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Catabolism of the Plant Secondary Metabolites Calystegins and Trigonelline by *Rhizobium Meliloti**

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Abstract

We look for plant secondary metabolites able to play a nutritional role for the soil bacterium *Rhizobium meliloti*. Genes responsible for catabolism of a family of novel compounds, calystegins, were localized on a cryptic plasmid of *R. meliloti*. Calystegins do not seem to be involved in the symbiotic relationship with legumes, but they could provide a rather specific nutrient source for *R. meliloti* in the rhizosphere of non-host plants. Genes responsible for the catabolism of trigonelline, a betaine abundant in legumes, have been mapped on the symbiotic plasmid pSym, between two sets of symbiotic genes. This could suggest a potential role for these genes in symbiosis.

Introduction

Rhizobia are soil bacteria able to induce the formation of nodules on the roots of leguminous hosts, in which they fix nitrogen. Many tools have been developed to analyse the genetic basis of these symbiotic properties, and *Rhizobium* is one of the best characterized plant-associated bacteria. However, rather little is known about its free-living state in the soil, preceeding or following the infection of its lguminous host. It has been reported that the growth of *Rhizobium* populations is strongly stimulated in the rhizospheres of legumes, and sometimes in the rhizospheres of non-leguminous plants (Nutman, 1965). The cause of this stimulation is thought to depend in some

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way on the nutrients present in root exudates. These nutrients can be ubiquitous, like products of intermediary metabolism, or else specific to a limited number of plant species, like secondary metabolites. While there is probably an intense competition between microorganisms of the rhizosphere for the catabolism of the former nutrients, we suggest that the latter are not catabolized by the majority of soil microorganisms. According to this hypothesis, it would seem logical that if such a secondary metabolite were present in plant root exudates, bacteria which have evolved the corresponding catabolic functions would have a selective advantage in the rhizosphere of this plant. We looked for plant secondary metabolites which were likely to play this role of specific nutritional mediators for the genetically well-characterized soil bacterium *R. meliloti*.

In this paper, we describe the catabolism by *R. meliloti* of two types of plant secondary metabolites: A family of novel compounds called calystegins, and trigonelline, a betaine particularly abundant in legumes.

Material and Methods

Extraction and visualisation of calystegins

Crude extracts of *Calystegia sepium* were submitted to high voltage electrophoresis, and stained with silver nitrate as previously described (Tepfer et al., 1988a). Prior to catabolic tests, calystegin extracts were biologically enriched by 40 h of incubation in the presence of a log-phase culture of *Agrobacterium tumefaciens* T37, as has been described for the enrichment of opine preparations (Guyon et al., 1980). Catabolic tests were performed according to Tepfer et al., (1988a).

Microbiological techniques

The conditions used for bacterial growth and conjugation experiments (Truchet et al., 1984), and the details of genetic manipulations (Rosenberg and Huguet, 1984) were as described previously. Trigonelline used as the sole carbon and nitrogen source was added at 0.5 g/l to the minimal medium of Vincent (1970) which was deficient in carbon and nitrogen. Transfer and curing of pRme41a has been previously described (Rosenberg and Huguet, 1984). Plasmid pRme41a purified DNA was prepared as described by Jouanin et al., (1981). Transposon mutagenesis of the *cac* and *trc* regions was performed as described by Debellé et al., (1986). The transposable elements used for this mutagenesis were Tn5, miniMudIIlac (Castilho et al., 1984), and a derivative of Tn5 able to generate transcriptional fusions (Keller et al., 1988).

Plant cultures

The symbiotic properties of *R. meliloti* were determined *in vitro* by inoculating legumes grown on nitrogen-free agar slants as described elsewhere (Truchet et al., 1984).

Results

(*A*) *Calystegins*

Discovery

We screened crude extracts of roots and leaves from about one hundred plant species for energy-rich secondary metabolites which were present in sufficient quantity to make them possible nutritional mediators. Such compounds have been found in underground organs and root exudates of two members of the *Convolvulaceae*, *Calystegia sepium* and *Convolvulus arvense*, and one Solanaceous plant, *Atropa belladona* (Tepfer et al., 1988). The structure of the two major compounds, which we call calystegins A and B, has been determined (Tepfer et al., 1988b; Goldmann et al., submitted for publication). They are small N-heterocyclic molecules with a tropane-like backbone. Formulas are given in Table 1.

Table 1. Molecular weights and formulas for three calystegins

Compound	MW	Formula
A_3	159	$C_7H_{13}NO_3$
B_1	175	$C_7H_{13}NO_4$
B_2	175	$C_7H_{13}NO_4$

Catabolism of calystegins by Rhizobium meliloti

Various bacteria previously known to associate with plants (*Rhizobium*, *Bradyrhizobium*, *Agrobacterium*, *Azospirillum*, and *Pseudomonas*) were screened for their ability to degrade calystegins. Out of the 44 strains tested, only *R. meliloti* strain 41 was able to catabolize calystegins (Cac^+ phenotype), which suggests that calystegin catabolism could be restricted to a limited number of soil microorganisms. *R. meliloti* 41 can grow on a minimal medium supplemented with calystegins as the sole source of carbon and nitrogen.

