Symbiotic Plasmid Rearrangement in a Hyper-recombinant Mutant of *Rhizobium leguminosarum* biovar *phaseoli*

By GLORIA SOBERÓN-CHÁVEZ* AND REBECCA NÁJERA

Centro de Ingeniería Genética y Biotecnología, Universidad Nacional Autónoma de México,
Apdo. Postal 510-3 Col. Miraval 62271, Cuernavaca, Morelos, México

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The symbiotic plasmid of *Rhizobium leguminosarum* biovar *phaseoli* strain CFN23 has been previously shown to undergo a series of genetic rearrangements that modify the bacterial symbiotic phenotype. A hyper-recombinant derivative of strain CFN23 was isolated. This derivative (strain CFN2300) has a similar phenotype to that reported for *Escherichia coli* mutants defective in DNA metabolism. The frequency of CFN23 symbiotic plasmid deletion is increased in the CFN2300 background, suggesting that homologous genetic recombination is involved in the generation of this genetic rearrangement.

INTRODUCTION

Rhizobium leguminosarum biovar *phaseoli* is a soil bacterium that nodulates beans and fixes nitrogen in this association. As in other fast-growing rhizobia, the genetic information for the bacterium's symbiotic phenotype is plasmid-encoded (Lamb *et al.*, 1982). The symbiotic plasmid (Sym plasmid) of *R. leguminosarum* biovar *phaseoli* strain CFN23 suffers a series of genetic rearrangements that modify its symbiotic phenotype. One of these rearrangements is responsible for the loss at high frequency of the nodulation ability of strain CFN23 (Soberón-Chávez *et al.*, 1986).

The nitrogenase structural genes (*nif* genes) of *R. leguminosarum* biovar *phaseoli* are reiterated (Quinto *et al.*, 1982) and the nucleotide sequences of the three copies of the nitrogenase reductase gene (*nifH*) that are encoded by the Sym plasmid are identical (Quinto *et al.*, 1985). The involvement of these reiterated sequences in the genetic rearrangements described has not been established.

Some *Escherichia coli* mutants defective in DNA metabolism have an abnormally high frequency of homologous genetic recombination. Mutations in the genes coding for DNA polymerase I (*polA*), exonuclease VII (*xsc*), helicase II (*uvrD*), DNA ligase (*lig*), DNA adenine methylase (*dam*) and deoxyuridine triphosphatase (*dut*) genes have been selected on the basis of higher frequency of genetic recombination (Konrad & Lehman, 1974; Konrad, 1977; Chase & Richardson, 1977; Arthur & Lloyd, 1980).

We describe here the isolation of an *R. leguminosarum* biovar *phaseoli* CFN23 derivative cured of an indigenous plasmid (p23a) different from the Sym plasmid. Plasmid p23a is not stably maintained in the CFN23 derivative originally isolated as cured of this plasmid (strain CFN2300). We present the characterization of strain CFN2300, showing that it has a higher frequency of homologous recombination than CFN23. The frequency of one of the CFN23 Sym plasmid rearrangements was determined in the CFN2300 genetic background.

METHODS

Bacterial strains and plasmids. These are shown in Table 1.

Microbiological procedures. All *R. leguminosarum* biovar *phaseoli* strains were grown on PY medium (Noel *et al.*, 1984) at 30 °C and *E. coli* cells on LB medium (Miller, 1972) at 37 °C. The antibiotic concentrations used were as

