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The study of resistance marker rearrangement in *Spiroplasma citri* mutants provides evidence of transfer of chromosomal information followed by recombination. This is the first report of such a transfer in vivo in the mollicutes—that is, in the smallest self-replicating organisms. The double-resistant phenotypes obtained are stable even without selection pressure. The mechanism of gene transfer is insensitive to deoxyribonuclease, requires contact, and possibly, areas of fusion of the cell membranes; it shares properties with the transfer by protoplast fusion in Gram-positive bacteria. The extensive degenerative evolution of mollicutes has retained, in *S. citri*, bacterial functions of chromosomal transfer and recombination.

SPIROPLASMA CITRI IS A HELICAL mollicute, pathogenic for citrus trees (*1*). Mollicutes are mainly characterized by absence of cell wall and by a small sized genome (*2*). They are the simplest and smallest self-replicating organisms and may consequently be considered as minimum living systems (*3*).

Little is known about the genetics of mollicutes because of the absence of mutations that can be used as chromosomal markers (*4*). An in vitro transformation of *Acholeplasma laidlawii* and *Mycoplasma pulmonis* with Tn916 has been reported (*5*). More recently, a conjugal transfer of this transposable element from bacteria to *Mycoplasma* was obtained in mixed cultures of *Streptococcus faecalis* and *Mycoplasma hominis* (*6*). However, nothing is known about in vivo chromosomal gene exchange in mollicutes. To determine the existence of a spontaneous transfer of chromosomal information in *S. citri*, we used chromosomal mutants selected for resistance to vanadium oxide or arsenic acid (*7*, *8*).

The isolation of ultraviolet-induced mutant strains (Ars^R1, resistant to arsenic acid, and Van^R2, resistant to vanadium oxide) from *S. citri* R8A2 (ATCC 27577) has been described (*7*). All strains were triply cloned by conventional procedures (*9*), and stability of phenotypes was verified after five subculturings (about 20 generations) in nonselective BSR medium (*10*). *Spiroplasma citri* cells were grown at 32°C in liquid or solid (0.5% agar) BSR medium or in BSR medium supplemented with 0.5 m*M* arsenic acid (Merck) or 1 m*M* vanadium oxide (Merck).

To study transfer of chromosomal information, we mixed 1 ml of a cell culture of the mutant strain Ars^R1 in middle logarithmic phase of growth (26 hours; 10^9 cell/ml) with 1 ml of a culture of the mutant strain Van^R2 in the same phase of growth (26 hours; 2×10^8 cell/ml). After the mixture was incubated at 32°C for 3, 9, 18, or 42 hours, samples were recovered and diluted, and 100 µl of convenient dilutions were plated (in petri dishes 50 mm in diameter) on BSR medium, to determine the number of colony-forming units (CFU), and on selective medium containing the two toxic products, to determine the number of double-resistant colonies. The double-resistant colonies may have two different origins: spontaneous mutations in single-resistant cells or exchange of genetic material between the two single mutant strains. In all the experiments, the spontaneous mutation frequencies were determined in control cell cultures of each single mutant strain. Assay conditions for the control cell cultures were the same as for the mixture.

Transfer of chromosomal information was also investigated when 50 Kunitz units of deoxyribonuclease I (DNase I) and 20 µl of $MgSO_4$ (1*M*) were added per milliliter of culture medium, in mixtures and in control cultures. Persistence of the DNase activity was verified after 60 hours of incubation of the mixture.