The genetic basis of this catabolism was studied. The *R. meliloti* 41 genome is composed, in addition to the chromosome, of 3 different plasmids (Rosenberg et al., 1981): the symbiotic plasmid pSym, another megaplasmid of very high molecular weight, and a self-transmissible cryptic plasmid of 225 kb, called pRme41a. We showed that a strain cured of this latter plasmid is no longer able to catabolize calystegins. Furthermore, transfer of pRme41a to this cured strain, or to a strain of *Agrobacterium tumefaciens*, confers the ability to catabolize calystegins to these recipient strains. This result showed that the *cac* genes are located on this cryptic plasmid pRme41a. Restriction fragments resulting from a partial digest of this plasmid were cloned into the broad host-range vector pRK290 (Ditta et al., 1980), and

mutagenized. Insertional and deletional mutations were obtained, which resulted in three different phenotypes: no degradation of calystegins (Cac-), degradation delayed (Cac^d), or degradation accompanied by the accumulation of a neutral compound (Nca) likely to result from a block in the catabolic pathway. Insertions conferring this Nca phenotype were mapped within a 10 kb region, while the whole set of *cac* genes is spread over a region of 30 to 40 kb (see Fig. 1).

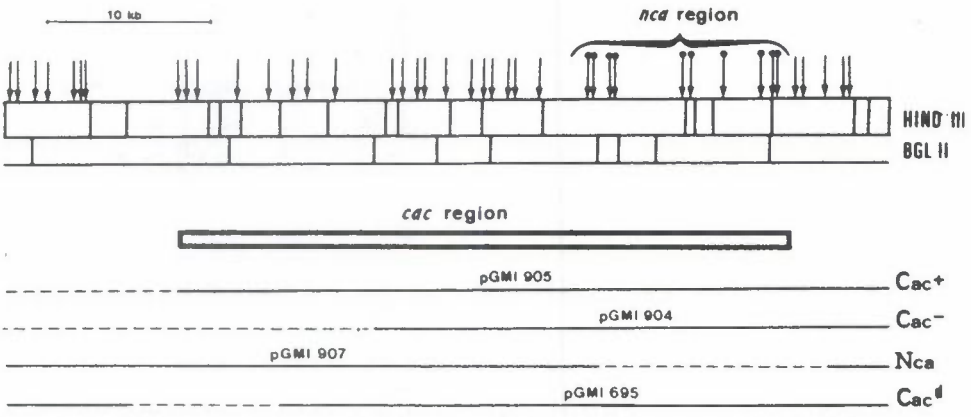


Figure 1. Insertions and deletions into the *cac* region of pRme41a that alter calystegin catabolism. Deleted regions in plasmids pGM1905, pGM1904 and pGM1695 are represented by interrupted lines. The phenotypes of strains carrying the various plasmids are indicated on the right. Arrows represent Tn5 insertions: simple arrow = Cac^+; arrow beginning with a dot = Nca (Neutral compound accumulation).

Ecological significance of calystegin catabolism

The *cac* genes do not seem to be involved in the control of symbiosis, since we were not able to detect calystegins in any of *R. meliloti* leguminous hosts tested (Data not shown). Furthermore, the symbiotic properties of the strain cured of pRme41a are not altered, at least not under the conditions of our experiment, i.e. inoculation of aseptic seedlings cultivated *in vitro*. Thus, it seems more likely that *cac* genes are involved in the survival of the strain during its saprophytic life in the rhizosphere of a non-host plant.

According to our working hypothesis, if calystegins are specific nutritional mediators, Cac^+ bacteria should exhibit a selective advantage in the rhizosphere of calystegin-producing plants. To try to answer this question, naturally occuring wild-type bacteria originating from the rhizospheres of plants which do or do not synthesize calystegins were screened for their ability to degrade calystegins. We found that about 25% of the bacteria isolated from the former rhizosphere were Cac^+, whereas no Cac^+ bacteria could be detected in the latter.

(B) Trigonelline

In our search for secondary metabolites involved in plant-bacteria interactions, our attention was focused on a compound which seems to exhibit some of the characteristics of a nutritional mediator: Trigonelline is a betaine, derived from nicotinic acid by addition of a methyl group to the heterocyclic nitrogen (Fig. 2). This compound is present in a number of plants, but is particularly abundant in legumes (Tramontano et al., 1986).

