Transcriptional activity of the symbiotic plasmid of *Rhizobium etli* is affected by different environmental conditions

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Global patterns of transcriptional activity of the symbiotic plasmid (pSym) of *Rhizobium etli* were studied under a variety of environmental conditions, including some relevant to the symbiotic process. ³²P-labelled single-stranded complementary DNAs synthesized from total RNA were used as hybridization probes against an ordered collection of cosmid clones that covered the whole pSym. Our results showed that, under aerobic conditions, discrete regions of the pSym are differentially transcribed depending on the carbon and nitrogen sources employed. In general, poor carbon or nitrogen sources allowed greater expression than rich ones. Time-course experiments with the *nod* gene inducer genistein led us to the identification of new regions responsive to this flavonoid. Widespread transcription was observed during microaerobiosis, but not under aerobic conditions, indicating that oxygen concentration is a major effector of transcriptional activity in the pSym. This response was reduced, but not suppressed, in a *nifA* mutant, indicating the location of regions whose transcription may depend on other oxygen-sensitive regulators. During symbiosis, almost the entire pSym was actively transcribed and the transcription pattern was similar to that observed during microaerobiosis. The experimental approach described allowed the identification and localization of specific regions in the pSym whose expression depends on defined environmental stimuli.

Keywords: *Rhizobium etli*, symbiotic plasmid, pSym, nodulation, transcription

INTRODUCTION

In *Rhizobium* species, more than fifty different genes that participate in nodulation and nitrogen fixation have been identified (Fischer, 1994; Schultze *et al.*, 1994; Van Rhijn & Vanderleyden, 1995). Current research is mostly devoted to clarifying the role of specific genes in definite steps of the *Rhizobium*–legume interaction (Carlson *et al.*, 1994; Schultze *et al.*, 1994). In several *Rhizobium* species, most of these genes are clustered in a sector of a single large plasmid, the so-called symbiotic plasmid or pSym. Complete physical maps of the pSymb of some *Rhizobium* species have been published (Girard *et al.*, 1991; Perret *et al.*, 1991); genes involved in the symbiotic process cover

a relatively small zone of these megaplasmids. It is commonly assumed that some of the remaining genetic information is required for stable maintenance of the plasmid, or that it participates in other, as yet undescribed, processes.

The identification of many symbiotic genes has been achieved mainly through loss of function approaches, such as random transposon mutagenesis and/or gene fusion techniques. Cloning and sequencing of interesting regions and their surroundings has been useful, due to the observed clustering of genes involved in the symbiotic process (Sanjuan *et al.*, 1993). However, application of these approaches to the identification of regions responding to different environmental stimuli usually requires new mutant hunts or extensive sequencing efforts. Moreover, these strategies usually do not detect genes with a leaky phenotype when mutated. This situation may arise

Abbreviation: ³²P-sscDNA, ³²P-labelled single-stranded complementary DNA.

either when active reiterations (a common situation in *Rhizobium* species) or alternative pathways occur. These strategies may also fail to detect genes with an ancillary, albeit important, role in the symbiotic interaction. These limitations probably result in an underestimation of the actual number of genes participating in bacteria-plant interactions.

A global approach for the detection of transcriptional activity under specific conditions offers an alternative to circumvent the above problems. This strategy relies on the quantitative detection of regions undergoing active transcription under diverse experimental conditions. Application of this strategy to sectors of the genome where a comprehensive map is available allows a positional evaluation of gene activity in response to the stimulus to be tested (Chuang *et al.*, 1993). A similar approach was recently employed on the pSym of *Rhizobium* sp. NGR234 (Fellay *et al.*, 1995) to identify regions whose expression is enhanced by plant root exudates or specific flavonoids.

The use of this technique, coupled with the existence of a complete map of the pSym of *Rhizobium etli* (Girard *et al.*, 1991), allows us to evaluate the extent of transcriptional activity under different conditions. In this work, we report quantitative maps of gene expression in the pSym in response to growth on several carbon and nitrogen sources, flavonoid induction and microaerobic conditions, and during symbiosis.

