

## Evaluation of a strategy for identifying nodulation competitiveness genes in *Rhizobium leguminosarum* biovar *phaseoli*

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*Rhizobium leguminosarum* biovar *phaseoli* strain KIM5s is consistently much more competitive than strain CE3 in nodulation of beans (*Phaseolus vulgaris* L.) in the laboratory and in the field. To identify genes that contribute to the competitiveness of KIM5s, we transferred a cosmid library containing KIM5s DNA into CE3 and applied the transconjugants to bean plants to allow the plants to enrich for those with enhanced nodulation competitiveness. The nodule isolates were then applied to plants for further enrichment. Of 75 isolates from nodules sampled after the two enrichments, 9 were more competitive than CE3. For example, when outnumbered in the inocula 40-fold by a reference strain, these nine strains typically occupied 15–40% of the nodules compared with 0–3% for CE3. However, when these strains were cured of the cosmids, they remained highly competitive, demonstrating that the enhanced competitiveness of the strains was not associated with the cosmids. We found no evidence for cosmid insertion into the chromosome or for cosmid-induced genetic changes in these cured strains. We found some evidence suggesting that their altered competitiveness was due to spontaneous genetic changes that did not involve the cosmids. Although these highly competitive variants remain genetically uncharacterized, they may provide insight into bacterial traits that contribute to, or detract from, successful nodulation competitiveness.

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### Introduction

The success of a micro-organism living among other micro-organisms depends on genes that contribute to its survival or successful competitiveness. By identifying these genes and the traits they confer, we will begin to understand the survival strategies of micro-organisms in specific environments. Plant-associated bacteria provide interesting models for studying the determinants of ecological fitness, since the plant may provide a highly selective habitat for the bacteria.

The approach most commonly taken to identify determinants of ecological fitness is to test whether specific phenotypes identified in culture affect survival or competition. For example, in studies of the colonization fitness of plant-associated bacteria, Haefele & Lindow (1987) tested mutants for a role of motility in epiphytic colonization by *Pseudomonas syringae*, and Anderson *et*

*al.* (1988) tested for a role in agglutination by a plant glycoprotein in root colonization by *P. putida*. Although this predictive approach has led to the identification of characteristics involved in bacterial behaviour in natural habitats, other approaches may be useful to understand these complex phenotypes. By exploiting the selective pressures exerted by specific habitats and applying modern molecular techniques, we should be able to identify genes that affect bacterial behaviour in a specific environment and then identify their functions. The attraction of this shotgun approach is that it has the power to extend beyond the predictive abilities of the researchers.

Bacteria of the genus *Rhizobium* are ideal candidates for studying competitive behaviour, because the root nodules that they induce in symbiotic association with legumes usually contain pure cultures of the successful competitor. Root nodules therefore could provide a source of highly competitive genetic variants enriched from the rhizosphere population.

Through mutant analysis, phenotypic alterations identified in culture have been correlated with changes in competitiveness. Such studies have shown that competitiveness is affected by motility and chemotaxis (Ames

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& Bergman, 1981; Caetano-Anollés *et al.*, 1988; Liu *et al.*, 1989; Mellor *et al.*, 1987; Zdor & Pueppke, 1991), cell surface polysaccharides (Araujo & Handelsman, 1990; Bhagwat & Keister, 1990; Handelsman *et al.*, 1984; Milner *et al.*, 1992; Trinick & Hadobas, 1989; Ugalde *et al.*, 1986; Zdor & Pueppke, 1991; Lagares *et al.*, 1992), bacteriocin production (Triplett & Barta, 1987), succinate-sensitivity (Urban, 1988), and rate of nodule induction (Hahn & Hennecke, 1988). Such studies have also demonstrated that the ability to fix nitrogen (Amarger, 1981; Hahn & Struder, 1986; Sanjuan & Olivares, 1991*b*) and to metabolize particular aromatic compounds (Rynne *et al.*, 1988) does not affect competitiveness under the conditions tested.

Although successful competitiveness is likely to be conferred by multiple, possibly interacting, mechanisms that are controlled by many genes, the above studies demonstrate that altering a single gene can result in a detectable change in competitiveness. A strategy for identifying such genes, which is complementary to the predictive approach, is to identify randomly generated genetic changes that either contribute to or detract from competitiveness and then determine the cellular functions of the genes involved. An analogous approach was used to identify and functionally characterize *nod* genes in *Rhizobium* spp. (Long *et al.*, 1982; Kondorosi, 1989), and *vir* (Douglas *et al.*, 1985; O'Connell & Handelsman, 1989), *avr* (Ronald & Staskawicz, 1988; Swanson *et al.*, 1988), and *hrp* (Lindgren *et al.*, 1986; Grimm & Panopoulos, 1989) genes in plant-pathogenic bacteria. In the only previous study to apply this shotgun strategy to nodulation competitiveness, McLoughlin *et al.* (1987) generated 600 random Tn5 mutants of *R. fredii* and identified four that were reduced in competitiveness. Unfortunately, they were unable to identify the physiological function of the gene(s) involved. An alternative to generating random mutants would be to transfer genes from one strain to another and screen for altered competitiveness. Although this requires that all of the genes involved in a single mechanism of competitiveness be clustered and that these genes be functionally dominant in the recipient, success with this approach directly provides clones of the gene(s) of interest.

We define nodulation competitiveness as the relationship between the ratio of strains in the inoculum and the ratio of nodules occupied by each strain. In a previous evaluation of the relative competitiveness of two *R. leguminosarum* bv. *phaseoli* strains, KIM5s and CE3, we showed that KIM5s is consistently much more competitive than CE3 in nodulating beans in the laboratory and in the field (Beattie *et al.*, 1989). This consistency is critical for a genetic study of nodulation competitiveness, because it facilitates identifying genetically altered strains whose competitiveness deviates from that of the parent

strain and may make it possible to identify genes in the laboratory that are also important for competitiveness in the field. In this paper, we report our attempts to transfer genes that influence nodulation competitiveness from KIM5s into CE3.