To study the possible role of molecules or virus particles in cultures in effecting the transfer, we replaced 1 ml of the cell culture of one mutant strain in the mixture by 1 ml of the same mutant strain culture that had previously been centrifuged (11,000*g* for 20 minutes at 4°C) (Sorvall SS.1) and filtered on a Millipore membrane of 0.22-µm porosity to eliminate *S. citri* cells. To study the role of contact between the two mutant cells, we carried out experiments in a U-tube with two compartments. The two cultures (50 ml of each mutant strain) were separated by a membrane of 0.22-µm porosity. The effect of fusing agents was tested on mixtures and control cultures in BSR medium containing polyethylene glycol (PEG) 6000 (Merck), or in the presence of calcium and magnesium ions. In that case, 0.05 volume of 20 m*M* KH_2PO_4, 0.05 volume of 1*M* $CaCl_2$, and 0.02 volume of 1*M* $MgSO_4$ were successively added to the contents of the tubes.

In all experiments, results were expressed as the frequency of double-resistant colonies in the population of CFU obtained on nonselective BSR medium. In Table 1 are reported the values of double-resistant colonies frequency in the mixture (*T*) and the

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Table 1. Transfer of chromosomal information in *S. citri*. Cells were from *S. citri* cultures in middle logarithmic phase of growth. Each experimental value is the average of three independent experiments. Standard errors are indicated.

Mixture conditions	Incubation time of the mixtures (hours)	Frequency of A^RV^R colonies in mixture (*T*)	Frequency of A^RV^R colonies obtained by spontaneous mutation		Frequency of A^RV^R colonies obtained by transfer of genetic information $T - (S1 + S2)$
			Control Ars^R1 (*S1*)	Control Van^R2 (*S2*)	
Ars^R1 + Van^R2	0	$(1 \pm 0.6) \times 10^{-8}$	$\leq 1 \times 10^{-9}$	$(1 \pm 0.1) \times 10^{-8}$	0
	3	$(1 \pm 0.8) \times 10^{-8}$	$\leq 1 \times 10^{-9}$	$(1 \pm 0.1) \times 10^{-8}$	0
	9	$(7 \pm 1.2) \times 10^{-8}$	$\leq 1 \times 10^{-9}$	$(3 \pm 0.5) \times 10^{-8}$	$(4 \pm 1.7) \times 10^{-8}$
	18	$(1 \pm 0.1) \times 10^{-4}$	$\leq 1 \times 10^{-9}$	$(3 \pm 2.0) \times 10^{-8}$	$(1 \pm 0.1) \times 10^{-4}$
	42	$(1 \pm 0.5) \times 10^{-4}$	$\leq 1 \times 10^{-9}$	$(7 \pm 1.2) \times 10^{-8}$	$(1 \pm 0.5) \times 10^{-4}$
Ars^R1 + Van^R2	18	$(1 \pm 0.7) \times 10^{-4}$	$\leq 1 \times 10^{-9}$	$(3 \pm 0.9) \times 10^{-8}$	$(1 \pm 0.7) \times 10^{-4}$
+ DNase	42	$(9 \pm 0.8) \times 10^{-5}$	$\leq 1 \times 10^{-9}$	$(4 \pm 1.4) \times 10^{-8}$	$(9 \pm 0.8) \times 10^{-5}$
Ars^R1 + filtered	18	$\leq 1 \times 10^{-9}$	$\leq 1 \times 10^{-9}$	$(3 \pm 0.9) \times 10^{-8}$	0
supernatant from Van^R2	42	$\leq 1 \times 10^{-9}$	$\leq 1 \times 10^{-9}$	$(6 \pm 0.9) \times 10^{-8}$	0
Van^R2 + filtered	18	$(3 \pm 1.2) \times 10^{-8}$	$\leq 1 \times 10^{-9}$	$(3 \pm 0.9) \times 10^{-8}$	0
supernatant from Ars^R1	42	$(6 \pm 2.1) \times 10^{-8}$	$\leq 1 \times 10^{-9}$	$(5 \pm 2.4) \times 10^{-8}$	$(1 \pm 4.5) \times 10^{-8}$

spontaneous mutation frequencies *S1* and *S2* in the control cultures of each single mutant, after various incubation times. The difference between *T* and the sum *S1* + *S2* represents the frequency of the double-resistant colonies obtained by gene transfer between the two types of cells in the mixture. This difference was equal to zero until 9 hours of incubation; it became clearly positive between 9 and 18 hours of incubation. After 18 hours, the difference was maximal and equal to 1×10^{-4}. This high frequency may be easily distinguished from spontaneous mutation frequencies, which are always less than 1×10^{-7}. Accordingly, the excess of double-resistant organisms in the mixture may be attributed to a transfer of genetic material between the two single-resistant strains.

