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***Intercellular communication in bacteria nodulating plants of the
family Leguminosae***

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

January 31st, 2008

Elisa Polone

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Summary

In order to achieve a better understanding of the nodulation patterns of rhizobia in their symbiosis with legume hosts, and with the aim to examine their signalling behaviour in cell-to-cell communication, a series of experimental projects were carried out. In first instance the microbial inhabitants of 831 pea root nodules formed on nine plants, sown in different field soil parcels, were isolated and characterized by PCR-based electrophoretic fingerprinting using the BOXA1R primer. Band profiles were analyzed by GelComparII image analysis software converting differences into a numerical matrix yielding their similarity dendrogram in terms of genetic fingerprint distances. The level of strain-specific association with individual plant or soil plots has been assessed. As 85% of the profiles result singletons, having been found in only one nodule, the overall diversity of the site appears particularly high. Estimates of the total diversity at biovar level were obtained by nonparametric estimators pointing to a value over 1300 types. Such richness was compared with the much lower one recorded eight years earlier on the same plots and was put in relation with the repeated host cropping occurred in between. Moreover, the position of each nodule within the root apparatus, in terms of root rank order and distance from the crown, had been recorded in digitized images and the existence of topological and temporal patterns in each strain's nodulation process has been inspected. The fingerprinting quality of BOX-PCR in terms of reproducibility and sensitivity, was compared to that obtainable by other primers as *ISRh1* *outbound primers*.

The same fingerprint-characterized strains were screened for the production of Quorum Sensing signals consisting in short-medium- (C_4 - C_8) and long- (C_{14}) chained N-acyl homoserine lactones (AHLs) using, respectively, the two reporter systems: *Chromobacterium violaceum* CV026 (violacein pigment induction) and *Rhizobium leguminosarum* A34 (colony growth inhibition). The majority of the natural *Rhizobium leguminosarum* strains were found to be quorum-signalling positive. The occurrence of isolates negative to one or both phenotypes however shows that those traits are not absolute requirements for host nodulation

In a different study we examined the root nodule symbionts of eight species of wild legumes collected in Sardinia. Interestingly, unlike the case of cultivated legumes, the

recovery on plates of the rhizobial occupant could not be obtained under any of the conditions used, while at the same time a number of different endophytic taxa were rescued and their taxonomic identity was determined by 16S nucleotide sequencing. By direct PCR analysis from the nodule tissue, we were also able to show the presence of the nonculturable rhizobia inside the same nodules.

In parallel, other two studies were conducted. AHL-mediated quorum sensing communication was quantified at single cell resolution through a red-fluorescing AHL-producing and a green-fluorescing AHL-sensor strain in a 3-dimensional system by using computer-assisted microscopy (CMEIAS). The average effective 'calling distance' from the single cell producer capable of inducing the gfp-tagged reporter cells, resulted 46.8 μm . Moreover, in relation to the possible involvement of AHL signals in different phenotypes, a series of plant-interacting strains, among which *Rhizobium leguminosarum*, were tested for their ability to maintain viability in stressful situations (nutrient and oxygen limitations). Some of the tested strains lost culturability in different of the imposed conditions. However viable cells could be detected by staining microscope-based techniques (*BacLight*®, acridine orange and CTC), demonstrating that the treated bacteria changed into a viable but nonculturable (VBNC) form; none of the AHL Quorum Sensing signals tested was effective in promoting the transition to the VBNC state nor in recovering cells to culturability, suggesting that the two phenotypical frameworks of QS and VBNC do not share signalling paths.

Riassunto

Al fine di valutare dal punto di vista dell'assetto spazio-temporale le modalità di nodulazione di popolazioni naturali di rizobi nella loro simbiosi con le leguminose ospiti, e con l'intento di esaminarne i meccanismi di segnalazione nella comunicazione intercellulare, è stata messa in atto una serie di progetti sperimentali. In primo luogo i batteri ospitati in 831 noduli radicali di pisello formati su nove piante, seminate in diverse parcelle di suolo, sono stati isolati e caratterizzati tramite una tecnica di tipizzazione elettroforetica basata sulla reazione a catena della DNA polimerasi (PCR) utilizzando il primer BOXA1R. I profili sono stati analizzati con il programma di analisi d'immagine GelComparII convertendone le differenze in una matrice numerica che ha dato luogo al loro dendrogramma di similarità in termini di distanza genetica. Questa analisi ha permesso inoltre di stabilire il grado di ceppo-specificità dei batteri associati ai diversi individui di pianta e gli effetti di separazione fisica delle stesse, quando appartenenti a parcelle di terreno distinte.