## Methods

**Bacterial strains and plasmids.** These are shown in Table 1. Antibiotics, when needed, were added to the media at the following concentrations: spectinomycin (Sp), streptomycin (Sm), and kanamycin, 200 µg ml<sup>-1</sup> vancomycin, 5 µg ml<sup>-1</sup> and tetracycline (Tc), 20 µg ml<sup>-1</sup>.

**Construction of genomic libraries of KIM5s.** Standard DNA manipulations such as isolation and restriction digestion of DNA and agarose gel electrophoresis were performed as described by Ausubel *et al.* (1987). We constructed genomic libraries of KIM5s by partially digesting genomic DNA with *Sau*3A, size-fractionating it on a 10–40% linear sucrose gradient, and cloning fragments in the range 20–30 kb into the cosmid vectors pLAFR3 (Staskawicz *et al.*, 1987) and pLA2917 (Allen & Hanson, 1985). Clones were stored individually in 15% (v/v) glycerol at –80 °C. We isolated cosmids by the method of Birnboim (1983) and determined the average insert size in each library by examining *Bam*HI restriction patterns of cosmid DNA.

We transferred the libraries into CE3 by the method of Triplett (1988). Each mating mixture contained 48 cosmid clones in *Escherichia coli* strain DH1, the recipient CE3, and *E. coli* strain DH5α(pRK2013). CE3 transconjugants were selected on BSM agar (Bergersen, 1961) containing Sm and Tc.

**Plant growth conditions and preparation of inocula.** Seeds of all *Phaseolus vulgaris* L. (bean) cultivars were the gift of K. Kmiecik, Department of Horticulture, University of Wisconsin-Madison, USA. Seeds were surface-sterilized immediately before planting by rinsing them for 30 s in ethanol, 3 min in 1% (w/v) sodium hypochlorite, and 3 min in sterile water. Unless otherwise indicated, seeds of the common black bean cultivar WBR22-34 were planted in sterilized sand and vermiculite (1:1 by volume) in glass tubes (Araujo *et al.*, 1986) and grown in a growth chamber as described previously (Beattie *et al.*, 1989).

Inocula were prepared by growing each strain to early stationary phase in yeast extract/mannitol (YM) broth (Wacek & Brill, 1976) at 28 °C. An inoculum (1 ml) containing 10<sup>7</sup>–10<sup>8</sup> cells was applied directly to each seed before covering. Three weeks after planting, nodules were removed, surface-sterilized, and nodule bacteria were plated as described previously (Beattie & Handelsman, 1989).

**Cosmid stability in culture and during nodulation.** We examined the stability of the cosmids in five independent transconjugants from each library. We grew two replicates of each transconjugant for approximately 10 generations in YM broth at 28 °C and enumerated the bacteria on YM agar with and without Tc. We calculated the mean percentage of Tc-resistant cells in the ten cultures that represented each library. To evaluate cosmid stability *in planta*, we applied each transconjugant to two seeds of cultivar Puebla 152, crushed five nodules from each plant, and enumerated the bacteria from each nodule on YM agar with and without Tc. We calculated the mean percentage of Tc-resistant cells in the 50 nodules that represented each gene library.

**Enrichment for transconjugants with enhanced competitiveness.** We used the host plant to enrich for highly competitive strains from a mixture of strains. To evaluate the selective power of the plant with strains KIM5s and CE3, we applied a 1:50 mixture of KIM5s and CE3

Table 1. *Bacterial strains and plasmids used in this study*

| Designation  | Relevant characteristics*  | Source or reference                            |
|--|--|--|
| <i>Rhizobium leguminosarum</i> bv. <i>phaseoli</i> |  |  |
| KIM5s  | Spontaneous Sp <sup>r</sup> derivative of KIM5, Cmp <sup>++</sup>                                    | Beattie <i>et al.</i> (1989)                   |
| CE3  | Spontaneous Sm <sup>r</sup> derivative of CFN42  | Noel <i>et al.</i> (1984)                      |
| CE3013   | CE3::Tn5, Sm <sup>r</sup> , Km <sup>r</sup> , Cmp <sup>o</sup>                                       | R. S. Araujo & J. Handelsman, unpublished data |
| CE3(pOWR1)   | CE3 transconjugant isolated after two cycles of plant nodulation, Cos <sup>+</sup> Cmp <sup>++</sup> | This study                                     |
| CE3(pOWR2)   | Same as CE3(pOWR1), Cos <sup>+</sup> Cmp <sup>++</sup>   | This study                                     |
| CE3(pOWR14)  | Same as CE3(pOWR1), Cos <sup>+</sup> Cmp <sup>++</sup>   | This study                                     |
| CE3(pOWR15)  | Same as CE3(pOWR1), Cos <sup>+</sup> Cmp <sup>++</sup>   | This study                                     |
| CE3(pOWR52)  | Same as CE3(pOWR1), Cos <sup>+</sup> Cmp <sup>++</sup>   | This study                                     |
| CE3(pOWR58)  | Same as CE3(pOWR1), Cos <sup>+</sup> Cmp <sup>++</sup>   | This study                                     |
| CE3(pOWR71)  | Same as CE3(pOWR1), Cos <sup>+</sup> Cmp <sup>++</sup>   | This study                                     |
| CE3(pOWR73)  | Same as CE3(pOWR1), Cos <sup>+</sup> Cmp <sup>++</sup>   | This study                                     |
| CE3(pOWR75)  | Same as CE3(pOWR1), Cos <sup>+</sup> Cmp <sup>++</sup>   | This study                                     |
| CE3(pOWR3)   | Same as CE3(pOWR1), Cos <sup>+</sup> Cmp <sup>o</sup>  | This study                                     |
| CE3(c#)  | CE3(pOWR#) cured of the cosmid pOWR#   | This study                                     |
| CE3'(pOWR#)  | CE3 containing the pOWR# cosmids isolated from transconjugants after two cycles of plant nodulation  | This study                                     |
| Plasmids   |  |  |
| pLAFR3   | Broad host range cosmid vector, Tc <sup>r</sup>  | Staskawicz <i>et al.</i> (1987)                |
| pLA2917  | Broad host range cosmid vector, Tc <sup>r</sup>  | Allen & Hanson (1985)                          |
| prK2013  | Conjugation helper plasmid, Km <sup>r</sup>  | Figurski & Helinski (1979)                     |
| pOWR#  | pLAFR3 or pLA2917 containing a 15 to 30 kb insert of KIM5s genomic DNA                               | This study                                     |