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used are listed in Table 1. *Rhizobium* strains were grown at 30 °C in PY rich medium (Noel *et al.*, 1984) or in Y minimal medium with different carbon and nitrogen sources, each at a concentration of 10 mM (Bravo & Mora, 1988). Carbon sources were sodium succinate, glucose or glycerol; nitrogen sources were ammonium chloride, potassium nitrate, sodium glutamate or glutamine. *Escherichia coli* strains were grown at 37 °C in LB medium (Miller, 1972). Antibiotics were added at the following concentrations: chloramphenicol (Cm), 25 µg ml⁻¹ (*E. coli*) or 10 µg ml⁻¹ (*R. etli*); kanamycin (Km), 30 µg ml⁻¹ (*R. etli*); nalidixic acid (Nal), 20 µg ml⁻¹ (*R. etli*); spectinomycin (Sp), 50 µg ml⁻¹ (*E. coli* or *R. etli*); tetracycline, 10 µg ml⁻¹ (*E. coli*) or 5 µg ml⁻¹ (*R. etli*).

Genetic manipulations. Plasmid pGC479 is a pSUP202 derivative (Simon *et al.*, 1983) that carries a 2170 bp *Hind*III fragment containing the *nifA* gene of *R. etli* CFN42 (GenBank accession no. U31630). As a first step in the generation of a mutation in the *nifA* gene from *R. etli*, a 394 bp fragment from *nifA* (position 1165–1559) was excised from pGC479 by digestion with *Eco*RI. A 2.0 kb Ω Sp/Sm (Prentki & Krisch, 1984) cassette was then ligated in its place, thus generating pGC480. Double recombinants carrying the *nifA*_Δ:: Ω Sp/Sm allele in *R. etli* were obtained by standard genetic procedures (Martínez *et al.*, 1990). This replacement was confirmed by Southern hybridization (Ausubel *et al.*, 1987) using the *nifA* gene of *R. etli* as a probe, and also by plant tests in which this strain was completely ineffective for nitrogen fixation. The resulting *nifA* mutant strain was designated CFNX247.

Construction of a strain lacking the pSym but harbouring the wild-type *nifA* gene on a replicating plasmid was done as

follows. The strain CFNX248 was constructed by a triparental mating using *R. etli* CFNX89 as the recipient and HB101/pNC206 (Table 1) as cointegrative plasmid for HB101/pGC479; cointegration between these plasmids occurred through recombination among ampicillin resistance genes shared by these plasmids. The derivatives were selected as Nal^r Cm^r Km^r transconjugants. They were analysed by Southern blot hybridization and plasmid profiles (Eckhardt, 1978).

Growth conditions. Cultures were grown to mid-exponential phase in PY medium. Cells were collected by centrifugation (at 6000 g in a Sorvall SS34 rotor), washed with sterile minimal medium and concentrated 100-fold. Fresh Y minimal medium containing the desired carbon and nitrogen sources was inoculated with these suspensions at an initial OD₅₄₀ of 0.05. For growth under microaerobic conditions, sodium succinate and ammonium chloride were used as carbon and nitrogen sources, respectively. Twenty milliliters of these cultures were incubated either in 150 ml bottles closed with a cotton stopper (aerobic conditions), or in 150 ml bottles closed with an airtight stopper and previously flushed with several volumes of an oxygen/argon (1:99, v/v) mixture (microaerobic conditions). Both types of cultures were grown with shaking (200 r.p.m.) for 8 h at 30 °C. Cells were then collected by centrifugation (6000 g) at 4 °C and pellets were stored at -70 °C until RNA isolation.