Table 1. *Bacterial strains and plasmids*

Strain or plasmid	Relevant characteristics†	Source or reference
<i>R. leguminosarum</i> biovar <i>phaseoli</i> *		
CFN23	Wild-type strain; Nod ⁺ <i>nifH</i> ⁺	Soberón-Chávez <i>et al.</i> (1986)
CFN231	Nod ⁺ <i>nifH</i> ⁺ ; spontaneous Str ^r mutant	This work
CFN2300	Nod ⁻ Δ <i>nifH</i> ; cured of plasmid p23a; Km ^s Str ^r	This work
CFN2305	Nod ⁺ <i>nifH</i> ⁺ ; strain CFN2300 with Sym plasmid from strain CFN2315; Km ^r Str ^r	This work
CFN2306	Nod ⁻ Δ <i>nifH</i> ; strain CFN2300 with plasmid p23a from strain CFN2353; Km ^r Str ^r	This work
CFN2307	Nod ⁺ <i>nifH</i> ⁺ ; strain CFN2300 with Sym plasmid from strain CFN2314; Km ^r Str ^r	This work
CFN2313	Nod ⁺ <i>nifH</i> ⁺ ; Tn5 inserted in plasmid p23a from strain CFN231; Km ^r Str ^r	This work
CFN2314	Nod ⁺ <i>nifH</i> ⁺ ; Tn5 <i>mob</i> inserted in Sym plasmid; Km ^r Rif ^r	Soberón-Chávez <i>et al.</i> (1986)
CFN2315	Nod ⁺ <i>nifH</i> ⁺ ; Tn5 <i>mob</i> inserted in Sym plasmid; Km ^r Rif ^r	Soberón-Chávez <i>et al.</i> (1986)
CFN2316	Nod ⁺ <i>nifH</i> ⁺ ; spectinomycin-resistance cassette inserted in <i>nifH</i> gene of <i>nif</i> region <i>a</i> ; Sp ^r Rif ^r	This work
CFN2350	Nod ⁻ Δ <i>nifH</i> ; Rif ^r	Soberón-Chávez <i>et al.</i> (1986)
CFN2353	Nod ⁻ Δ <i>nifH</i> ; strain CFN2350 with plasmid p23a from strain CFN2313; Km ^r , Rif ^r	This work
CFN2370	Nod ⁻ Δ <i>nifH</i> ; Str ^r	Soberón-Chávez <i>et al.</i> (1986)
<i>E. coli</i>		
1830	<i>pro met</i> (pJB4JI)	Beringer <i>et al.</i> (1978)
HB101	<i>recA13 hsdS20 rpsL</i>	Boyer & Roulland-Dussoix (1969)
Plasmids		
p23a	135 kb CFN23 indigenous plasmid	This work
pLS151	Vector for <i>nifH</i> sequences; carries Sp ^r and Tc ^r	Soberón-Chávez <i>et al.</i> (1986)
pRK2013	Tra ⁺ Km ^r (not transposable); unable to replicate in <i>Rhizobium</i>	Guiney & Helinski (1979)
pJB4JI	pPHIJI::Mu::Tn5; Km ^r Ge ^r ; unable to replicate in rhizobia	Beringer <i>et al.</i> (1978)
pRK404	Mobilizable plasmid; able to replicate in <i>Rhizobium</i> ; Tc ^r	Ditta <i>et al.</i> (1985)

* All the strains of *Rhizobium leguminosarum* biovar *phaseoli* are derivatives of strain CFN23.

† Nod, ability to nodulate beans; *mob*, gene encoding the ability to be mobilized by some transferable plasmid; *nifH*, nitrogenase reductase gene; Tra⁺, conjugative transfer ability. Resistance to streptomycin (Str^r or *rpsL*), kanamycin (Km^r), rifampicin (Rif^r), spectinomycin (Sp^r), tetracycline (Tc^r) and gentamicin (Ge^r) and susceptibility to kanamycin (Km^s) are indicated; Str^r and Rif^r are encoded on the chromosome; all other antibiotic resistances are plasmid-encoded. *E. coli* HB101 is unable to recombine, or to modify or restrict DNA. *pro* and *met*, proline and methionine auxotrophies.

follows: gentamicin 40 µg ml⁻¹, kanamycin 60 µg ml⁻¹, rifampicin 30 µg ml⁻¹, spectinomycin 100 µg ml⁻¹ and streptomycin 200 µg ml⁻¹. To isolate strain CFN2300, two cycles of ampicillin (1 mg ml⁻¹) and cycloserine (2 mg ml⁻¹) enrichment were used in the presence of 4 µg kanamycin ml⁻¹. Tn5 insertions were selected as kanamycin-resistant transconjugants from the mating between strains 1830 and CFN23 (Table 1); plasmid pJB4JI is unable to replicate in rhizobia so the kanamycin-resistant transconjugants must contain the Tn5 inserted in their genome. The Tn5 insertion in plasmid p23a was identified by its ability to be transferred by conjugation to strain CFN2350 (Table 1), and the position of Tn5 was later corroborated by DNA hybridization using Tn5 as probe (data not shown).