La diversità complessiva del sito si presenta particolarmente elevata considerando che 85% dei profili risultano singoli, essendo stati reperiti in non più di un nodulo.

Stime della diversità totale della specie batterica sono state ottenute con estimatori non parametrici che indicano valori superiori a 1300 tipi distinti. Una tale ricchezza è stata comparata con quella molto più bassa registrata otto anni prima nelle stesse parcelle, che è stata messa in relazione con la ripetuta coltura dell'ospite effettuata nell'intervallo di tempo tra i due progetti. Inoltre, avendo tenuto conto all'atto dell'isolamento, della posizione di ciascun nodulo all'interno dell'apparato radicale in termini di radice di appartenenza, ordine della stessa e distanza dal colletto, nonché dimensioni, è stato possibile valutare la presenza di eventuali modalità topologiche, morfologiche o temporali, ricorrenti nel processo di nodulazione di ogni determinato ceppo. La qualità di fingerprinting della PCR BOX, in termini di riproducibilità e sensibilità, è stata confrontata con quella ottenibile da altri primer quali ISRh1 *outbound*.

Gli stessi ceppi così caratterizzati sono stati esaminati per la produzione di molecole segnale N-acil-omoserina-lattone (AHLs) quorum sensing a catena medio-corta (C₄-C₈) o lunga (C₁₄) usando, rispettivamente, i due sistemi reporter *Chromobacterium violaceum*

CV026 (induzione del pigmento violaceina) e *Rhizobium leguminosarum* A34 (inibizione di crescita cellulare). La maggioranza dei ceppi naturali di *Rhizobium leguminosarum* sono risultati positivi. Comunque l'esistenza di isolati negativi ad uno o entrambi i fenotipi indica che questi tratti non sono requisiti assoluti per la nodulazione dell'ospite.

In un diverso studio si sono esaminati i simbionti di noduli radicali di otto specie di leguminose selvatiche collezionate in Sardegna. Inaspettatamente, a differenza del caso di leguminose coltivate, non è stato possibile ottenere la crescita su piastra degli occupanti rizobici, mentre diversi taxa di batteri endofiti sono stati isolati dagli stessi noduli e identificati tassonomicamente tramite sequenziamento dei geni per l'rRNA ribosomale 16S. Da analisi di PCR diretta di tessuto nodulare è stato altresì possibile dimostrare la presenza di rizobi non coltivabili all'interno degli stessi noduli.

In parallelo, due ulteriori studi sono stati eseguiti. La comunicazione Quorum Sensing mediata da molecole AHL è stata quantificata a livello di risoluzione di singola cellula tramite un ceppo AHL- produttore a fluorescenza rossa e un ceppo sensore a fluorescenza verde in un sistema tridimensionale in microscopia seguita da analisi di immagine computer-assistita (CMEIAS). La media delle distanze di comunicazione dalla singola cellula produttrice efficaci nell'indurre le cellule sensori è risultata 46.8 µm. Quale ulteriore progetto, in relazione al possibile coinvolgimento delle molecole segnale AHL in diversi fenotipi, una serie di microrganismi interagenti con piante, tra cui *Rhizobium leguminosarum*, sono stati analizzati circa la capacità di mantenere la vitalità in condizioni di stress (limitanti per ossigeno e nutrienti). Alcuni dei ceppi analizzati hanno perso la coltivabilità in diverse delle condizioni imposte. Sono state nel contempo enumerate le cellule vive e/o attive attraverso colorazioni osservabili in microscopia a fluorescenza quali (*BacLight*®, arancio di acridina e CTC), quantificando i passaggi a forme vitali ma non coltivabili (VBNC); nessuna delle molecole AHL testate si è dimostrata efficace nel promuovere la transizione allo stato VBNC né il ripristino di coltivabilità delle cellule, suggerendo che nei due fenomeni di QS e VBNC non sembrano manifestarsi intersezioni di circuiti e cascate di segnalazione.

