calystegins A and B. (B) A repeat of the 0.5-mg/ml calystegin concentration experiment shown in panel A, with the addition of a strain (GMI14, lightly shaded bars) containing a Tn*5* insertion into pRme41a (pGMI35) that interferes with calystegin catabolism and calystegin-dependent growth.

differential growth was accompanied by the removal of calystegins from the medium by Cac^+ bacteria (Fig. 4A). We conclude that calystegins are selective agents for Cac^+ bacteria under these conditions. This conclusion is supported by the observation (Fig. 4B) that a single Tn*5* insertion into pRme41a interferes with calystegin catabolism and calystegin-dependent growth. Detailed studies of the *cac* gene(s) on pRme41a reveal complexity both of organization and function (C. Boivin, C. Rosenberg, and J. Dénarié, manuscript in preparation).

Search for a relationship between calystegins, pRme41a, and symbiotic nitrogen fixation. The symbiotic properties of *R. meliloti* AK631 (Cac^+) and GMI13 (Cac^-) were studied on various *R. meliloti* hosts: *Medicago sativa* var. Gemini, *M. lupulina*, *M. rugosa*, *M. truncatula*, *M. tornata*, *M. littoralis*, *Melilotus alba*, and *Trigonella foenum-graecum*. No significant difference in nodulation ability or nitrogenase activity was detected (data not shown). Thus pRme41a does not carry unique genes (not reiterated elsewhere in the genome) essential for nodulation and symbiotic nitrogen fixation. Roots and nodules from these plants inoculated with AK631 and GMI13, as well as from uninoculated plants, were screened for calystegins (as were similar treatments of *M. sativa* var. Magali, *M. aculeata*, *M. scutellata*, *M. laciniata*, and *M. arabica*). All results were negative.

Although *C. sepium* is not a known host for *R. meliloti*, we examined the possibility of a symbiotic nitrogen fixation relationship between the two organisms by inoculating pot-grown plants in the greenhouse under conditions of nitrogen starvation. Although *R. meliloti* 41 induced nodules on *M. sativa* var. Gemini and compensated for nitrogen starvation, no beneficial effect was observed in parallel inoculations on *C. sepium* (data not shown). Inoculations in vitro of *C. sepium* with *R. meliloti* 41 did not lead to detectable acetylene reduction (data not shown). These experiments do not, however, rule out other relationships between *R. meliloti* 41 and *C. sepium*.

DISCUSSION

Our search for plant metabolic mediators in the rhizosphere led to the discovery of secondary metabolites which we call calystegins. They are abundant in the underground organs and root exudates of *C. sepium*, *Convolvulus arvensis* (*Convolvulaceae*), and *Atropa belladonna* (*Solanaceae*). Calystegins were not detected in 102 other plant species. The synthesis of these compounds is thus rare in the plant kingdom. Similarly, calystegin catabolism appears to be a rare property in a sample of 42 rhizosphere bacteria, previously isolated from various plant organs or rhizospheres for their ability to associate with plants and alter their growth: calystegin catabolism was detected in only one of these strains (*R. meliloti* 41) and was encoded in the plasmid pRme41a. Furthermore, calystegins provide a carbon and nitrogen source to *R. meliloti* 41, but not to a derivative cured of pRme41a. The rarity of the calystegin synthesis functions (Cas) and catabolic functions (Cac), the abundance of calystegins in underground plant organs, and

the ability of calystegins to selectively stimulate the growth of Cac$^+$ bacteria all suggest that calystegins play a role in rhizosphere ecology through nutritional selection.

Diverse bacteria harbor plasmids that confer the catabolism of exotic substances in their environment (see reference 51 for a review) and are thus considered to have ecological significance. These plasmids are not essential for growth but do confer a selective advantage when exotic nutrient sources (e.g., toluene) or deleterious substances (e.g., antibiotics) are present. Plasmid-encoded catabolic functions in the *Rhizobiaceae* strains have also been assigned possible ecological roles. The Ti and Ri *Agrobacterium* plasmids confer the ability to catabolize opines produced by plant cells and organs genetically transformed by a segment of one of these plasmids (for a review, see reference 25). This catabolic function, coupled with genetic transformation, has been termed "genetic colonization" (36). Similarly, *R. meliloti* L5-30 contains on its pSym plasmid a region responsible for the catabolism of a substance, called rhizopine, present in the alfalfa root nodules elicited by this strain (24). It is not known whether rhizopine is synthesized by the bacterial partner or in plant cytoplasm, or whether its catabolism (or that of the opines) confers any selective advantage under natural conditions.