Matings were done by plating a 1:1 mixture of the donor and recipient strains on PY medium and leaving this cross overnight. The mixture was then diluted and plated onto selective medium. The viable counts of both parents were determined from the same mixture of bacteria; three to five matings were done in parallel experiments. When we determined the frequency of transfer of the same marker to different recipient strains, a single culture of donor bacteria was used. Tn5*mob* insertions were mobilized with plasmid pRK2013 (Table 1).

Minimal inhibitory concentration (MIC) is the drug concentration at which bacteria were no longer able to form colonies when streaked on plates.

Isolation and manipulation of DNA. Isolation of DNA, radioactive labelling and Southern blot hybridization were done as reported by Quinto *et al.* (1982). The *nifH* probe was described previously (Quinto *et al.*, 1985).

Plasmid visualization and purification. Plasmids were visualized as described by Eckhardt (1978). Sym plasmid was purified as described previously (Soberón-Chávez *et al.*, 1986). It was radioactively labelled and used as probe on the plasmid profile of strain CFN23.

Determination of DNA homologous recombination. Plasmid pLS151 is an intermediary vector derived from plasmid pSUP205 (Simon *et al.*, 1983) which carries an *R. leguminosarum* biovar *phaseoli* nitrogenase reductase gene (*nifH*) interrupted by a DNA fragment conferring spectinomycin resistance (Soberón-Chávez *et al.*, 1986). pLS151 is unable to replicate in rhizobia, and when introduced into an *R. leguminosarum* biovar *phaseoli* strain it can be maintained only as a cointegrate with *nifH* genes present in the bacteria. Thus by measuring the frequency of spectinomycin-resistant transconjugants from the mating between *E. coli* HB101(pLS151) and an *R. leguminosarum* biovar *phaseoli* strain, the recombination frequency between pLS151 and *nifH* genes of that strain is determined. In the cells where only one crossover occurs the spectinomycin-resistant transconjugants retain the tetracycline resistance of pLS151. This case represents more than 90% of the transconjugants. Strain CFN2316 (Table 1) contains the *nifH* gene of *nif* region *a*, interrupted by the spectinomycin-resistance cassette. It was selected as a double recombinant between strain CFN232 (Rif^r derivative of strain CFN23) and pLS151; the presence of the cassette was corroborated by the DNA hybridization using *nifH* as probe (data not shown).

Measurements of nodulation and nitrogen fixation. These were made as reported previously (Soberón-Chávez *et al.*, 1986).

RESULTS AND DISCUSSION

Characterization of plasmid p23a

R. leguminosarum biovar *phaseoli* strain CFN23 contains three indigenous plasmids, of 135, 400 and >450 kb (Fig. 1). The 400 kb plasmid has been shown to be the symbiotic plasmid (Soberón-Chávez *et al.*, 1986). The 135 kb plasmid (p23a) shares DNA homology with the Sym plasmid, but does not contain the structural genes of the nitrogenase enzyme (*nif* genes) (Fig. 1).

A Tn5 insertion in p23a was isolated (see Methods); the strain carrying this plasmid is called CFN2313 (Table 1). The frequency of transfer of kanamycin resistance between strain CFN2313 and the Sym-deleted CFN23 derivative, strain CFN2350 (Table 1) is 1×10^{-2} . The transconjugants did not inherit the ability to nodulate beans nor the nitrogenase genes (*nifH*) (see, for example, strain CFN2353, Table 1).

Isolation of strain CFN2300

Strain CFN2300 is a kanamycin-sensitive derivative of strain CFN2313 (Table 1). It was isolated after two cycles of ampicillin enrichment for kanamycin sensitivity, and the approximate frequency of its appearance was 1×10^{-6} . Strain CFN2300 lacks plasmid p23a, is unable to nodulate beans, and lacks DNA homology with *nifH* genes (Fig. 2). The frequency of CFN23 Sym plasmid deletion that removes *nifH* genes and some of the genes required for nodulation is 1% (Table 2; Soberón-Chávez, 1986), and so it seemed very likely that strain CFN2300, besides lacking plasmid p23a, carried a deleted Sym plasmid.