Genistein induction. Cultures grown for 1 d on PY agar plates were used to inoculate a minimal medium with the following composition: sucrose, 5 mM; KNO₃, 1 mM; K₂HPO₄, 1 mM; KH₂PO₄, 7 mM; MgSO₄, 1 mM; NaCl, 2 mM; CaCl₂, 0.1 mM; biotin, 0.4 mM; ferric citrate, 0.2 mM; H₃BO₃, 46 µM; MnSO₄, 9 µM; ZnSO₄, 0.076 µM; CuSO₄, 0.3 µM; Na₂MoO₄, 0.4 µM. Cultures were inoculated at an initial OD₆₀₀ of 0.1 and grown with shaking (200 r.p.m.) for 4 h at 30 °C. Genistein was then added at a final concentration of 1.2 µM, with further incubation for 1, 4, 8 or 24 h. Control cultures were treated similarly, but without genistein addition. At the appropriate times, samples were withdrawn and cells were collected by centrifugation (6000 g) at 4 °C and pellets were stored at -70 °C until RNA isolation. To verify the production of nodulation factors, parallel 1 ml cultures were labelled 3 h after genistein addition with 0.2 µCi (7.4 kBq) D-[1-¹⁴C]glucosamine, and grown for 12 h. Culture supernatants were analysed by thin-layer chromatography as described previously (Mendoza *et al.*, 1995).

Plant growth. Surface-sterilized *Phaseolus vulgaris* cv. Negro Jamapa seeds (kindly provided by PRONASE) were germinated under sterile conditions in trays containing vermiculite. After 3 d at 30 °C, the seedlings were transferred to 1 l plastic pots filled with sterile vermiculite and inoculated with 1 ml of an overnight culture (in PY medium) of *R. etli* CFN42. Plants were grown in a greenhouse and watered periodically with a N-free nutrient solution (Vincent, 1970) as previously described (Romero *et al.*, 1988). After 25 d, nodules were picked out from the roots, washed briefly with diethyl-pyrocyanate-treated water and surface-sterilized. They were then immediately frozen in a dry-ice-ethanol bath and stored at -20 °C until further use.

Bacteroid purification. Bacteroids were purified from root nodules (3 g, fresh wt), obtained as described above, by centrifugation through self-generated Percoll gradients (Reibach *et al.*, 1981). Purified bacteroids were utilized immediately for RNA purification.

RNA purification. Total RNA extraction from purified bacteroids and from cells grown under aerobic or microaerobic conditions was done by the acid phenol procedure, employing a commercial kit (RNAid kit, BIO101). RNA was quantified by absorbance measurements and electrophoresed under de-

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
Strain		
<i>Rhizobium etli</i>		
CFN42	Wild-type strain	Quinto <i>et al.</i> (1985)
CFNX89	CFN42 derivative lacking the pSym	Brom <i>et al.</i> (1992)
CFNX247	CFN42 derivative carrying the <i>nifA_Δ::ΩSp/Sm</i> allele	This work
CFNX248	CFNX89 derivative carrying a cointegrate plasmid pNC206/pGC479 (<i>nifA⁺</i>)	This work
<i>Escherichia coli</i>		
HB101	Host for recombinant plasmids	Boyer & Roulland-Dussoix (1969)
Plasmids		
pSUP202	Suicide vector, Ap ^r Cm ^r Tc ^r	Simon <i>et al.</i> (1983)
pNC206	A deleted RP4 derivative, Ap ^r Km ^r , self-replicating in <i>Rhizobium</i> spp.	A. Pühler, Universität Bielefeld, Germany.
pRK2013	Conjugation helper	Figurski & Helinski (1979)
pGC479	A pSUP202 derivative carrying the wild-type <i>nifA</i> gene	This work
pGC480	A pGC479 derivative carrying the <i>nifA_Δ::ΩSp/Sm</i> allele	This work
cGD collection	Ordered cosmid collection from the pSym of <i>R. etli</i> CFN42	Girard <i>et al.</i> (1991)

naturing conditions in agarose-formaldehyde gels (Ausubel *et al.*, 1987).

Synthesis of total ³²P-labelled single-stranded complementary DNA (³²P-sscDNA). This was done by the random hexamer priming method (Sambrook *et al.*, 1989). The reaction mixture contained 1 µg total RNA; 0.3 µg of a random hexanucleotide (Amersham); 0.5 mM dGTP, dATP and dTTP; 5 µM dCTP; 80 µCi [α-³²P]dCTP [6000 Ci mmol⁻¹ (222 TBq mmol⁻¹)]; 20 units placental ribonuclease inhibitor (Gibco BRL); 5 mM DTT; 400 units M-MLV Reverse Transcriptase (Gibco BRL) in the presence of first strand buffer (Gibco BRL); in a total volume of 30 µl. The reaction mixture was incubated for 90 min at 37 °C. To hydrolyse the RNA templates, EDTA, SDS and NaOH were added at final concentrations of 50 mM, 0.4% and 0.36 M, respectively, and the mixture was incubated for 30 min at 68 °C. For neutralization, Tris buffer (pH 7.4) and HCl were then added at final concentrations of 0.33 M and 0.2 M, respectively. Samples were precipitated with ethanol in the presence of tRNA as carrier, resuspended in 0.1 M NaOH and used as hybridization probes (A. Garay, personal communication).