\* Sp, spectinomycin; Sm, streptomycin; Km, kanamycin; Tc, tetracycline; <sup>r</sup>, resistant; Cos<sup>+</sup>, contains a cosmid; Cmp<sup>++</sup>, more competitive than CE3; Cmp<sup>o</sup>, similar to CE3 in competitiveness; # indicates the number assigned to the cosmid.

to five seeds, combined all of the nodules from the plants (approximately 40 nodules per plant), and counted bacteria in the crushed nodule suspension by plate dilution on YM agar containing either Sm or Sp. The suspension was applied to five more seeds, and the procedure was repeated.

To enrich for transconjugants with enhanced competitiveness, we applied pools of transconjugants, each containing 48 independent cosmids, to plants. Approximately 10<sup>7</sup> cells of each pool were applied to each of five seeds. All of the nodules from the five plants were combined (approximately 40 nodules per plant), surface-sterilized, and crushed, and the bacteria were grown on YM agar containing Tc. The bacteria from plants representing a pool of cosmids were resuspended in water and 10<sup>7</sup> cells were applied to a seed. For each pool of cosmids, one nodule isolate was selected for evaluation in a competitiveness assay.

**Competitiveness assay.** For nodulation competitiveness measurements, we prepared the inocula by diluting cultures of a test strain and a reference strain to the same optical density (OD<sub>600</sub> = 0.1–0.3) and mixing them in various ratios. We calculated the actual inoculum ratios by dilution plating each culture on selective media. Each inoculum mixture was applied to six seeds, and isolates from six nodules, selected from the crown region of each plant, were identified by their resistance to antibiotics (Beattie & Handelsman, 1989). We determined the proportion of nodules occupied by each strain singly and by both strains.

**Strain construction.** We isolated cosmid DNA from transconjugants by a modified boiling method (Holmes & Quigley, 1981). The cosmids

were transformed into *E. coli* strain DH5 $\alpha$  and transferred into a stock culture of CE3 by conjugation. CE3 was subjected to a mock mating procedure by the protocol described previously with a mixture of CE3 and *E. coli* strain DH5 $\alpha$ . To cure the transconjugants of cosmids, they were grown for 10 generations in YM broth and plated on YM agar. After replica plating on YM agar with and without Tc, Tc-sensitive colonies were chosen. Cosmid acquisition and loss were evaluated by Tc sensitivity and plasmid isolation followed by visualization on agarose gels (Ausubel *et al.*, 1987).

**Physiological characterization of strains.** The growth rate of each strain was measured in YM broth by plate dilution on a non-selective YM medium and by measuring OD<sub>600</sub> at selected intervals. The cultures were grown in batch culture at 28 °C with vigorous shaking.

The nodulation efficiency of the strains (Caetano-Anollés *et al.*, 1988) was evaluated by applying serial dilutions of a culture of each strain to each of three seeds and counting the nodules that were present after three weeks. Acetylene reduction was measured according to Somasegaran & Hoben (1985).

## Results

### *Construction of genomic libraries of KIM5s*

Since cosmids are often lost from rhizobia during nodulation (Gray *et al.*, 1990; Haugland *et al.*, 1984; Kahn & Timblin, 1984; Lambert *et al.*, 1987; Long *et al.*,

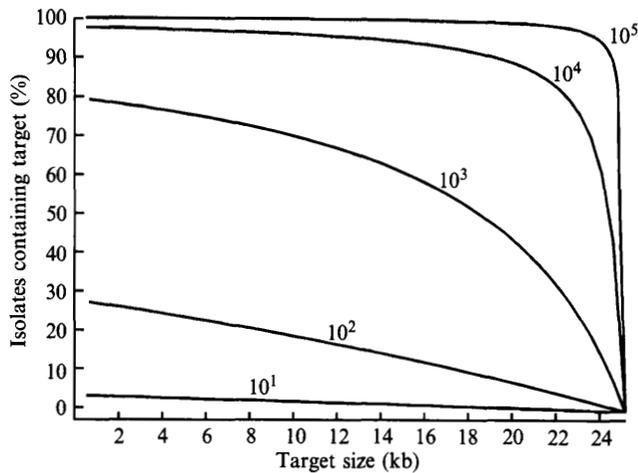


Fig. 1. Relationship between target size, enrichment power, and the expected frequency of isolates containing the target DNA after the plant enrichments. Target is the DNA region required for enhanced competitiveness;  $10^n$  is the enrichment power, i.e. the magnitude of the increased representation of a strain in the mixture of bacterial isolates selected after two plant passages compared with its representation in the original inoculum mixture. Calculations are based on an average insert size of 25 kb and a genome size of 6500 kb.