Chapter I

Analysis of nodulation patterns on natural *Rhizobium leguminosarum* bv. *viciae* populations

Introduction

The importance of nitrogen

The growth of all organisms depends on the availability of nutrient elements, among which nitrogen is a particularly critical one. It is required in large amounts as an essential component of proteins, nucleic acids and other cellular constituents. There is an abundant supply of nitrogen in the earth's atmosphere, nearly 79%, in the form of N₂ gas. However, N₂ is unavailable for use by most organisms as the triple bond between the two nitrogen atoms renders the molecule almost inert. In order for nitrogen to be used for growth it must be combined in the form of ammonium (NH₄⁺) or nitrate (NO₃⁻) ions. A relatively small amount of ammonia is produced by lightnings. Some ammonia is also produced industrially by the Haber-Bosch process, using an iron-based catalyst, very high pressures and temperatures. But the major conversion of N₂ into ammonia, and thence into proteins, is achieved by microorganisms. Biological nitrogen fixation is catalyzed as yet only by prokaryotes. The group of prokaryotes competent for such reaction is large and diverse and contains both eubacteria and archaea (Zehr, J. P. *et al.*, 2003). Prior to agriculture, biological fixation of atmospheric nitrogen is estimated to have accounted for about 90% of the 100 to 140 Tg of nitrogen fixed annually in terrestrial environments. The remaining 10% was fixed abiotically, primarily by lightnings. Now human activity, especially the generation of ammonium compounds for agricultural fertilizers, fossil fuel consumption, and increased planting of legumes, contributes an estimated 140 Tg of additional fixed nitrogen each year (Vitousek, *et al.*, 1997).

Nitrogen fixing bacteria

Biological systems which are capable of fixing nitrogen are historically classified as nonsymbiotic (free-living) or symbiotic, depending on the required involvement of one or more than one organism, respectively, in the process.

There is great diversity in the metabolic types of free-living microorganisms which are capable of biological nitrogen fixation. This includes about 20 genera of non-photosynthetic aerobic (*Azotobacter*, *Beijerinckia*) and anaerobic (*Clostridium*) bacteria and about 15 genera of photosynthetic cyanobacteria (blue-green algae) such as *Anabaena*.

Free-living, non-photosynthetic bacteria depend on soil organic matter as a food source whereas the photosynthetic microorganisms may derive their food from the products of photosynthesis.

The most important contribution to biological nitrogen fixation comes from the symbiotic association of certain microorganisms with the roots of higher plants, which leads to the development of a new organ on the roots of the plant, the legume root **nodule**, in which the bacteria fix dinitrogen in a microaerobic environment.

This process is, for the most part, restricted to a limited number of bacterial groups, including the genera *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Bradyrhizobium*, and *Azorhizobium* (collectively referred to as rhizobia) and *Frankia*. All but the last of these are from the α -proteobacterial *Rhizobiaceae* family and induce nodules on plants from the *Leguminosae* family. *Frankia* is a filamentous gram-positive actinomycete that induces nodules on a variety of woody plants from the Betulaceae, Casuarinaceae, Myricaceae, Elaeagnaceae, Rhamnaceae, Rosaceae, Coriariaceae, and Datisticaceae families (Benson, and Silvester, 1993). Rhizobia species and strains have characteristic host ranges (Dénarié *et al.* 1992, Perret *et al.* 2000), such that they nodulate a subset of legume species. The host range of rhizobia may be narrow (such as *Sinorhizobium meliloti* which nodulates *Medicago* and the related genera *Melilotus* and *Trigonella*) or broad (such as *Rhizobium* sp. NGR234 which is able to form nodules on species in over 35 genera). On the plant side, many members of the large family *Leguminosae* form nodules but some species show fidelity by being nodulated generally by only one or a few specific rhizobia (e.g. *Medicago* spp. by *S. meliloti*), whereas others show promiscuity in being nodulated by many rhizobia (e.g. *Macroptilium atropurpureum*) (Lonneke *et al.*, 2005).