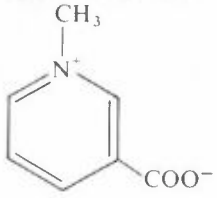
Compound	MW	Structure
Choline	121	$(CH_3)_3N^+CH_2CH_2OH$
Glycine betaine	134	$(CH_3)_3N^+CH_2COO^-$
Trigonelline	137	

Figure 2. Molecular weights and structures for three betaines

Most of the *Rhizobiaceae* tested were able to catabolize trigonelline. We showed that various *R. meliloti* strains were able to grow on a minimal medium with trigonelline as the sole source of carbon and nitrogen. Using a catabolic test, we showed that trigonelline was removed from the medium, concommitant with the growth of the culture.

In order to determine the location of the *trc* genes, which control trigonelline catabolism, we tested derivatives of *R. meliloti* RCR2011, carrying large deletions of the pSym. One of them, GMI766, for which a region of about 400 kb (including several clusters of symbiotic genes) had been deleted, was unable to catabolize trigonelline (Trc^-). Introduction into this strain of pGMI471, a plasmid carrying a 30 kb insert of pSym, restored the Trc^+ phenotype. To determine the precise location of the *trc* genes on this fragment, transposon mutagenesis was performed using a Tn5 derivative able to generate transcriptional fusions. We thus defined a 9 kb *trc* region, surrounded by two clusters of symbiotic genes: *nifAB* and the *fix*VI′ cluster described by Renalier et al. (1987).

The mutants affected in trigonelline catabolism fell into two classes according to their phenotype and their location: Mutants of class I were unable to grow on trigonelline as the sole carbon and nitrogen source, whereas mutants of class II showed only a delay in growth. Since the *trc* region is located on the pSym plasmid and surrounded by sets of symbiotic genes, we are presently investigating a possible modification of the symbiotic properties of the Trc^- mutants on various hosts.

Two other betaines, choline and glycine betaine (see Fig. 2) are also catabolized by *R. meliloti* (Le Rudulier and Bernard, 1986). pSym is probably implicated in their catabolism, since GMI766 cannot use them as carbon or nitrogen sources. The plasmid-controlled catabolism of betaines is not a general feature of the *Rhizobiaceae*, since an *Agrobacterium* strain cured of detectable plasmids (Rosenberg and Huguet, 1984) was still able to catabolize trigonelline.

Le Rudulier and Bernard (1986) have reported that choline and glycine betaine are potent osmoprotectants in *R. meliloti*. The osmoprotective effect of trigonelline is much weaker (Bernard et al., 1986). We confirmed this result in the case of *R. meliloti* 2011: The doubling time of a culture in the vincent minimal medium was 5 h 20′. In the same medium containing 0.5M of NaCl, the doubling time increased to 16 h. If trigonelline was added to this culture, the doubling time became 9 h 40′. This partial restoration reflects a weak osmoprotective effect, which is possibly limited by the toxic effect observed when trigonelline is present in the medium at concentrations over 1g/l.

Discussion

Calystegins and trigonelline belong to two different categories of plant secondary metabolites, which are both catabolized by *R. meliloti*. While trigonelline catabolism is very common among *Rhizobiaceae*, calystegin catabolism seems to be rare in soil bacteria, making calystegins good candidates for specific nutritional mediators. Competition experiments between Cac^+ and Cac^- isogenic strains should allow us to determine whether the presence of *cac* genes can actually confer a selective advantage to bacteria in the rhizosphere of calystegin producing-plants.

Calystegin catabolism does not seem to be related to symbiosis since: (i) no calystegin can be detected in *R. meliloti* leguminous hosts; and (ii) a strain cured of the pRme41a plasmid shows normal symbiotic properties. A possible role for these catabolic functions could be to enhance the survival of *Rhizobium* populations during their saprophytic life, in the absence of the leguminous host. As the presence of large cryptic plasmids is a general feature of *Rhizobiaceae* (Rosenberg et al., 1981), we suggest that some of these plasmids could be involved in the catabolism of plant exudates.

In contrast, trigonelline catabolism could be important to the symbiotic life of *Rhizobium*. Trigonelline is present in various hosts of *R. meliloti*, and moreover, genes controlling its degradation are located on the pSym plasmid between two sets of symbiotic genes.

It is noteworthy that *R. meliloti* genes controlling the catabolism of compounds present in non-host plants (calystegins) are located on a plasmid not involved in the symbiosis, while genes controlling the catabolism of a compound abundant in symbiotic hosts (trigonelline) are located on the pSym plasmid, closely linked to symbiotic genes.

In addition to a potential nutritional effect, several properties of trigonelline have been reported which could interfere with symbiosis.

Trigonelline is considered a plant hormone (Evans and Tramontano, 1981); it can induce *R. meliloti nod* gene expression (Schmidt et al., 1986); and it is a weak osmoprotectant.

We are currently examining the potential role of trigonelline catabolism in the colonization of the rhizosphere of legumes, nodulation, and nitrogen fixation.

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