1982), we constructed libraries in two cosmid vectors to increase the probability of producing cosmids that would be consistently recoverable from nodules. Libraries consisted of 1300 clones derived from pLAFR3 and 2700 clones derived from pLA2917. The average insert sizes were  $26.6 \pm 1.1$  kb (mean  $\pm$  SEM,  $n = 24$ ) and  $25.2 \pm 1.3$  kb ( $n = 24$ ) for the pLAFR3 and pLA2917 libraries, respectively.

#### Cosmid stability in culture and during nodulation

Maintenance of the cosmids was inferred by maintenance of tetracycline resistance (Tc<sup>r</sup>). After 10 generations in culture, the percentages of cells that contained either pLAFR3- or pLA2917-derived cosmids were  $69 \pm 15\%$  ( $n = 10$ ) and  $78 \pm 6\%$  ( $n = 10$ ), respectively. In nodules, the percentages were  $49 \pm 8\%$  ( $n = 50$ ) and  $60 \pm 4\%$  ( $n = 50$ ), respectively. Although the stability of pLAFR3-derived cosmids was more variable than that of pLA2917-derived cosmids, cosmids from either library were present in at least some of the bacteria isolated from any given nodule. Both libraries were therefore transferred into CE3 and screened for cosmids that confer enhanced competitiveness.

#### Relationship between target size, enrichment power, and the expected frequency of isolates containing the target DNA

The proportion of isolates that will contain cosmids conferring enhanced competitiveness after the plant

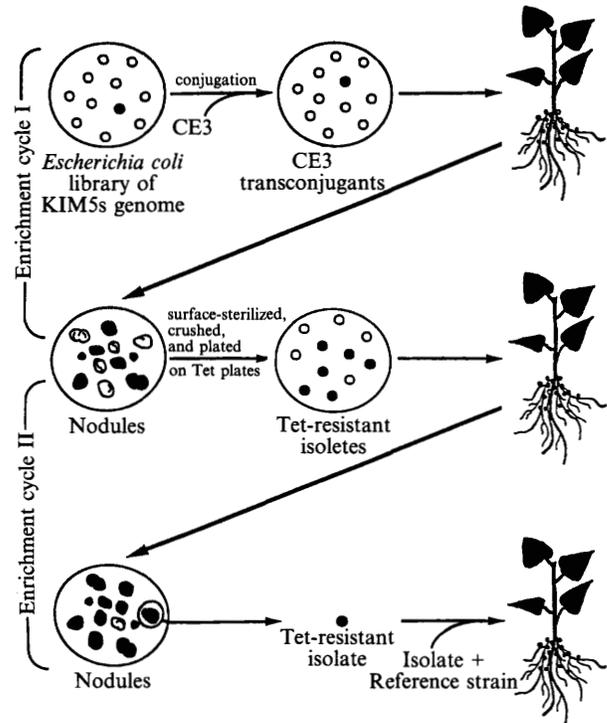


Fig. 2. Strategy for enriching for highly competitive CE3 transconjugants.

enrichments depends on two primary factors, the size of the required target DNA region and the enrichment power. The enrichment power will depend on the extent to which the target DNA enhances the competitiveness of the recipient. Assuming a completely random library, the proportion ( $P$ ) of cosmids containing the target DNA is related to the size of the target DNA ( $T$ ) as follows:  $P = (1 - T/I)(I/G)$ , where  $I$  is the average insert size in the library and  $G$  is the size of the genome from which the library was derived (Clarke & Carbon, 1976). Based on an average insert size of 25 kb in the KIM5s libraries and a genome size of KIM5s similar to that reported for *R. meliloti*, 6500 kb (Sobral *et al.*, 1991), the predicted percentage of isolates containing the target DNA after the plant enrichments increases dramatically as the enrichment power increases and/or as the size of the target DNA decreases (Fig. 1). Based on this relationship, if  $n$  isolates are selected after the enrichments, the probability that at least one will contain the target DNA is  $1 - (1 - p)^n$  where  $p$  is the predicted proportion of isolates containing the target at a given target size and enrichment power. For example, if 20 isolates are screened, the probability of identifying at least one with a cosmid conferring enhanced competitiveness is greater than 0.95 ( $> 0.95$ ) if the enrichment is  $10^3$ - to  $10^5$ -fold and the target is any size up to 24 kb.

Table 2. Evaluation of the role of cosmids in the enhanced competitiveness of strains derived from CE3

Each test strain was applied with CE3013 in approximately a 1:40 ratio (test strain: CE3013). The results are shown as the percentage of six nodules per plant occupied by the test strain alone (%T) and by either CE3013 alone or both CE3013 and the test strain (%C+B). Values are means from six plants. Values followed by the same letter do not differ significantly at  $P = 0.05$  when compared by a Fischer's LSD (least significant difference) on the arc sine square root transformation of the proportion of nodules occupied by the test strain.