Hybridization conditions and data analysis. Equal amounts of the cosmids that cover the whole pSym of *R. etli* CFN42 (quantified spectrophotometrically) were digested with *Bam*HI, electrophoresed in 1% (w/v) agarose gels and transferred to Nylon membranes (Hybond-N+, Amersham) (Ausubel *et al.*, 1987). DNA blots were hybridized in a solution containing 50% (v/v) formamide as described previously (Girard *et al.*, 1991). Hybridization signals were integrated by scanning the autoradiographs with a GS300 scanning densitometer using the GS-365W data system (Hoefer Scientific Instruments), taking care that measurements were within the linear range of the film.

RESULTS

Experimental design

In order to study the global transcriptional patterns of the pSym of *R. etli* CFN42 under different conditions, we took advantage of the physical map of this plasmid, previously established in our laboratory. This map was assembled from an ordered cosmid collection, comprising 85 *Bam*HI fragments, which are numbered consecutively on the circular map from an arbitrary start in the *nifHDK* region a (Girard *et al.*, 1991). Hybridization with probes representing the total RNA population from different conditions against the whole cosmid set allows us to determine the extent and localization of transcriptional activity on the pSym. To this end, total RNA was isolated from cultures grown under different conditions and from bacteroids (see Methods). These RNAs were used as templates for the synthesis of ³²P-sscDNAs, which were then employed as probes in hybridization experiments against the whole cosmid collection. In these experiments, signal intensity in the autoradiographs was taken as a measure of the relative abundance of the mRNA corresponding to a certain region, i.e. its degree of expression. Pairwise comparison between treatments allowed the determination of induction or repression ratios, which are defined as the quotient between the conditions to be compared. To make valid comparisons between the different data sets, densitometric scanings were normalized according to the total radioactivity in the probes, exposure time and amount of cosmid DNA loaded in the

Fig. 1. Detection of transcriptional patterns of the *R. etli* pSym and their quantification. The patterns were obtained using as probes ^{32}P -sscDNAs from strain CFN42 grown in succinate-ammonium medium under aerobic (a, b) or microaerobic (c, d) conditions. (a, c) Southern blot hybridizations of a set of cosmid clones from the pSym digested with *Bam*HI. Lanes: 1, cGD7 (bands 80–2); 2, cGD102 (bands 1–7); 3, cGD101 (bands 3–12); 4, cGD28 (bands 9–21); 5, cGD15 (bands 20–24); 6, cGD35 (bands 21–26); 7, cGD45 (bands 24–31). (b, d) Results of densitometric integration of hybridization signals for the whole pSym. Bar numbers indicate the position of specific fragments on the map (Fig. 3a).

gels, as described in Methods. An example of this type of analysis is shown in Fig. 1.

This experimental design assumes that the signals observed are due to transcription of sequences in the pSym, which are specifically detected through high-stringency hybridization against our cosmid collection. Although this is a reasonable assumption, reiteration of some potentially transcribable regions occurs in other replicons in the cell (Girard *et al.*, 1991). To exclude the possibility that detectable transcription originated from these reiterated regions, RNA was isolated from microaerobic cultures of strain CFNX248. This strain lacks the pSym but carries an active *nifA* gene on an autonomous, self-replicating plasmid (see Methods). Microaerobic conditions in the wild-type strain provoked a widespread, *nifA*-dependent transcription in the pSym (see below). If detectable transcription originated from reiterated DNA, hybridization signals should be observed with a probe made using microaerobically-grown CFNX248. No hybridization signals were observed with ^{32}P -sscDNA synthesized from this strain (data not shown). Thus, the

transcriptional activity detected in our experiments originated from sequences on the pSym, and not from reiterated regions located elsewhere in the genome.