The Sym plasmid from strain CFN2315 (Table 1) was transferred to strain CFN2300. The transconjugants from this mating (see strain CFN2305, Table 1) are able to nodulate beans and fix nitrogen as well as strain CFN2313 even though they lack plasmid p23a. Plasmid p23a was transferred back to strain CFN2300 and the resulting strain (strain CFN2306, Table 1) remained unable to nodulate beans. From these data we concluded that plasmid p23a, though sharing DNA homology with the Sym plasmid, is not required for the establishment of the symbiosis between *R. leguminosarum* biovar *phaseoli* strain CFN23 and common bean.

The presence of plasmid p23a has no effect on the bacteria's symbiotic phenotype tested under laboratory conditions, even though p23a has a high degree of DNA sequence homology with the Sym plasmid (Fig. 1). It is intriguing that a plasmid with similar characteristics to plasmid p23a – in terms of its size, transfer ability and DNA homology with the Sym plasmid – is present in about half of the Mexican isolates of *R. leguminosarum* biovar *phaseoli* (data not shown), and that it does not seem to have a role in the symbiosis.

Plasmid p23a is transferred at a high frequency, so we think that it might participate in the conjugal mobilization of the entire Sym plasmid or of part of it, acting as a 'vector' of symbiotic information. We have preliminary evidence suggesting that the transfer frequency of the Sym plasmid is enhanced by the presence of p23a, which might thus act as a helper for Sym plasmid transfer; this might be the reason for its wide distribution among *R. leguminosarum* biovar *phaseoli* strains.

Fig. 1. Plasmid electrophoretic profile of strain CFN23 (lane 1), and hybridization of this profile with the Sym plasmid as a probe (lane 2) and with a *nifH* probe (lane 3).

Fig. 2. Plasmid electrophoretic profile of strain CFN23 and derivatives (a) and their hybridization with a *nifH* probe (b). Lanes: 1, strain CFN23; 2, strain CFN2300; 3, strain CFN2370.

Characterization of strain CFN2300

The kanamycin resistance of strain CFN2306 is lost at a high frequency while that of strain CFN2353 or strain CFN2313 is not (Table 2). The instability of plasmid p23a in the CFN2300 genetic background suggested to us that strain CFN2300 contained a spontaneous mutation affecting the maintenance of this plasmid.

Certain *E. coli* strains mutant for genes whose products are involved in the SOS response have an increased sensitivity to nalidixic acid (Drlica, 1984). We tested the nalidixic acid sensitivity of strain CFN2300 relative to that of the wild-type CFN23 strain, and of its derivative with the deleted Sym plasmid (CFN2370, Table 1). Strain CFN2300 is nalidixic acid sensitive. This sensitivity is independent of the presence of plasmid p23a, since strain CFN2306 is sensitive to this antibiotic, even when grown in the presence of kanamycin where the maintenance of p23a is selected (Table 3). The nalidixic acid sensitivity of strain CFN2300 is not due to the deletion affecting its Sym plasmid, since strain CFN2370, which harbours a deleted Sym plasmid (Soberón-Chávez *et al.*, 1986), is as resistant to nalidixic acid as the wild-type strain CFN23, and strain CFN2305 is as sensitive as strain CFN2300 (Table 3). We have found that *R. leguminosarum* biovar *phaseoli* is naturally resistant to high levels of nalidixic acid (data not

Table 2. *Stability of plasmid p23a and the Sym plasmid in different genetic backgrounds*

Strain	Plasmid where Tn5 is inserted	Percentage of Km ^r derivatives*
CFN2305†	Sym plasmid (same insertion as in strain CFN2315)	20 (10–36)
CFN2313	Plasmid p23a	<1
CFN2306†	Plasmid p23a (same insertion as in strains CFN2313 and CFN2353)	5 (4–7)
CFN2307†	Sym plasmid (same insertion as in strain CFN2314)	<1
CFN2314	Sym plasmid	<1
CFN2315	Sym plasmid	1 (0.87–1.3)
CFN2353	Plasmid p23a	<1

* Means of three determinations; for results $\geq 1\%$, the range of values obtained is given in parentheses.