| Cosmids in original chromosomal background* |     |                     | Cosmids reintroduced into CE3† |     |                   | Original chromosomal background cured of cosmid‡ |     |                   |
|---|-----|---------------------|--------------------------------|-----|-------------------|--|-----|-------------------|
| Test strain                                 | % T | % C+B               | Test strain                    | % T | % C+B             | Test strain                                      | % T | % C+B             |
| CE3(pOWR3)                                  | 7   | 93 <sup>abc</sup>   | CE3'§                          | 3   | 97 <sup>a</sup>   | CE3(c3)  | 3   | 97 <sup>ab</sup>  |
| CE3(pOWR1)                                  | 26  | 74   <sup>cde</sup> | CE3'(pOWR1)                    | 0   | 100 <sup>a</sup>  | CE3(c1)  | 31  | 69 <sup>d</sup>   |
| CE3(pOWR2)                                  | 15  | 85 <sup>bcd</sup>   | CE3'(pOWR2)                    | 0   | 100 <sup>a</sup>  | CE3(c2)  | 42  | 58 <sup>ef</sup>  |
| CE3(pOWR14)                                 | 14  | 86 <sup>bcd</sup>   | CE3'(pOWR14)                   | 0   | 100 <sup>a</sup>  | CE3(c14)   | 31  | 69 <sup>def</sup> |
| CE3(pOWR15)                                 | 31  | 69 <sup>def</sup>   | CE3'(pOWR15)                   | 0   | 100 <sup>a</sup>  | CE3(c15)   | 46  | 54 <sup>f</sup>   |
| CE3(pOWR52)                                 | 23  | 77 <sup>cde</sup>   | CE3'(pOWR52)                   | 3   | 97 <sup>ab</sup>  | CE3(c52)   | 43  | 57 <sup>ef</sup>  |
| CE3(pOWR58)                                 | 28  | 72 <sup>cde</sup>   | CE3'(pOWR58)                   | 0   | 100 <sup>a</sup>  | CE3(c58)   | 47  | 53 <sup>f</sup>   |
| CE3(pOWR71)                                 | 70  | 30 <sup>g</sup>     | CE3'(pOWR71)                   | 3   | 97 <sup>ab</sup>  | CE3(c71)   | 50  | 50 <sup>g</sup>   |
| CE3(pOWR73)                                 | 33  | 67 <sup>def</sup>   | CE3'(pOWR73)                   | 3   | 97 <sup>ab</sup>  | CE3(c73)   | 31  | 69 <sup>de</sup>  |
| CE3(pOWR75)                                 | 37  | 63   <sup>ef</sup>  | CE3'(pOWR75)                   | 7   | 93 <sup>abc</sup> | CE3(c75)   | 33  | 67 <sup>de</sup>  |

\* Strains isolated after two cycles of plant nodulation.

† Strains constructed by introducing cosmids isolated from the above strains into the parent CE3.

‡ Strains constructed by curing the cosmids from the strains listed in the first column.

§ CE3' was a stock culture of CE3.

|| These values are from a separate experiment.

Table 3. Competitiveness of  $Cos^-Cmp^{++}$  strains derived from strain CE3 relative to strain KIM5s

| Test strain | Inoculum ratio* | Nodule occupancy† |         |                   |
|-------------|-----------------|-------------------|---------|-------------------|
|             |                 | % Test            | % KIM5s | % Both‡           |
| CE3         | 12:1            | 17                | 69      | 14 <sup>a</sup>   |
| CE3(c3)     | 16:1            | 8                 | 53      | 39 <sup>a</sup>   |
| CE3(c1)     | 11:1            | 76                | 7       | 17 <sup>bcd</sup> |
| CE3(c2)     | 14:1            | 61                | 22      | 17 <sup>bc</sup>  |
| CE3(c14)    | 13:1            | 89                | 6       | 6 <sup>cd</sup>   |
| CE3(c15)    | 9:1             | 82                | 16      | 3 <sup>bc</sup>   |
| CE3(c52)    | 10:1            | 58                | 23      | 18 <sup>bc</sup>  |
| CE3(c58)    | 16:1            | 42                | 27      | 32 <sup>bcd</sup> |
| CE3(c71)    | 11:1            | 69                | 19      | 11 <sup>bcd</sup> |
| CE3(c73)    | 9:1             | 100               | 0       | 0 <sup>d</sup>    |
| CE3(c75)    | 11:1            | 48                | 27      | 26 <sup>b</sup>   |

\* Ratio of test strain to KIM5s in the inoculum.

† Percentage of six nodules per plant occupied by the test strain alone (% Test), by KIM5s alone (% KIM5s), and by both the test strain and KIM5s (% Both). Values are means from six plants.

‡ Values followed by the same letter do not differ significantly at  $P = 0.05$  when compared by a Fischer's LSD on the arc sine square root transformation of the proportion of nodules occupied by the test strain.

It is  $> 0.95$  if the enrichment is 100-fold and the target is less than 14 kb, but it is only 0.52 if the enrichment is 10-fold and the target is as small as 1 kb.

### Evaluation of the selective power of the plant

To explore the power of the plant to select highly competitive strains from a mixture of strains, we measured the extent of the enrichment for KIM5s relative to CE3 after two plant passages. Application of a 1:50 (KIM5s:CE3) inoculum mixture to five bean seeds resulted in a ratio of 30:1 in the nodule isolate mixture, demonstrating a 1500-fold enrichment for KIM5s. After a second passage, this ratio was 1100:1, demonstrating an additional 37-fold enrichment for KIM5s, and an overall enrichment of  $5.6 \times 10^4$ . In two replicate enrichment series, each involving two plant passages, we observed a  $10^3$ - to  $10^5$ -fold enrichment for KIM5s relative to CE3.