The enzyme system: nitrogenase

The ability of a biological system to fix nitrogen is dependent on the presence of a particular enzyme system known as nitrogenase, which catalyzes the conversion of N₂ into a reduced form (ammonia combined with certain organic compounds) which can then be used for growth by microorganisms and higher life forms. The nitrogenase system consists of two different protein molecules (enzymes) which must function together in the nitrogen fixing process. One of the enzymes (azoferredoxin) is an iron-containing protein. The

second enzyme (molybdoferredoxin) contains both iron and molybdenum. The two components combine and function together as a single system.

A point of special interest is that the nitrogenase enzyme complex is highly sensitive to oxygen. It is inactivated when exposed to oxygen, because this reacts with the iron component of the proteins. Although this is not a problem for anaerobic bacteria, it could be a major problem for the aerobic species such as cyanobacteria (which generate oxygen during photosynthesis) and the free-living aerobic bacteria of soils, such as *Azotobacter* and *Beijerinckia*. These organisms have various methods to overcome the problem. For example, *Azotobacter* species have the highest known rate of respiratory metabolism of any organism, so they might protect the enzyme by maintaining a very low level of oxygen in their cells. *Azotobacter* species also produce copious amounts of extracellular polysaccharide (as do *Rhizobium* species in culture). By maintaining water within the polysaccharide slime layer, these bacteria can limit the diffusion rate of oxygen to the cells. In the symbiotic nitrogen-fixing organisms such as *Rhizobium*, the root nodules can contain oxygen-scavenging molecules such as **leghaemoglobin**, which confers a pink colour visible when the active nitrogen-fixing nodules of legume roots are cut open. It regulates the supply of oxygen to the nodule tissues.

Legume-rhizobia symbiosis

The symbiotic relationships formed between the nitrogen-fixing rhizobia and their legume hosts are the result of an intricate signalling network between the host and symbiont. As yet, many aspects of the signal exchange are still a mystery.

Nodulation is generally initiated only in a susceptible region of the root, behind the root tips. As the nodules start to develop and the roots continue to grow, the subapical root tip zone loses susceptibility and thus there is a control on nodule number.

Determinate and indeterminate nodules

The nodule that develops can generally be classified into two types. Indeterminate nodules are cylindrical and elongated due to a persistent apical meristem, and nodule organogenesis begins with cell divisions in the inner cortex and pericycle of the root. Determinate nodules are more spherical, with no persistent meristem, and cell divisions begin in the outer cortex. The type of nodule that develops is characteristic of the plant host rather than the nodulating

microsymbiont. There is also variation in the infection process. Many legumes (including those that form indeterminate nodules) are infected through root hairs, with rhizobia gaining access to the cortex via specific infection threads. Other legumes are infected by rhizobia that penetrate between epidermal cells (crack entry), particularly at the emergence sites of lateral roots, and infection threads may not be formed. Indeterminate nodulation is considered more advanced, has been better studied genetically and developmentally, and is characteristic of temperate legume genera such as *Pisum*, *Medicago*, *Vicia* and *Trifolium* (Lonneke *et al.*, 2005).

Adhesion of rhizobia to root hairs

In order to initiate a productive symbiosis, rhizobia must recognize and then respond to the presence of host plant roots. During growth in the rhizosphere of a host plant, rhizobia sense compounds such as flavonoids and betaines secreted by the host root and respond by inducing *nod* genes (Carlson *et al.*, 1994, Long 1996.). Genetic analysis of rhizobial mutants has established that host-range, infection, and nodulation depend on the above mentioned genes. These genes are the genetic determinants characterizing rhizobia and appear to have been acquired by taxonomically distinct bacteria through horizontal gene transfer (Moulin *et al.* 2001). Their encoded proteins specify the production of an excreted symbiotic signal called the **Nod factor**.

Rhizobia are capable of binding tightly to host root hairs. With *Rhizobium leguminosarum* this binding consists of two steps. The first is a weak, Ca²⁺-dependent binding step to root hairs that is mediated by a protein called **rhicadhesin**, which is thought to be present in most rhizobia (Smit *et al.*, 1987, Smit *et al.*, 1989).

