Production and Selection of *Spiroplasma citri* Mutants Inducing No Symptoms in Plants or Non-Transmissible to Plants by the Leafhopper Vector *Circulifer haematoceps*

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ABSTRACT. Mutants of *Spiroplasma citri* have been produced by random insertion of transposon Tn 4001 in the spiroplasmal genome. Two hundred fifty-seven mutants have been screened for their ability to be transmitted to young periwinkle plants by the leafhopper vector, *Circulifer haematoceps*, and for their ability to produce symptoms in the periwinkle plants. Two mutants were of particular interest. One mutant did not grow in the leafhoppers and was not transmitted by these insects to plants. The second mutant grew in the insects to titers as high as those achieved by the wild type spiroplasma and was transmitted by the insects to the plants where it reached the same high titers than those of the wild type. However, it did not induce disease symptoms. These results lead to identification of the spiroplasmal gene(s) involved in the transmissibility of *S. citri* by the insect and in plant pathogenicity.

Since the initial culturing of *Spiroplasma citri* Saglio in 1970, many studies have been devoted to the citrus stubborn disease agent (1, 2, 3, 4). The restriction map of the *S. citri* genome has been established (10), and more than 100 genes have already been located on the map. The origin of DNA replication (*oriC*) has been cloned and used to construct artificial plasmids which, upon transformation, replicate in the spiroplasma as extrachromosomal elements or integrate in the spiroplasmal genome (7, 9). These *oriC* plasmids can be used as vectors to introduce homologous or heterologous genes into *S. citri* (7).

Through these studies, we have learned a great deal about the molecular biology of *S. citri*, but not much about the interaction between the spiroplasma, its leafhopper vector and host plant. We have now developed a system by which it becomes possible to study the spiroplasmal genes required for the development of *S. citri* in the insect vector and for the production of disease symptoms in the plant, i.e. phytopathogenicity. If such genes exist, their inactivation should result in (i) the inability of *S. citri* to multiply within the leafhopper and (ii) in the absence of disease symptoms in the infected plants. To inactivate the genes, we have used transposon-induced mutagenesis. Since spiroplasmas, like all mollicutes, are phylogenetically related to Gram-positive bacteria, we have chosen transposon Tn 4001 from *Staphylococcus aureus*, a Gram positive bacterium. Tn 4001 contains the *aacA-aphD* gene which confers resistance to aminoglycosides such as gentamicin. Thus, a spiroplasmal transformant containing Tn 4001 is gentamicin resistant.

The Tn 4001 transposon is contained within a plasmid, pMUT, derived from p1SM1001 (kindly obtained from C. Minion [Veterinary Medical Research Institute, Iowa State University, Ames, IA 50011-163, USA]). For the transposon to become inserted in the spiroplasmal genome, plasmid pMUT must first be introduced into the spiroplasmal cell by electroporation-mediated transformation and, next, the transposon must move from the plasmid into the genome by transposition. We have selected a strain of *S. citri* with which the transformation and transposition events are more effi-

cient than with other strains. The selected strain is GII3, isolated in 1979 in Morocco from the leafhopper, *Circulifer haematoceps* (Mulsant & Rey) (8). Strain GII3 was chosen for a second reason; it is easily transmissible by the leafhopper *C. haematoceps* to the host plant, periwinkle *Catharantus roseus*. *C. haematoceps* is the major *S. citri* vector in the Old World (5).

The transposon approach for the production of *S. citri* mutants implies that the transposon becomes randomly inserted into the genome, so that each gene has a chance to become inactivated, including those searched for in this work. We have shown that Tn 4001 is indeed inserted randomly in the genome of strain GII3. Insertion of Tn 4001 into the genome of a GII3 cell renders this transformant resistant to gentamicin. This property is used to screen for the Tn 4001 transformants. However, in the presence of gentamicin, a certain number of non-transformed cells develop a so-called spontaneous resistance to the antibiotic. However, only the Tn 4001 mutants hybridize with a Tn 4001 derived probe, not the spontaneous mutants, and can, therefore, be easily screened.

In this way we have obtained 257 Tn 4001 mutants of *S. citri* strain GII3 (6). Each mutant was screened as follows for the loss of its ability to multiply in the *C. haematoceps* leafhopper or to produce disease symptoms in the periwinkle plant.