### Competitiveness of the selected nodule isolates

The strategy for the enrichment for transconjugants with enhanced competitiveness is illustrated in Fig. 2. Transconjugants containing 4000 independent cosmids were subjected to *in planta* enrichments in groups of 48 cosmids. A total of 75 nodule isolates, designated CE3(pOWR1) to CE3(pOWR75), were screened individually for enhanced competitiveness. We used the strain CE3013 as the reference strain in these competitions, since CE3013 was indistinguishable from CE3

Table 4. *Competitiveness of nodule isolates selected after plant passage of strains CE3(pLAFR3), CE3(pLA2917), CE3'(pOWR75) and CE3*

| Test strain       | Source* | Inoculum ratio† | % T‡ | % C + B | P§    |
|-------------------|---------|-----------------|------|---------|-------|
| CE3(pLAFR3)       | Control | 1:43            | 6    | 94      | 0.93  |
|                   | N1      | 1:31            | 3    | 97      |       |
|                   | N2      | 1:36            | 3    | 97      |       |
|                   | N3      | 1:39            | 4    | 96      |       |
| CE3(pLA2917)      | Control | 1:42            | 0    | 100     | 0.24  |
|                   | N1      | 1:47            | 6    | 94      |       |
|                   | N2      | 1:32            | 11   | 89      |       |
|                   | N3      | 1:35            | 11   | 89      |       |
| CE3'(pOWR75)      | Control | 1:45            | 4    | 96      | 0.85  |
|                   | N1      | 1:48            | 4    | 96      |       |
|                   | N2      | 1:36            | 6    | 94      |       |
|                   | N3      | 1:44            | 13   | 87      |       |
| CE3(pOWR75)       | Control | 1:53            | 61   | 39      | 0.003 |
| CE3               | Control | 1:24            | 0    | 100     | 0.47  |
| CE3 – mock mating | N1      | 1:28            | 0    | 100     |       |
|                   | N2      | 1:20            | 0    | 100     |       |
|                   | N3      | 1:23            | 6    | 94      |       |

\* Control, parent strain from stock culture; N, strain isolated from a nodule selected after the parent strain was subjected to two plant passages.

† Ratio of test strain to CE3013 in the inoculum.

‡ Percentage of six nodules per plant occupied by the test strain alone (%T) and by either CE3013 alone or both the test strain and CE3013 (% C + B). Values are means from six plants.

§ Obtained from an ANOVA (analysis of variance) on the arc sine square root transformation of the proportion of nodules occupied by the test strain. Separate ANOVAs were performed on each of the CE3 (pLAFR3) control strain and the following three nodule isolates, the CE3 (pLA2917) control strain and the following three nodule isolates, the CE3'(pOWR75) control strain and the following three nodule isolates, and the CE3 control strain and the following three nodule isolates.

|| P value from an ANOVA on the arc sine square root transformation of the proportion of nodules occupied by each CE3'(pOWR75), the following three nodules isolates, and CE3(pOWR75).

in competitiveness (data not shown), but could be differentiated from CE3 or the cosmid-containing derivatives by antibiotic resistance.

After three rounds of enrichment, we identified nine nodule isolates that were more competitive than CE3 in competitions with CE3013. Six contained pLA2917-derived cosmids; three contained pLAFR3-derived cosmids. In competitions between each of the nine test strains and a 40-fold excess of CE3013, the test strains typically occupied 15–40% of the nodules. When outnumbered 100-fold by CE3013 in the inocula, these strains occupied 4–23% of the nodules. In contrast, when CE3 was outnumbered 40- or 100-fold by CE3013, it typically occupied only 0–3% of the nodules. We designated the phenotype of the nine strains that had enhanced competitiveness as Cos<sup>+</sup>Cmp<sup>++</sup> (Table 1).

#### *Role of the cosmids in enhanced competitiveness*

To evaluate similarities in the cosmids of the nine Cos<sup>+</sup>Cmp<sup>++</sup> strains, the *EcoRI* DNA restriction digest patterns of the cosmids from the strains were compared. Other than the fragment containing vector DNA, no fragment was common to all or even most of the cosmids, and no two cosmids shared more than four

fragments of the same size. This demonstrates that the nine cosmids were distinct from each other.

We isolated the cosmids from the Cos<sup>+</sup>Cmp<sup>++</sup> strains and reintroduced them into a stock culture of CE3 to determine whether the cosmids conferred a competitive advantage on the recipients. We also cured the cosmids from the nine original Cos<sup>+</sup>Cmp<sup>++</sup> nodule isolates. Competitions were performed with each of these constructed strains and CE3013 at inoculum ratios of approximately 1:40 (Test strain:CE3013). Curing the Cos<sup>+</sup>Cmp<sup>++</sup> strains of their cosmids produced strains that remained highly competitive (Table 2). Furthermore, reintroducing the cosmids into CE3 did not enhance its competitiveness, since there were no significant differences in nodule occupancy by the test strain among the control strains CE3, CE3(pOWR3), CE3(c3), and all of the strains containing a reintroduced cosmid (Table 2). Together, these results indicate that the enhanced competitiveness of the nine Cos<sup>+</sup>Cmp<sup>++</sup> strains was not associated with the cosmids.

In three independent measurements, when outnumbered in the inocula approximately 40-fold by CE3013, the nine Cos<sup>-</sup> strains derived from the Cos<sup>+</sup>Cmp<sup>++</sup> strains occupied between 31–50% of the nodules. In contrast, at similar inoculum ratios, CE3

Table 5. *Competitiveness of nodule isolates selected after plant passage of cultures of strain CE3*