Following weak binding, a tight binding step that is mediated by the bacterial synthesis of cellulose fibrils is initiated (Smit *et al.*, 1986). The synthesis of these fibrils was shown to be required for *R. leguminosarum* to form biofilm-like caps on the tips of pea root hairs. Mutants that did not form the fibrils did not form caps, but they were able form nitrogen-fixing nodules, which indicated that capping and cellulose-mediated tip binding are not absolutely required for a successful symbiosis to occur. However, binding and capping may be needed for rhizobia to effectively colonize root hairs under natural conditions where competition for access to root surfaces and grazing pressure by eukaryotes are likely to be intense.

Host **lectins** have also been shown to play roles in rhizobial adhesion to plants that form determinate and indeterminate nodules. These lectins localize to root hair tips and are thought to help convey host-symbiont specificity by binding simultaneously to the plant cell wall and to saccharide moieties on the surfaces of compatible bacteria (Diaz *et al.*, 1986, Diaz *et al.*, 1995, Hirsh 1999).

Results such as these suggest that cell-cell contact and specific binding of compatible bacteria to root tips are important for infection, because they result in the exposure of infectible root hair tips to the proper symbiont and hence to a high localized concentration of the Nod factors (van Rhijn *et al.*, 2001).

Nod factors

The Nod factor is a lipooligosaccharide signal consisting of a chitin backbone, four to five *N*-acetylglucosamine units in length, with a lipid attached to the nonreducing end and host-specific modifications on the backbone consisting in different chemical substitutions on the sugar residues and/or variations in the structure of the acyl chain.

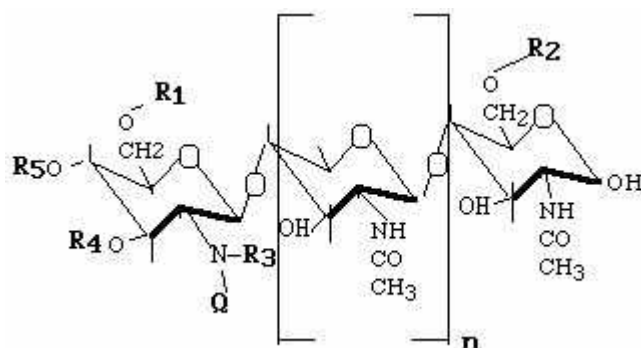


Figure I.1 – General structure of Nod factors produced by rhizobia. n: oligomerization degree, R₁₋₅ and Q: substitutions.

The variation in the amount and structures of Nod factors produced by a rhizobial species is a key factor determining its host range (Perret *et al.*, 2000). Nod factor initiates many of the developmental changes seen in the host plant early in the nodulation process, including root hair deformation, membrane depolarization, intracellular calcium oscillations, and the initiation

of cell division in the root cortex, which cells are normally quiescent and establishes a meristem and a focus of newly divided cells called the nodule primordium.

Root hair deformation and curling

Nod factor alters the growth of the epidermal hairs on the surface of the roots such that they curl.

This reorientation of hair tip growth forms an infection pocket. Within this infection pocket, the rhizobia are able to establish an infection site.

Gage explains in his review (Gage, 2004) that the most susceptible root hairs are those that have nearly finished growing (root hair zone II, fig. I.2).