Carbon and nitrogen sources modulate transcriptional activity in the pSym

To evaluate the role of nutritional stimuli on pSym expression under aerobic conditions, transcript levels were analysed after growth in a variety of carbon and nitrogen sources. As shown in Fig. 1(a), growth of the wild-type strain in a C- and N-rich medium (succinate plus ammonium) was very restrictive in terms of transcriptional activity. Only two regions (bands 30 and 56) were expressed at a detectable level, while the rest of the plasmid was not expressed. This profile was taken as the baseline expression pattern of the pSym. To discern between effects caused by different N or C sources, media containing succinate and glutamine, glutamate or KNO_3 were used. For comparison of the effects between the different treatments, expression values obtained in any given treatment were divided by the corresponding values

obtained under the baseline condition (succinate plus ammonium), thus deriving induction ratios. Growth on glutamine (Fig. 2a) led to higher induction than ammonium. In contrast, growth on a moderate (glutamate, Fig. 2b) or poor (KNO_3 , Fig. 2c) nitrogen source provoked a gradual increase in induction ratios. Growth on KNO_3 led to the activation of 16 regions, with induction ratios ranging from 2.5 to 7.3. Growth on medium containing ammonium and glucose (Fig. 2d) or glycerol (Fig. 2e) as alternative carbon sources promoted higher transcriptional activity than succinate, leading to the activation of 10 regions at induction ratios ranging from 2.5 to 9.6. Thus, transcriptional activity in the pSym is modified by the type of nitrogen and carbon sources employed.

Temporal order of pSym expression in response to flavonoid induction

Flavonoid compounds are components of root exudates which are important mediators in the chemical communication between bacteria and leguminous plants. Flavonoids are responsible for the induction of genes involved in the nodulation process, but it has been suggested that they are also involved in other steps of the symbiotic interaction, such as competitiveness (Bhagwat & Keister, 1992; Sadowsky *et al.*, 1988). To identify the extent and location of pSym regions induced by the isoflavone genistein, cultures were grown in sucrose- KNO_3 minimal medium (see Methods) and expression values were obtained at different times after genistein addition. As a control, expression values were obtained from parallel cultures treated similarly, but without added genistein. Induction or repression ratios are expressed as the quotient of the values obtained in the presence vs the absence of genistein. Four different regions, differing in temporal order after induction, could be recognized (Fig. 2f–i). Members of region I, spanning bands 1–11, showed an early pattern of induction. Expression was detectable 1 h after genistein addition, achieving maximal levels 24 h after the onset of the induction period. In contrast, regions II–IV (bands 22–26; 43–51 and 80–85, respectively) were clearly induced only after 24 h. Under our conditions, production of Nod factors was detectable at 15 h after induction (data not shown). All the nodulation genes so far identified in *R. etli* are contained in region I and in part of region IV (see Fig. 3a and Discussion); however, regions II and III mark the location of new, isoflavone-induced sectors that might be involved in nodulation or other flavonoid-controlled processes.

Transcriptional activity of the pSym is regulated by oxygen concentration

Oxygen limitation is an important metabolic effector of expression of many genes involved in nitrogen fixation. The effect of this environmental variable was explored by growing the wild-type strain in a medium containing succinate and ammonium, under both aerobic and microaerobic conditions (see Methods). Extensive expression of

genes borne by the pSym was observed under microaerobic conditions: 67 out of 85 *Bam*HI bands were expressed (Fig. 1b). This is more clearly seen when induction ratios (microaerobic vs aerobic levels) are considered. As shown in Fig. 2j, oxygen limitation provokes widespread induction of expression in the pSym, with ratios ranging from 2 to 28. Transcription of most of the *nif* and *fix* genes in *Rhizobium* is dependent on the *nifA* gene product, under both microaerobic and symbiotic conditions (Fischer, 1994). A comparison of the induction ratios achieved under microaerobic conditions in the wild-type strain (Fig. 2j) and in the *nifA* mutant strain (Fig. 2k) allowed us to classify the different pSym regions according to their degree of dependency on the *nifA* gene product. Thirty-seven fragments showed an absolute dependency for this gene, i.e. they were expressed in the wild-type strain but not in the *nifA* mutant strain. Twenty-nine fragments showed lower induction ratios in the *nifA* mutant strain, suggesting a partial dependency for this transcriptional activator. The extent of this dependency varied widely, since the induction ratios ranged from 1.2 to values 10-fold larger in the wild-type as compared to the mutant strain.