Whatever the natural role of calystegins, their specific catabolism by *R. meliloti* 41 appears to be independent of nitrogen-fixing symbiosis. *R. meliloti* 41 cured of pRme41a engages in normal symbiosis with *Medicago* hosts. Furthermore, we were unable to detect calystegins in root or nodule extracts of 12 *Medicago* hosts, and only one of the eight *R. meliloti* strains tested was Cac$^+$ (Fig. 2). Thus if calystegins and *cac* genes are involved in rhizosphere ecology, they probably do not directly act on symbiotic nitrogen fixation. We would point out that *Rhizobium* is a common soil bacterium found in the rhizospheres of plants other than legumes (7, 50). pRme41a could be implicated in commensalistic, rather than symbiotic, associations with plants, enabling Cac$^+$ bacteria to catabolize calysteginlike compounds in the rhizospheres of plants liberating such molecules.

The presence of large plasmids of unknown functions is a general feature in members of the family *Rhizobiaceae* (14, 16, 17, 29). Some of these plasmids are not essential for tumor or nodule induction (16, 17, 31). pRme41a is one of the best characterized of these cryptic plasmids. It contains extensive homology to other cryptic plasmids of *R. meliloti* (pRmeL5-30, pRme102F51, and pRme1322), and it belongs to the same incompatibility group as pATC58, a large *A. tumefaciens* plasmid not necessary for tumor induction (31). pRme41 also bears homology to Ti plasmids (16), including regions encoding transfer functions and origins of replication. pRme41a is self-transmissible to other *R. meliloti* strains (16). It would seem consistent that catabolic functions with ecological significance would be encoded in self-transmissible plasmids. We have now assigned a catabolic function to pRme41 and propose that other "cryptic" *Rhizobiaceae* plasmids could carry catabolic genes implicated in the saprophitic (ex planta) existence of the bacterium. Other large, non-pSym, self-transmissible *Rhizobium* plasmids carry genes responsible for the synthesis of bacteriocins, compounds probably important in interstrain competition (14, 17, 48). It is perhaps not coincidental that bacterial functions known to play a role in plant infection and to modify plant growth, creating an in planta ecological niche for the bacterium (functions governing nodule formation), are encoded on a different replicon, pSym.

Small molecules synthesized by plants are thought to be involved in the control of plant-bacterium relationships. Phenols, such as acetosyringone, are released from wounded tissues and induce transcription of *A. tumefaciens* virulence (*vir*) genes (38). These molecules also serve as chemoattractants to agrobacteria (1). Flavonoids from legume root exudates induce transcription of *nod* genes of rhizobia (12, 27). These compounds would appear to inform the bacterium of the proximity of an appropriate host. They act at low concentrations (10 nM to 1 μM). In contrast, calystegins, like the opines, would act through nutritional selection, thus at much higher concentrations than the signal molecules described above.

The question of the natural role of calystegins is being addressed by testing soil samples, obtained from the rhizospheres of plants which do or do not synthesize calystegins, for the presence of Cac$^+$ bacteria. These tests show that a large percentage of the bacteria isolated from the former rhizospheres are Cac$^+$, whereas no Cac$^+$ bacteria could be found in the latter rhizospheres (V. Fleurie, B. Message, and D. Tepfer, unpublished results). Studies of the chemical structures of calystegins have been completed (A. Goldmann, J. Y. Lallemand, C. Decoins, M. Maille, E. Charpin, and A. Lepingle, manuscript in preparation), indicating that they are novel, low-molecular-weight, nitrogen-containing, bicyclic compounds that may be amenable to chemical synthesis.

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