† CFN2300 genetic background.

Table 3. *Drug sensitivity of strain CFN2300 and its derivatives*

Strain	Minimal inhibitory concentration*			
	EMS (%, v/v)	Nal ($\mu\text{g ml}^{-1}$)	Rif ($\mu\text{g ml}^{-1}$)	Amp ($\mu\text{g ml}^{-1}$)
CFN2300	0.04	200	5	10
CFN2305	0.04	200	ND	ND
CFN2306	0.04	200	5	10
CFN2315	0.09	> 400	ND	ND
CFN2370	0.09	> 400	5	10

* EMS, ethyl methane sulphonate; Nal, nalidixic acid; Rif, rifampicin; Amp, ampicillin; ND not determined.

shown). This explains the high levels of resistance of the strains used in this work. Even strain CFN2300 and its derivatives, which are reported as being sensitive to nalidixic acid, are able to grow, at a reduced rate, in concentrations as high as $180 \mu\text{g ml}^{-1}$. The concentration at which growth of the wild-type strain is arrested was not determined, but it is higher than $400 \mu\text{g ml}^{-1}$.

Bacteria bearing a mutation in one of the genes which participate in DNA replication, repair or methylation have an increased sensitivity to different mutagens. We determined the sensitivity to ethyl methane sulphonate (EMS) of strain CFN2300 and its derivatives compared to the wild-type strain, and were able to show that the mutation present in strain CFN2300 renders it sensitive to this mutagen, and that this sensitivity is independent of the presence of plasmid p23a or of the undeleted Sym plasmid (Table 3).

To rule out the possibility that the increased sensitivity to EMS and nalidixic acid was due to increased membrane permeability of strain CFN2300, we determined the sensitivity of this strain to ampicillin and rifampicin; we chose these antibiotics because *E. coli* mutants defective in DNA metabolism do not show an increased sensitivity to them. The mutation present in strain CFN2300 does not affect the level of resistance of this strain to ampicillin or rifampicin (Table 3), suggesting that this mutation affects DNA metabolism.

E. coli derivatives bearing a defect in some genes involved in DNA metabolism, such as *lig*, *polA*, *dam*, *xsc*, *xth*, *uvrE* or *uvrD*, exhibit an abnormally high frequency of homologous recombination, Zeig *et al.* (1978). We determined the frequency of homologous recombination of strain CFN2305 (the CFN2300 derivative containing the Sym plasmid) compared to that of strain CFN2315 (Table 1) by measuring the frequency of recombination between the *nifH* genes present in both strains and a cloned *nifH* gene carrying a segment of DNA conferring spectinomycin resistance (plasmid pLS151). Strain CFN2305 has a frequency of homologous recombination 7.1 times higher than that of strain CFN2315 (Table 4). The frequency of insertion of pLS151 in the absence of *nifH* genes was determined, to discriminate between homologous genetic recombination and random insertion of this plasmid. When strain CFN2300 was used as recipient for plasmid pLS151, we found a frequency of 1.9×10^{-8}

Table 4. *Frequency of homologous recombination*

Strain	Frequency* of Sp ^r transconjugants in matings with HB101(pLS151)
CFN2315	$3.5 \pm 0.7 \times 10^{-6}$ (100%)
CFN2305	$2.5 \pm 0.3 \times 10^{-5}$ (710%)
CFN2300	$1.9 \pm 1.4 \times 10^{-8}\dagger$ (0.5%)

* Mean of five determinations, $\pm$ SE. Percentage values relative to CFN2315 are shown in parentheses.

† This value represents the CFN2300 spontaneous mutation rate to spectinomycin resistance, since all the bacteria are sensitive to tetracycline.

spectinomycin-resistant bacteria and all of them were sensitive to tetracycline (Table 4), showing that they were not transconjugants. We conclude that the frequency of pLS151 insertion not due to homologous genetic recombination is less than the CFN2300 spontaneous mutation rate to spectinomycin resistance.