These results indicate that (i) oxygen limitation leads to a dramatic induction in the transcriptional activity of the pSym, and (ii) that the NifA transcriptional activator partially controls this response.

General transcriptional activity of the pSym during symbiosis

The transcription pattern of the pSym during symbiosis was only studied in the wild-type strain, due to the low recovery of bacteroids from nodules induced by the *nifA* mutant. To this end, ^{32}P -sscdNA was synthesized from total bacteroid RNA of the wild-type strain. This ^{32}P -sscdNA was then used as a probe. Under symbiotic conditions, almost all the plasmid was actively transcribed. In fact, the absolute transcriptional levels during this stage were the highest among the conditions tested. As shown in Fig. 2(l), induction ratios during symbiosis vs aerobic conditions (succinate plus ammonium) may be as high as 46-fold. Two contiguous regions in the pSym, spanning fragments 80–85 and 1–29, contain most of the fragments with a high induction during symbiosis (Fig. 2l); many of the symbiotic genes detected in this organism are located here (see Discussion).

The induction ratios observed during symbiosis (Fig. 2l) are qualitatively similar to the ones seen during microaerobiosis (Fig. 2j). To further explore the observed similarity in transcription patterns, data were plotted as the quotient of the values during symbiosis vs microaerobiosis. As shown in Fig. 2(m), 41 fragments of the pSym were induced during symbiosis at levels well above the ones observed under low oxygen conditions. Fourteen fragments were expressed at the same level in both conditions; fifteen fragments that showed expression under low oxygen tensions were repressed in bacteroids.

Fig. 3. Transcriptional activity in the *R. etli* pSym. (a) Linear *Bam*HI map of the 390 kb pSym of *R. etli* CFN42, indicating the location of specific symbiotic genes. The location of these genes was compiled from data given by Girard *et al.* (1991); Vázquez *et al.* (1991, 1993); and our own unpublished results. Exact locations for specific genes are: *nod box*, *nodA* and *nifHDK a* (band 1); *fixNO* (band 3); *fixQP* (band 4); *nod box*, ORF 1–2 and *nodB* (band 7); *nodC* (band 8); *nodJ* (band 10); *nodD1* (bands 10–11); *nolE* (band 11); *nifH c* (band 13); *fixABCX*, *nifA* and *nifB* (band 21); *nifHDK b* (band 25); *melA* (bands 55–56); *nod box*, *nodD3*, *nodD2*, *nod box* and *nolP* (band 85). Numbers indicate the corresponding *Bam*HI fragments. (b) Extent of transcriptional activity under each environmental condition tested. In this figure, only bands that showed an induction ratio over 2.5 were considered.

DISCUSSION

How many regions in a symbiotic plasmid are actively expressed during symbiosis? How much of this information is regulated by other physiological stimuli? In this paper we approached these questions through the determination of the global transcription patterns in the pSym. Our analyses were greatly aided by a sensitive technique that allows the quantitative detection of the transcriptional activity in specific genomic regions (Chuang *et al.*, 1993). The validity of this approach is supported by the fact that the transcriptional activity detected originates from the pSym (see Results).

Two limitations need to be considered to analyse these

experiments. First, it is not generally possible to equate transcriptional activity of a region with the activity of single genes due to the possibility that a region may contain several transcriptional units. A second limitation is that if transcripts are generated from a multigene family located exclusively in the same replicon (such as the *nifHDK* reiterations in the pSym), the total transcriptional activity detected may be due to any or all the members of the family. Nevertheless, this approach is valuable for the identification and localization of regions which respond to defined environmental stimuli.