To verify the stability of the double-resistant phenotype of the colonies obtained from the mixture, 20 CFU of Ars^RVan^R cells were triply cloned in nonselective BSR medium and transferred ten times (48 hours of incubation for each transfer) in nonselective medium. For each clone that was transferred ten times, the numbers of CFU and of double-resistant strains have always been equal. This result shows that the double-resistant strains are stable even on a medium without selection pressure, and accordingly, that there is no segregation of the resistance phenotype. It allows the elimination of two possibilities: (i) "cross-feeding," in which double resistance is the result of a cooperation between single-resistant cells in mixed clumps resulting from cellular aggregation, and (ii) the presence of the two chromosomal molecules in the same polyploid cell. The stability of the double-resistant phenotype indicates that the transfer is definitive and is followed by a stable event such as recombination, as shown for other organisms (*11*).

The presence of DNase in the mixture has no effect in transfer (Table 1). Accordingly, the transfer does not require the presence of free DNA in culture medium.

Spiroplasma citri may be infected by virus whose known properties are very similar to those of bacteriophages (*12*). *Spiroplasma citri* strain R_8A_2, used in our experiments, has never revealed the presence of virus particles by appearance of plaque lysis or of extrachromosomal DNA molecules when total DNA was analyzed by agarose gel electrophoresis. Results of experiments searching for a possible role of undetected viral particles (for details of procedure, see above) have shown that the double-resistant colony frequencies obtained corresponded to the spontaneous mutations frequencies (Table 1). Accordingly, viral particles do not appear to be involved in the transfer of the genetic material studied.

The double-resistant colony frequencies in the two cultures of single mutant strains incubated for 18, 42, and 60 hours on the two sides of the membrane of the U-tube were equal to the double-resistant colony frequencies of each respective control culture. Consequently, a contact between the two mutant cells seems necessary to transfer genetic information.

High concentrations of PEG have been extensively used to increase fusion of Gram-positive bacterial protoplasts (*13*, *14*). When PEG was added in *S. citri* mixtures in final concentrations of 40% (w/v) and 20% (w/v), the already high frequency of double-resistant colonies obtained by transfer and recombination (1×10^{-4}) was increased by factors of 2 and 4, respectively, and in a poorly reproducible way. In the same way, the fusion-like effect of centrifugation (11,000*g* for 20 minutes at 4°C) (Sorvall SS.1) of a mixture of the two mutant strains, followed by suspension of the cell pellet in the supernatant, increased the frequency of double-resistant colonies by a factor of 2.5. These results suggest that the mechanism of chromosomal gene transfer involves a step of membrane fusion.

The divalent cations calcium ($CaCl_2$) and magnesium ($Mg\,SO_4$), used as fusing agents for plant protoplasts (*15*), had no effect on genetic marker transfer in *S. citri*.

Table 2 shows results of mating experiments between cells collected at three growth phases. Only *S. citri* from cultures in middle logarithmic phase of growth (26 hours) was able to transfer genetic markers, indicating that this transfer depends on the physiological state of the cultures.

The insensitivity to DNase of the transfer, the absence of participation of viral particles, and the necessity of a contact between cells and, more precisely, of a membrane fusion step, as suggested by the influence of fusing agents, clearly distinguish chromosomal gene transfer in *S. citri* from the DNase-sensitive mechanism of bacterial transformation (*16*) and from transduction which involves viral particles (*17*).