Infection of the leafhopper was carried out by intra-abdominal injection of strain GII3 (in SP4 medium) into female leafhoppers. Each mutant was injected into 20 insects. The injected insects were caged on two young periwinkle plants (10 insects per plant) and kept at 30°C in the day (16 hr) and 25°C at night (8 hr). After 2 weeks, the insects were killed and the periwinkles were kept for an additional period of 4 weeks under the same conditions of temperature and light. During the 4-week-period, symptom development was recorded.

After 2 weeks, leaf symptoms were already well-developed for 255 of the 257 mutants, and, after 4 weeks, the plants were severely stunted in comparison with healthy control plants or control plants having been in contact with insects injected with spiroplasma-free SP4 medium. The plants infected with one or the other of the 255 mutants were indistinguishable from those infected with the wild type GII3 spiroplasma.

Two groups of two plants showed no symptoms after 4 weeks, by which time plants infected in parallel with the wild type strain were severely affected. One group corresponded to mutant GMT 470 and the other to mutant GMT 553.

The two plants corresponding to GMT 470 were found to be free of spiroplasmas, indicating that transmission of this mutant to the plant by the insects did not occur. The multiplication of GMT 470 in leafhoppers injected with this mutant was then studied over a period of 15 days during which time the injected leafhoppers were kept on their favored host plant, an ornamental species of stock (*Matthiola incana* (L.) R. Brown). It was found that the mutant did not multiply in and disappeared from the insects. This result explains why mutant GMT 470 could not be transmitted to the periwinkle plants.

The two plants corresponding to GMT 553 were infected with the spiroplasmal mutant. Hence, experiments were conducted to study the multiplication, in the periwinkle plant, of the GII3 mutant in comparison with that of the wild type GII3. It was found that the wild type spiroplasma reached its highest titer in the plant (10^7 spiroplasmas per 0.2 g of leaf midribs) within 2 weeks, a time at which the plants showed severe symptoms. Mutant GMT 553

reached the same high titers within 4 weeks, a period during which no disease symptoms could be observed. These results indicate that periwinkles which contain similar amounts of spiroplasmas in their leaves show severe symptoms with wild type GII3 but no symptoms with GII3 mutant GMT 553. However, the symptomless GMT 553-infected plants did not remain asymptomatic, eventually developing symptoms after 4 weeks. Eighteen weeks later they were severely affected and the spiroplasmas isolated from these plants behaved like wild type spiroplasmas. These results are to be expected since, in the plant where no gentamicin is present, the mutant spiroplasma is not submitted to the antibiotic pressure and eventually loses the transposon which is no longer required. The mutant, therefore, reverts to the wild type spiroplasma.

The presence of GMT 553 in the plant indicates that its transmission to the plant by the leafhopper did occur. Multiplication of GMT 553 in the leafhopper was studied and found to be indistinguishable of that of the wild type GII3. This explains why the mutant could be transmitted to the plant, in contrast to GMT 470 which did not multiply in the insect and could not be transmitted to the plant.

The genes in which Tn 4001 has become inserted in GMT 470 and GMT 553 are under investigation. In the case of GMT 553, the gene affected codes for a protein that has close similarity with repressor proteins of bacterial phosphoenolpyruvate-sugar-phosphotransferase systems (PTS). Preliminary results indicate that this gene seems to be part of a transcriptional unit coding for additional components of PTS, and that the corresponding mRNA is not expressed in the GMT 553 mutant.

This is the first time that mutants of *S. citri* have been obtained. A genetic approach is now available to elucidate the mechanism of such basic properties as motility and helical morphology of spiroplasmas, their transmissibility by leafhoppers to plants and their phytopathogenicity.

Note added in proof. The work summarized in this short communication has now been published:

Foissac, X., C. Saillard, and B. M. Bové. 1997. Random insertion of transposon Tn4001 in the genome of *Spiroplasma citri* strain GII3. Plasmid 37: 80-86.

Foissac, X., C. Saillard, J. L. Danet, P. Gaurivard, C. Paré, F. Laigret, and B. M. Bové. 1997. Mutagenesis by insertion of Tn4001 into the genome of *Spiroplasma citri*: characterization of mutant affected in plant pathogenicity and transmission by the leafhopper vector *Circulifer haematoceps* to the plant. Mol. Plant-Microbe Interact 10: 454-461.

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