Fig. 3 shows a correlation between the quantitative maps of transcriptional activity obtained in this work and the

Fig. 2. Comparison of the levels of induction in the *R. etli* pSym under different conditions. For each fragment, induction ratios were calculated as a quotient of absolute transcript levels (Fig. 1) between the conditions to be compared. For repression, the reciprocal of the induction ratios were plotted. For these comparisons, a value corresponding to the minimal detection level (A_{580} 0.01) was assigned to unexpressed regions. (a–e) Induction by glutamine (a), glutamate (b), nitrate (c), glucose (d) and glycerol (e) compared with the wild-type strain grown in succinate-ammonium medium under aerobic conditions. (f–i) Induction by genistein at 1 h (f), 4 h (g), 8 h (h) and 24 h (i) after addition compared with the corresponding control without genistein addition. (j, k) Microaerobic induction in the wild-type strain (j) and in the *nifA* mutant strain (k) compared with the wild-type strain grown under aerobic conditions. (l, m) Symbiotic induction in the wild-type strain compared to the wild-type strain grown under aerobic (l) or microaerobic (m) conditions. Bar numbers indicate the position of specific regions on the map (Fig. 3a).

location of specific genes on the pSym. It is noteworthy that physiological signals, such as the type of the carbon and nitrogen sources, exert significant control on the transcriptional activity of the pSym (Fig. 3b). In general, poorly utilized carbon or nitrogen sources allow a greater expression than rich ones. The expressed regions appear to be dispensable, since curing of the pSym does not affect growth rates under these conditions. *Ex planta* expression of some symbiotic genes in other *Rhizobium* species, such as *nodD3* (Dusha & Kondorosi, 1993), *nifHDK* and *fixABCX* (Szeto *et al.*, 1987), is affected by the nitrogen source. In fact, it has been proposed that nitrogen status may control nodulation by *R. etli* via the *ntrC* gene product (Mendoza *et al.*, 1995). Although the expression of some fragments under C- or N-limited conditions might be explained by correspondence with known symbiotic genes, many of these fragments map in zones of the pSym lacking identifiable *nod* or *fix* genes. The functional status of these fragments is still uncertain, but they represent N- and C-controlled regions that might be relevant for either saprophytic or symbiotic life-styles.

Four different regions were actively transcribed after induction with the isoflavone genistein. One of these (region I, Fig. 2i) contains most of the nodulation genes identified in *R. etli* (Fig. 3a). Complementation of a pSym-cured strain with a cosmid clone containing region I leads to nodule formation (Cevallos *et al.*, 1989); thus, this region is sufficient for nodulation. Region I shows an early pattern of expression, detectable 1 h after induction and achieves maximal levels after 24 h. These data are in agreement with experiments employing transcriptional fusions with members of region I, such as *nodA* and *nodBC*, where high-level expression was observed after 24 h (Vázquez *et al.*, 1991). All the new regions identified (regions II–IV, Fig. 2i) show a late pattern of inducibility, suggesting that these regions might be important in other flavonoid-influenced processes, such as efficiency of nodulation (Schultze *et al.*, 1994), competitiveness (Bhagwat & Keister, 1992; Sadowsky *et al.*, 1988), chemotaxis (Aguilar *et al.*, 1988) or resistance to plant phytoalexins (Parniske *et al.*, 1991).

A similar approach was recently employed with the pSym of *Rhizobium* sp. NGR234 (Fellay *et al.*, 1995). Although the spatial order of equivalent zones is different from the pSym of CFN42, four flavonoid-inducible regions were also observed. Three of these were induced only late after flavonoid addition. Contrasting with our data, a locus similar to our region I (containing *nodABC* and other host-specificity genes) was expressed only shortly after flavonoid induction. This region becomes transcriptionally silent late after induction. Thus, both the topological arrangement and the temporal order of expression of equivalent zones may vary depending on the bacterial host.

