Attenuation of Symbiotic Effectiveness by *Rhizobium meliloti* SAF22 Related to the Presence of a Cryptic Plasmid

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Several wild-type strains of *Rhizobium meliloti* isolated from alfalfa nodules exhibited different plasmid profiles, yet did not differ in growth rate in yeast-mannitol medium, utilization of 43 different carbon sources, intrinsic resistance to 14 antibiotics, or detection of 16 enzyme activities. In contrast, three measures of effectiveness in symbiotic nitrogen fixation with alfalfa (shoot length, dry weight, and nitrogen content) indicated that *R. meliloti* SAF22, whose plasmid profile differs from those of the other strains tested, is significantly less effective than other wild-type strains in symbiotic nitrogen fixation. Light microscopy of nodules infected with strain SAF22 showed an abnormal center of nitrogen fixation zone III, with bacteria occupying a smaller portion of the infected host cells and vacuoles occupying a significantly larger portion of adjacent uninfected host cells. In contrast, the effective nodules infected with other wild types or plasmid pRmSAF22c-cured segregants of SAF22 did not display this cytological abnormality.

In many wild-type strains of rhizobia, a large portion of the genome resides on plasmids. In *Rhizobium meliloti*, the genes coding for nodule formation and nitrogen fixation and certain steps in exopolysaccharide synthesis are located in symbiotic megaplasmids designated pSym. Together with pSym coexist other cryptic plasmids which vary in number and have molecular weights ranging from $3 \times 10^6$ to $200 \times 10^6$ (3, 7, 18). Little is known about the functions of these plasmids in *Rhizobium* spp., although their stability (31) suggests that they are important. Some investigators have established a relationship between certain cryptic plasmids and exopolysaccharide production (2, 17), melain production (11), utilization of certain carbon sources (1, 26), and bacterial growth (8, 10, 27). Other attempts have been made to link the occurrence of cryptic plasmids and certain symbiotic characteristics such as competitiveness (6, 18), infectivity (23), and effectiveness (2, 14, 16, 18, 24, 27). Whereas some studies indicate a positive involvement of certain cryptic plasmids in both saprophytic and symbiotic activities of *Rhizobium* spp., others suggest a negative involvement, and in most cases such involvement is not even found. For *R. meliloti* GR4, it was found that one of the cryptic plasmids (pRmeGR4b) has a positive effect on nodulation capacity (28), and a region of approximately 5 kb has been characterized and named nfe (nodule formation efficiency) (23). Recent studies have shown that copies of symbiotic genes are present in certain cryptic plasmids (4, 14, 20, 23), which could account for their positive role in symbiotic functions.

The aim of the present work was to investigate the involvement of cryptic plasmids of *R. meliloti* SAF22 in selected phenotypic characteristics expressed in vitro and in root nodule symbiosis with its usual host, alfalfa. (Part of this work was presented at the 12th International Congress on Nitrogen Fixation [29]).

Several wild-type strains of *R. meliloti* were isolated by standard methods (30) from effective nodules of alfalfa cultivated at different locations in the province of Salamanca, Spain. Their plasmid profiles were analyzed by vertical (15) and horizontal (19) electrophoresis, using 0.7% agarose gels, and calibrated with the reference strain *R. meliloti* GR4, which harbors two cryptic plasmids of 140 and 114 MDa (28). Wild-type strains of *R. meliloti* had pSym plasmids with a molecular weight above $1,400 \times 10^6$ and exhibited three different plasmid profiles distinguished by the presence or absence of various smaller sized cryptic plasmids (Fig. 1A). Strain SAF11 lacked cryptic plasmids (profile 3), strain SAN02 had a cryptic plasmid of 140 MDa (pRmeSAN02a) (profile 2), and strain SAF22 harbored three cryptic plasmids of 100 (pRmeSAF22a), 114 (pRmeSAF22b), and 140 (pRmeSAF22c) MDa (profile 1). The nomenclature system of Casse et al. (9) was used for naming the plasmids.

Two types of plasmid-cured segregants of strain SAF22 were obtained by growth on TY medium (5) at 40 and 42°C for 3 to 11 days (32) (Fig. 1B). One segregant, represented by strain SAF547, still harbored pSym and pRmeSAF22a but was cured of both pRmeSAF22c and pRmeSAF22b. Another segregant (strain SAF5118) was cured of only pRmeSAF22c. Heat-cured segregants of pRmeSAF22a were not obtained.