| Test strain* | Ratio of test strain to CE3013 in the inoculum |                    |           |                   |
|--------------|--|--------------------|-----------|-------------------|
|              | 1:10-1:20                                      |                    | 1:23-1:35 |                   |
|              | % T†   | % C+B‡             | % T†      | % C+B‡            |
| CE3          | 3  | 97 <sup>ab</sup>   | 9         | 91 <sup>abc</sup> |
| CE3          | 6  | 94 <sup>abc</sup>  | 3         | 97 <sup>ab</sup>  |
| N1           | 0  | 100 <sup>a</sup>   | 0         | 100 <sup>a</sup>  |
| N2           | 3  | 97 <sup>ab</sup>   | 4         | 96 <sup>abc</sup> |
| N3           | 4  | 96 <sup>abc</sup>  | 6         | 94 <sup>abc</sup> |
| N4           | 6  | 94 <sup>abc</sup>  | 0         | 100 <sup>a</sup>  |
| N5           | 6  | 94 <sup>abc</sup>  | 4         | 96 <sup>abc</sup> |
| N6           | 6  | 94 <sup>abc</sup>  | 10        | 90 <sup>abc</sup> |
| N7           | 8  | 92 <sup>abc</sup>  | —         | —                 |
| N8           | 15   | 85 <sup>abcd</sup> | 0         | 100 <sup>a</sup>  |
| N9           | 19   | 81 <sup>d</sup>    | 0         | 100 <sup>a</sup>  |
| N10          | 20   | 80 <sup>d</sup>    | 6         | 94 <sup>abc</sup> |
| N11          | 33   | 67 <sup>d</sup>    | 14        | 86 <sup>c</sup>   |

\* CE3 was from a stock culture; N, strain isolated from a nodule selected after a culture of CE3 was subjected to two plant passages.

† Percentage of six nodules per plant occupied by the test strain alone (%T) and by either CE3013 alone or both the test strain and CE3013 (%C+B). Values are means from six plants.

‡ Values followed by the same letter do not differ significantly at  $P = 0.05$  when compared by a Fischer's LSD on the arc sine square root transformation of the proportion of nodules occupied by the test strain.

typically occupied 0–3% of the nodules. These  $\text{Cos}^- \text{Cmp}^{++}$  strains also occupied significantly more nodules than did CE3 or CE3(c3) in competitions with KIM5s (Table 3).

#### *Molecular characterization of the $\text{Cos}^- \text{Cmp}^{++}$ strains*

Several genetic events could have resulted in the enhanced competitiveness of the  $\text{Cos}^- \text{Cmp}^{++}$  strains. To determine whether part or all of each cosmid had integrated into the genome of the  $\text{Cos}^-$  strains, cosmids from the  $\text{Cos}^+ \text{Cmp}^{++}$  strains were used to probe *EcoRI*-digested genomic DNA from CE3 and the  $\text{Cos}^- \text{Cmp}^{++}$  strains. In every case, the probe hybridized with fragments of the same size and intensity in CE3 and in the cured strains (data not shown and Beattie, 1991).

To test whether the enhanced competitiveness of the  $\text{Cos}^- \text{Cmp}^{++}$  strains resulted from cosmid-induced genetic changes, we subjected three replicate cultures each of CE3'(pOWR75), CE3(pLAFR3), and CE3(pLA2917) to two plant passages and measured the competitiveness of selected nodule isolates. We chose CE3'(pOWR75) to test whether pOWR75, which did not affect competitiveness when simply reintroduced into CE3 (Table 2), could induce a change in competitiveness during plant passage. We found that none of the isolates selected after two plant passages occupied significantly more nodules in competition with CE3013 than did the respective

parent strains (Table 4). If cosmid-induced changes in competitiveness occurred at a frequency of  $10^{-2}$  or greater, then the probability of finding at least one  $\text{Cmp}^{++}$  isolate out of three was  $> 0.95$ , assuming an enrichment power of  $\geq 10^3$ . Similarly, if cosmid-induced changes occurred at frequencies of  $10^{-3}$  or  $10^{-4}$ , then  $P > 0.88$  or  $P > 0.25$ , respectively. Although we may not have examined enough cosmids to detect low frequency cosmid-induced changes ( $\leq 10^{-3}$ ), such low frequency changes were unlikely to be responsible for the enhanced competitiveness of the original  $\text{Cmp}^{++}$  isolates. Assuming an enrichment power of  $\geq 10^3$ , the probability of identifying at least one  $\text{Cmp}^{++}$  isolate among the original 75 isolates would be reduced from greater than 0.1 to well below 0.07 if the frequency at which  $\text{Cmp}^{++}$  isolates arose were reduced from  $10^{-2}$  to  $10^{-3}$ .

To test whether the enhanced competitiveness of the  $\text{Cos}^- \text{Cmp}^{++}$  strains resulted from cosmid-independent spontaneous changes that were induced and/or enriched for by plant passage, we subjected 11 replicate cultures of CE3 to two cycles of plant passage and measured the competitiveness of selected nodule isolates. Three isolates, N9, N10, and N11, appeared to be moderately enhanced in competitiveness (Table 5). All three occupied significantly more nodules than did CE3 at the lower inoculum ratio, i.e. 10- to 20-fold excess of CE3013 ( $P < 0.05$ ). Although none of the three strains were enhanced in competitiveness to the same extent as the  $\text{Cos}^- \text{Cmp}^{++}$  strains, these data show that genetic variants with a moderate increase in competitiveness arose spontaneously. To test for a role of the conjugation procedure in inducing or enriching for genetic changes that cause increased competitiveness, we subjected three replicate cultures of CE3 to a mock-mating protocol and to two cycles of plant passage. None of the selected isolates were  $\text{Cmp}^{++}$  (Table 4).

#### *Physiological characterization of the $\text{Cos}^- \text{Cmp}^{++}$ strains*

CE3, CE3(c3), and all nine  $\text{Cos}^- \text{Cmp}^{++}$  strains grew in a rich medium in batch culture with doubling times between 2.29 and 2.40 h. There were no significant differences among the strains in growth rate or in the number of bacteria required to induce nodules. Additionally, acetylene reduction assays showed that CE3, CE3(c3), and the nine  $\text{Cos}^- \text{Cmp}^{++}$  strains were all capable of fixing nitrogen (Beattie, 1991).