It is commonly thought that the pattern of expression of nitrogen fixation genes under microaerobic conditions resembles the pattern observed during endosymbiotic stages. In fact, it has been recently proposed that oxygen limitation is a key determinant of the symbiotic pattern of

nitrogen fixation gene expression in alfalfa nodules (Soupène *et al.*, 1995). Our results show that oxygen concentration is one of the main effectors for induction of transcriptional activity in the pSym. Contrasting with the low expression of the pSym under most aerobic conditions, microaerobic status leads to a dramatic increase in the expression level for most of the fragments (Fig. 3b). Some of the induced fragments correspond to sectors that contain different *nif* and *fix* genes (for instance, both fragments 1 and 25 contain *nifHDK* operons, fragment 21 contains *fixABCX* and *nifA* genes, Fig. 3a). However, functions encoded in many of the induced fragments are still unknown.

It is important to note that this microaerobic induction is dependent, either partially or totally, on the presence of an active *nifA* gene (Fig. 2k). This dependency suggests that many induced fragments might have a role, either essential or ancillary, during the symbiotic process. Nevertheless, a detectable fraction of transcriptional activity in this condition is independent of *nifA*. It is possible that the remaining activity might be dependent on other oxygen-sensitive regulators, such as *fixK* (Fischer, 1994).

Symbiotic conditions gave the highest induction in the pSym. Our results show that most of the plasmid regions are expressed during symbiosis (Fig. 3b). Two contiguous regions account for most of the highly expressed fragments in this condition. Although some symbiotic genes, such as *nifHDK*, *fixABCX* and *nifA*, have been located in these fragments, the function of the others (Fig. 3a) is largely unknown.

Considering the level of expression of these regions, it is plausible to think that they may be particularly relevant for symbiosis. In support of this, deletions that remove a sector comprising fragments 1–25 lead to an inability to nodulate (Romero *et al.*, 1991). In contrast, deletions that affect fragments 30–81 are still able to nodulate and to fix nitrogen (unpublished data). The only other highly expressed region in the pSym is fragment 55, which apparently contains the *melA* gene (unpublished data); this gene is expressed during both microaerobic and symbiotic stages (Hawkins & Johnston, 1988).

Although our data are consistent with the role of oxygen as one of the main effectors of symbiotic expression, some discrepancies still remain. Our data for the symbiotic induction pattern resemble, but are not identical to, the microaerobic induction pattern. A comparison of both conditions (Fig. 2m), revealed that induction ratios were the same (i.e. close to one) for only fourteen expressed fragments. The rest of the fragments were either more induced or even repressed during symbiosis.

Microaerobic cultures similar to the ones we employed are commonly used to evaluate the level of expression of known symbiotic genes *in vitro*. Our comparisons are useful to evaluate the agreement between these conditions. Although there is a good qualitative agreement, there are important differences. It is now well established that the O₂ concentration in the infected nodule tissue is about 50 nM (Kuzma *et al.*, 1993). Although the actual

concentration of dissolved oxygen was not determined in our microaerobic cultures, calculations based on the published Ostwald coefficients for oxygen suggest that it was approximately 12 μM . The simplest explanation for the observed differences between the microaerobic and symbiotic induction patterns is that they are solely due to differences in oxygen concentration. Another explanation is that there are other controls, superimposed on the one exerted by oxygen concentration, which act to determine the level of expression during symbiosis. Resolution of these alternatives must wait for quantitative determinations of expression levels under nanomolar oxygen concentrations.

Previous attempts to evaluate the global transcriptional activity of a pSym were restricted to some defined pSym sectors (David *et al.*, 1987) or to isolated highly expressed regions from genome banks (Scott-Craig *et al.*, 1991). However, a recent report from Fellay *et al.* (1995) evaluated the transcriptional activity of the whole pSym of *Rhizobium* sp. NGR234 under flavonoid or root exudate induction, employing an approach similar to the one described in this paper. Our data extend the usefulness of this approach, providing quantitative information on transcriptional activity on a different pSym under a variety of environmental conditions.

An additional application of this approach is in the evaluation of the effect of mutations in global transcriptional regulators, such as *nifA*. This type of analysis allows a rapid evaluation of the number and position of regions controlled by a complex regulatory circuit, either in a positive or a negative mode. We believe that the resolute power achieved through a quantitative global approach will make it extremely useful for the analysis of complex genomes.

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