Panicker and Minkley (*18*) proposed that the stable mating stage that necessarily precedes transfer in conjugation of *Escherichia coli*, might be a stage of membrane fusion. This proposal suggests that chromosomal gene transfer in *S. citri* and in Gram-positive bacteria may be related to conjugation in Gram-negative bacteria. However, no pilus and no F factor have yet been found in *Spiroplasma* or in Gram-positive bacteria. Accordingly, gene transfer in *S. citri* appears to be closely related to that in Gram-positive bacteria, especially by protoplast fusion (*13*, *14*), and not to conjugation (*18*). This result strengthens the previously reported phylogenetic origin of mollicutes from Gram-positive bacteria (*19*). The degenerative evolution appears to have maintained the mechanism of DNA transfer and recombination as two functions of these simple free-living organisms.

Table 2. Influence of the physiological stage of the culture on the transfer of chromosomal information in *S. citri*. Each experimental value (*T*, *S1*, or *S2*) is the average of three independent experiments. Standard errors are indicated.

Phase of growth	Frequency of A^RV^R colonies in the mixture (*T*) at indicated hours			Frequency of A^RV^R colonies obtained by spontaneous mutation at indicated hours						Frequency of A^RV^R colonies obtained by transfer of genetic information at indicated hours		
				Control Ars^R1 (*S1*)			Control Van^R2 (*S2*)					
	0	18	42	0	18	42	0	18	42	0	18	42
Middle logarithmic (26 hours)	$(1 \pm 0.5) \times 10^{-8}$	$(2 \pm 0.5) \times 10^{-4}$	$(2 \pm 0.5) \times 10^{-4}$	$\leq 1 \times 10^{-9}$	$\leq 1 \times 10^{-9}$	$\leq 1 \times 10^{-9}$	$(1 \pm 0.8) \times 10^{-8}$	$(3 \pm 0.5) \times 10^{-8}$	$(7 \pm 1.2) \times 10^{-8}$	$(1 \pm 1.3) \times 10^{-8}$	$(2 \pm 0.5) \times 10^{-4}$	$(2 \pm 0.5) \times 10^{-4}$
Late logarithmic (43 hours)	$(1 \pm 0.6) \times 10^{-8}$	$(6 \pm 1.7) \times 10^{-8}$	$(9 \pm 1.6) \times 10^{-8}$	$\leq 1 \times 10^{-9}$	$\leq 1 \times 10^{-9}$	$\leq 1 \times 10^{-9}$	$(2 \pm 0.8) \times 10^{-8}$	$(8 \pm 2.1) \times 10^{-8}$	$(7 \pm 2.1) \times 10^{-8}$	0	0	$(2 \pm 3.7) \times 10^{-8}$
Stationary (65 hours)	$(7 \pm 1.7) \times 10^{-8}$	$(6 \pm 1.9) \times 10^{-8}$	$(9 \pm 0.8) \times 10^{-8}$	$\leq 1 \times 10^{-9}$	$\leq 1 \times 10^{-9}$	$\leq 1 \times 10^{-9}$	$(6 \pm 0.9) \times 10^{-8}$	$(6 \pm 2.1) \times 10^{-8}$	$(9 \pm 0.5) \times 10^{-8}$	$(1 \pm 2.6) \times 10^{-8}$	0	0

Chromosomal gene transfer as described in this report may be used for the mobilization of *S. citri* chromosomes and consequently the establishment of a mapping system. Moreover, the development of *Spiroplasma* genetics, studied in vivo or in vitro through recombinant DNA technology, will provide genetic tools to investigate problems specific to *Spiroplasma*, such as helical morphology and pathogenicity, as well as the nature of genes providing the minimal requirements for the life of these simple organisms.

Finally, this report is a contribution to Morowitz's proposal (*20*) for a thorough understanding of all the functions of these simplest of prokaryotic cells.

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21. We thank E. Ron for helpful comments on the manuscript. This research was supported by grants from the Conseil Scientifique de l'Université de Bordeaux II and from the Institut National de la Recherche Agronomique.