## Discussion

To identify nodulation competitiveness genes in *R. leguminosarum* bv. *phaseoli*, we attempted to transfer genes that confer enhanced competitiveness from a superior competitor, strain KIM5s, to an inferior

competitor, strain CE3. We identified nine highly competitive derivatives. Surprisingly, we found that the enhanced competitiveness of these nine strains did not appear to be due to the introduced DNA.

We found no evidence of cosmid insertion or of genetic changes induced by the cosmids in the Cos<sup>-</sup>Cmp<sup>++</sup> strains (i.e. the strains that were cured of the cosmids but remained highly competitive). Our studies were limited by the fact that all of the cosmids used in these studies were isolated after the original trans-conjugants were subjected to the enrichments. Therefore, we cannot eliminate the possibility that these cosmids were altered during the course of the enrichments by a process such as loss of a region by homologous recombination or transposition.

The most plausible explanation for the altered competitiveness of the Cos<sup>-</sup>Cmp<sup>++</sup> strains is that it resulted from spontaneous genetic changes. In experiments designed to reconstruct the events that produced the nine highly competitive strains, we identified several isolates that arose spontaneously and were more competitive than CE3, although none were as competitive as the original nine Cos<sup>-</sup>Cmp<sup>++</sup> strains. That none were highly competitive could be due to the absence of conditions necessary to induce such mutations or to chance, since the probability of identifying at least one Cmp<sup>++</sup> isolate among the 11 from the CE3 enrichments was only 0.75, based on finding Cmp<sup>++</sup> isolates at a frequency of 12% among the original 75 isolates. To identify at least one Cmp<sup>++</sup> isolate when such isolates arose at a frequency of 12%, with  $P > 0.95$ , we would need to screen at least 24 isolates.

Spontaneous changes that might have caused the Cmp<sup>++</sup> phenotype include point mutations, insertion of mobile elements, and genomic rearrangements. Although insertion sequences that transpose at frequencies of  $10^{-2}$  to  $10^{-3}$  have been reported in several *Rhizobium* spp. (Dusha *et al.*, 1987; Priefer *et al.*, 1980; Ruvkun *et al.*, 1982), none have been reported in *R. leguminosarum* bv. *phaseoli*. Genomic rearrangements have been shown to occur at frequencies as high as  $10^{-2}$  in the parent strain of CE3, CFN42 (Brom *et al.*, 1991; Romero *et al.*, 1991). We examined the Cos<sup>-</sup>Cmp<sup>++</sup> strains but did not find evidence of rearrangements with endpoints in the *nifH* gene (Beattie, 1991), which are known to occur at a high frequency in CFN42 (Romero *et al.*, 1991).

Genetic alterations that result in increased competitiveness have been observed previously. Studies have correlated the presence of a plasmid (Bromfield *et al.*, 1985; Hynes, 1990; Martínez-Romero & Rosenblueth, 1990; Sanjuan & Olivares, 1991*b*) and the presence of multiple copies of a gene (Sanjuan & Olivares, 1991*a*) to increased competitiveness. Others have correlated the inactivation of genes involved in EPS production (Zdor

& Pueppke, 1991) and succinate sensitivity (Urban, 1988) with increased competitiveness. Recently, Bhagwat & Keister (1992) used an elegant subtractive hybridization strategy to clone a gene that affects competitiveness of *B. japonicum*. In addition to the many genetic changes reported to increase competitiveness, our study suggests that genetic changes that increase competitiveness may occur spontaneously.

We may not have identified nodulation competitiveness genes in KIM5s for several reasons. First, genes that confer enhanced competitiveness simply may not exist in KIM5s. Perhaps the competitiveness of KIM5s is due to the *lack* of certain factors present in CE3. Second, if such genes do exist, they may be scattered throughout the KIM5s genome, or they may not be functionally dominant in CE3. And third, if such genes were successfully transferred to CE3, they may have been expressed only at a low level or they may have resulted in only a moderate increase in competitiveness. We may not have identified them in this study because the increases in competitiveness that they caused were much smaller than the increases we observed, and therefore we did not choose the appropriate trans-conjugants for further study. If KIM5s contained a region of DNA that could be transferred to CE3 and conferred competitiveness equal to that of KIM5s, the probability that we would have identified it among the 75 isolates screened was  $> 0.99$ . Therefore, assuming that our library was random and fairly complete, such a region probably does not exist in KIM5s.

The  $10^3$ - to  $10^5$ -fold enrichment for KIM5s from a mixture of KIM5s and CE3 demonstrates that the host plant can be a very powerful tool for enriching for highly competitive strains. Theoretically, the success of the direct cloning and enrichment approach depends on the extent to which the target DNA enhances the competitiveness of the recipient strain. As shown in Fig. 1, even a  $10^3$ -fold enrichment should result in at least 50% of the isolates containing the target DNA, if the required target DNA region were as large as 18 kb. This corresponds to a probability of greater than 0.99 that at least one isolate would contain the target region if 75 isolates are screened. However, as we found, other factors can influence the results. It should be possible to directly isolate cosmids containing genes that can confer enhanced competitiveness, such as *tfx* (Triplett, 1990), *nfe* (Sanjuan & Olivares, 1991*b*), and *nifA* (Sanjuan & Olivares, 1991*a*). Therefore, the lack of success of the approach with KIM5s and CE3 does not reflect on its potential for success with other strains.

In summary, in an attempt to transfer genes that confer enhanced nodulation competitiveness from a superior competitor of *R. leguminosarum* bv. *phaseoli* to an inferior competitor, we isolated several highly

competitive variants, whose altered competitiveness was not associated with the introduced DNA. Since they were very similar to their parent, any physiological differences between the variants and their parent are likely to be associated with, and may be causal to, their enhanced competitiveness. These variants, therefore, may provide insight into bacterial traits that affect successful nodulation competitiveness.

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