The effects of alterations in these cryptic plasmids on various physiological traits were examined in vitro. For growth studies in YMB (30), inocula were grown for 30 h and transferred in 0.5-ml aliquots to 250-ml flask cultures shaken at 150 rpm. Cultures were monitored for growth by measuring the optical density of 1-ml aliquots at 650 nm and by viable plate counts on YMA (30). For studies on utilization of various carbon sources (glucose, galactose, fructose, mannose, L-arabinose, ribose, D-xyllose, L-rihamnose, maltose, sucrose, cellulose, lactose, gentiobiose, trehalose, melezitose, raffinose, turanose, esculin, N-acetylglucosamine, β-methylxylloside, glycerol, adonitol, mannitol, inositol, sorbitol, D- and L-arabitol, D-xyllose, D-sorbitose, D-lucose, D- and L-fucose, methyl-α-D-glucoside, mannoside, amygdalin, salicin, inulin, xylitol, gluconate, glyceron, 2-keto-gluconate, and 5-keto-gluconate), bacterial suspensions were prepared in carbon-free Robertsen medium (22), inoculated in API 50 strips (BioMérieux, Marcy
The strains used in this study, including SAF22 and its segregants SAF5118 and SAF547, did not differ in growth rate in YMB, in utilization of 43 different carbon sources, in intrinsic resistance to 14 antibiotics, or in activities of the various enzymes tested.

Next, the symbiotic phenotypes of these strains were examined. Surface-sterilized seeds of Medicago sativa cv. Aragón were germinated into humid air in the dark. For studies of nodulation kinetics, seedlings of uniform size were transferred to agar slopes (one plant per tube) containing 10 ml of the nitrogen-free medium of Rigaud and Puppo (21). Plants were grown in a chamber with mixed incandescent and fluorescent illumination (400 microeinstein m\(^{-2}\) s\(^{-1}\); 400 to 700 nm) programmed for a 16-h photoperiod, 25:17°C day-night cycle, and 50 to 60% relative humidity. After 2 days, plants (10 per strain) were each inoculated with 1 ml of a standardized bacterial suspension containing 10\(^6\) cells. Root nodules were counted every 48 h from the start of their appearance over 10 consecutive days. All strains were similar in ability to induce root nodules, indicating that curing of pRmeSAF22e alone or in combination with pRmeSAF22b does not affect the ability of wild-type strain SAF22 to nodulate this host.

For studies of symbiotic effectiveness, plants were grown in Leonard jars containing sterile vermiculite as a support and irrigated with Rigaud and Puppo (21) medium adjusted to pH 7. Each strain was inoculated into five jars, each containing 10 plants. After 40 days of incubation in the growth chamber, plants were uprooted and analyzed for the number of nodules on roots and for shoot length, dry weight, and nitrogen content according to Association of Official Analytical Chemists methods (15). A statistical study was applied to the data obtained, using Student’s t test. The quantitative evaluation of symbiotic phenotypes for R. meliloti strains on alfalfa is presented in Table 1. Wild-type strains SAP11 and SAN02 were equally effective in symbiotic nitrogen fixation, as measured by the increase in lengths, dry weights, and nitrogen contents of inoculated plants. In contrast, wild-type strain SAF22 was significantly less effective in symbiotic nitrogen fixation as measured by each of these parameters. By comparison, segregant SAF5118, heat cured of plasmid pRmeSAF22c, and segregant SAF547, heat cured of plasmid pRmeSAF22c and pRmeSAF22b, were significantly more effective in symbiotic nitrogen fixation than the parent strain SAF22. The symbiotic effectiveness of these plasmid-cured segregants was similar to that of the other two wild-type strains, SAP11 and SAN02. The symbiotic studies performed with the alfalfa host indicated that curing of cryptic plasmid pRmeSAF22c results in a restoration of nitrogen-fixing nodules to a fully effective symbiosis.

The morphological features of alfalfa nodules induced by these strains were examined by microscopy. Ten nodules (typ-
FIG. 2. Light micrographs of cross sections through the center of alfalfa nodules infected with different strains of *R. meliloti*: (A and B) SAP11; (C and D) SAF22; (E and F) SAF547. Bar, 20 μm.
Plasmids in effect of inoculant strain SAF22 on other host genotypes. ProSAF22 and establish detrimental, neutral, or even beneficial for the nitro-
gen fixation zone III was normal in alfalfa nodules infected with
strain SAF22 (not shown). Interestingly, none of these abnormalities were found in nodules infected with the plasmid-cured segregant SAF547 (Fig. 2E and F) or SAF5118 (not shown).

These data indicate that R. meliloti wild-type strain SAF22 has the genetic capability to develop fully effective root nodules on alfalfa, but this phenotype is attenuated by its cryptic plasmid pRmSAF22c, which interferes with the normal nodulation development necessary for sustaining a fully effective nitrogen-fixing symbiosis. Due to the presence of pRmSAF22c, strain SAF22 is not fully genetically compatible with M. sativa cv. Aragón, and elimination of this cryptic plasmid improves the symbiotic performance of this inoculant strain. The curing of a cryptic plasmid leading to enhanced effectiveness in nitrogen-fixing ability has been previously found in certain strains of R. loti (18). Now we are testing whether cryptic plasmid pRmeSAF22c is detrimental, neutral, or even beneficial for the effectiveness of inoculant strain SAF22 on other host genotypes. This information should help to explain the divergent results in the literature on the influence of certain cryptic plasmids in Rhizobium spp. on the development of a nitrogen-fixing symbiosis with legumes.

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