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; Schultz 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; Schultz 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 pSym 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...
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 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 HindIII fragment containing the nifA gene of R. etli CFN42 (GenBank accession no. U31630). As a first step in the generation of a mutant in the nifA gene from R. etli, a 394 bp fragment from nifA (position 1165–1559) was excised from pGC479 by digestion with EcoRI. A 2.0 kb ΔSp/Stm (Prenkti & Krisch, 1984) cassette was then ligated in its place, thus generating pGC480. Double recombinants carrying the ΔSp/Stm 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 CFN89 as the recipient and HB101/pNC206 (Table 1) as cointegrative plasmid for HB101/pGC479; recombination between these plasmids occurred through recombinant plasmids (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 OD₆₀₀ 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 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 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 inoculated to a minimal medium with the following composition: sucrose, 5 mM; KNO₃, 1 mM; KH₂PO₄, 1 mM; KH₂PO₄, 7 mM; MgSO₄, 1 mM; NaCl, 2 mM; CaCl₂, 0.1 mM; biotin, 0.4 μM; ferrous citrate, 0.2 mM; H₂BO₃, 46 μM; MnSO₄, 0.9 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 [2-¹⁴C]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 11 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 23 d, nodules were picked out from the roots, washed briefly with diethyl-pyrocarbonate-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, Bifi101). RNA was quantified by absorbance measurements and electrophoresed under de-
naturting 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]dTTP [6000 Ci mmol−1 (222 TBq mmol−1)]; 20 units plasmidial ribonuclease inhibitor (Gibco BRL); 5 mM DTT; 400 units M-MLV Reverse Transcriptase (Gibco BRL) in the presence of first strand buffer (Gibco BRL); 35 mmol l−1 NaOH. The mixture was incubated for 90 min at 37 °C. To hydrolyse the RNA templates, EDTA, SDS and NaOH were added to 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 cosmid that cover the whole pSym of R. etli CFN42 (quantified spectrophotometrically) were digested with BamHI, electroforezed 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 5% (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 BamHI 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 scannings were normalized according to the total radioactivity in the probes, exposure time and amount of cosmid DNA loaded in the

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<th>Strain or plasmid</th>
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<td><strong>Strain</strong></td>
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<td>Brom et al. (1992)</td>
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<td>CFN42 derivative carrying the nif/A::ΩSp/Sm allele</td>
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<td>CFNX248</td>
<td>CFNX89 derivative carrying a cointegrate plasmid pNC206/pGC479 (nif-A')</td>
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<td>pRK2013</td>
<td>Conjugation helper</td>
<td>Figurski &amp; Helinski (1979)</td>
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<td>A pSUP202 derivative carrying the wild-type nif-A gene</td>
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<td>pGC480</td>
<td>A pGC479 derivative carrying the nif/A::ΩSp/Sm allele</td>
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<td>Ordered cosmid collection from the pSym of <em>R. etli</em> CFN42</td>
<td>Girard et al. (1991)</td>
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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 BamHI. 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 reiterations 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 (KNO₃, Fig. 2c) nitrogen source provoked a gradual increase in induction ratios. Growth on KNO₃ led to the activation of 16 regions, with induction ratios ranging from 2.5 to 73. 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 96. 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-KNO₃, 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 this stage were the highest among the conditions tested. As shown in Fig. 2(j), 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).

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 BamHI 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 Rhi zobium 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. 2i) 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, 32P-sscDNA was synthesized from total bacteroid RNA of the wild-type strain. This 32P-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. 2. For legend see facing page.
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
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 saproporphic 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. These three 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 (Soupée 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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