Strains CFN2305 and CFN2315 are equally efficient as recipients of mobilized small plasmids. The frequencies of their inheritance of plasmid pRK404 using *E. coli* HB101 as donor strain (Table 1) are 6×10^{-3} and 7.4×10^{-3} respectively; thus the difference in the frequency of their inheritance of the pLS151 spectinomycin resistance seems to be due to differences in their recombination rates.

Several hyper-recombinant *E. coli* mutants show elevated mutagenesis. In parallel experiments, we determined the spontaneous mutation rate to rifampicin resistance of strains CFN2300 and CFN2370 as $4.1 \pm 0.8 \times 10^{-7}$ and $4.0 \pm 1.1 \times 10^{-8}$, respectively (means $\pm$ SE; $n = 3$). This result gives additional evidence in support of our hypothesis of CFN2300 being a hyper-recombinant mutant.

The CFN2300 phenotype presented here is similar to that of certain *E. coli* mutants affected in DNA replication or repair (De Lucia & Cairns, 1969; Gross & Gross, 1969; Kinsbury & Helinski, 1973; Lehman & Chien, 1973; Konrad & Lehman, 1974; Chase & Richardson, 1977; Drlica, 1984; Bassett & Kushner, 1985; Charon *et al.*, 1985; Smith, 1988), but the precise defect of strain CFN2300 remains to be established.

Sym plasmid deletion in the genetic background of strain CFN2300

Strain CFN2300 has a higher frequency of homologous genetic recombination than strain CFN2315 (Table 4). Thus if the Sym plasmid deletion reported (Soberón-Chávez *et al.*, 1986) is a product of recombination between reiterated DNA sequences, this genetic rearrangement should be increased in the CFN2300 background.

The Tn5*mob* insertion present in the Sym plasmid of strain CFN2315 (Table 1) is lost when the nodulation ability and *nifH* genes are deleted, whereas the Tn5*mob* present in the Sym plasmid of strain CFN2314 (Table 1) is not affected by the deletion of this plasmid (Soberón-Chávez *et al.*, 1986).

The frequency of Sym plasmid deletion in the CFN2300 background was measured by determining the frequency of kanamycin-sensitive derivatives of strain CFN2305 (Table 1) compared to that of strain CFN2315; Sym plasmid deletion was increased 20-fold in the CFN2300 background (Table 2).

In order to distinguish between Sym plasmid deletion and its segregation, the frequency of kanamycin-sensitive derivatives of strain CFN2307 (Table 1) was determined; CFN2307 is a CFN2300 derivative containing the CFN2314 Sym plasmid, thus its kanamycin resistance is not affected by the Sym plasmid deletion. The results obtained (Table 2) show that the Sym plasmid is stably replicated in the CFN2300 background. To rule out the possibility that increased Sym plasmid deletion in the CFN2300 background was due to the absence of plasmid p23a, we determined the rate of Sym plasmid deletion in the genetic background of strain CFN2306. This was done by measuring the frequency of spectinomycin-sensitive (Sp^c) derivatives from strain CFN2306 carrying the CFN2316 Sym plasmid. These bacteria were

grown in the presence of kanamycin, thus selecting for the presence of plasmid p23a. We found a frequency of 28% Spc^r cells; thus the increased frequency of Sym plasmid deletion in the CFN2300 background does not seem to be related to the absence of plasmid p23a.

Strain CFN23 presents a genetic rearrangement that results in loss of the bacterium's nodulation ability and deletion of the nitrogenase structural genes without an apparent change in the Sym plasmid molecular mass (Soberón-Chávez *et al.*, 1986). It has been proposed that this rearrangement is due to homologous recombination between reiterated DNA sequences, which are very common among *Rhizobium* isolates (Flores *et al.*, 1987). The enhanced frequency of Sym plasmid deletion in the CFN2300 genetic background led us to the conclusion that recombination between homologous DNA sequences present in this plasmid might indeed be involved in the generation of the deletion leading to the instability of CFN23 symbiotic properties.

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