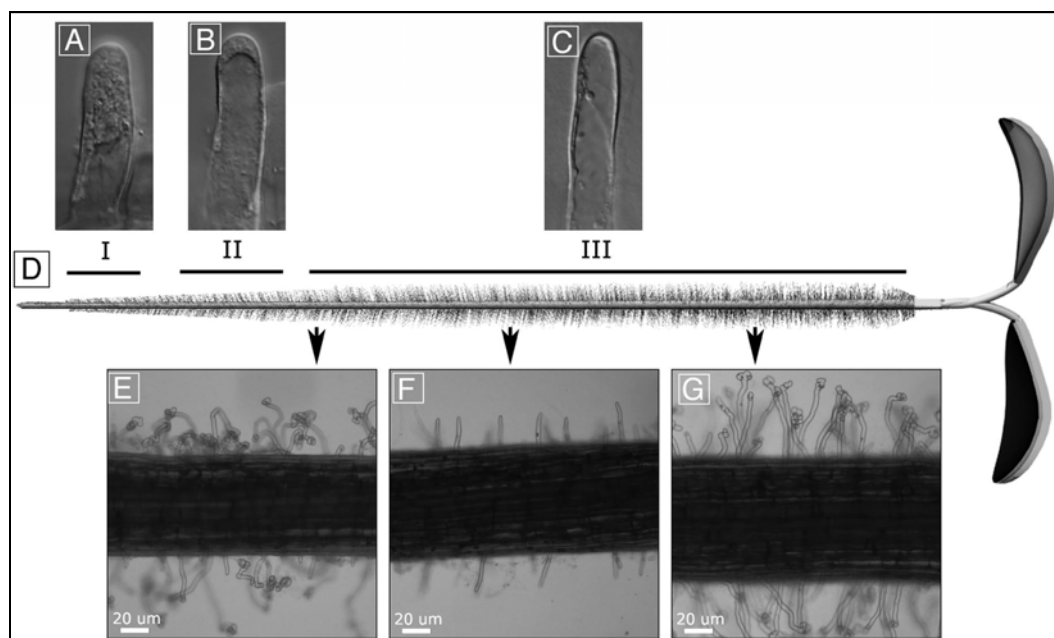


Figure I.2 – Root hair morphology. (A to C) Typical root hairs from zones I, II, and III, respectively, of an uninoculated alfalfa plant. (D) Diagram of an alfalfa seedling, showing the locations of root hair zones I, II, and III. Modified from Gage, 2004.

Root hairs that have finished growing (root hair zone III) and root hairs that are actively growing with a strongly polarized internal organization (root hair zone I) are refractory to the deforming activity of Nod factor. Zone II root hairs are terminating growth and are different morphologically from actively growing root hairs in zone I. They do not display the large plug

of cytoplasm below the root hair tip, the large vacuole is nearer the tip. Nod factor induced deformation of zone II root hairs begins with root hair tips swelling isodiametrically; this is followed by the establishment of a new growing tip that resembles highly polarized, actively growing tips of zone I root hairs (de Ruijter *et al.*, 1998, Heidstra *et al.*, 1994, Miller *et al.*, 1999, Sieber and Emons, 2000). Thus, purified Nod factor can induce new tip growth in zone II cells. It is not yet clear why vesicle deposition becomes temporarily isodiametric following exposure to Nod factor.

It is interesting to consider why zone I and zone III cells do not deform in response to the addition of purified Nod factor. Zone III cells may be unable to deform because they have a secondary cell wall or because they no longer have the machinery in place to catalyze tip growth. Zone I cells may not respond to Nod factor by deforming because they are already highly polarized and actively growing (Lhuissier *et al.*, 2001).

When added to the external medium, purified compatible Nod factors are sufficient to cause root hair deformation and branching, but they are not sufficient to cause the formation of tightly curled root hairs (shepherd's crooks) that are usually the sites of bacterial entry into plants. It had been hypothesized that the reason for this is that a localized source of Nod factor is needed to continually redirect the off-axis tip growth needed to form a tight curl (Ridge, 1992, Van Batenburg *et al.*, 1986). This idea has intuitive appeal. Recent experiments in which purified Nod factor from *S. meliloti* was applied in a highly localized fashion to root hairs of *M. truncatula* have shown that a point source of Nod factor can cause root hairs to grow into structures resembling shepherd's crooks (Esseling *et al.*, 2003).

The responsiveness of root hairs to deform in the presence of Nod factor can be modulated by plant hormones such as ethylene, which inhibits Nod factor signal transduction and can influence the degree of root hair deformation and the frequency of productive infections (Oldroyd *et al.*, 2001). Thus, changes in ethylene levels, in ethylene signal transduction, or in other hormone signalling systems during root growth may explain the observed variability in root hair responsiveness to Nod factor (Gage, 2004).

Infection thread

The rhizobia subsequently infect the root hair by a tubular structure called the **infection thread**, which is initiated by the invagination of the plasmamembrane and deposition of

new membrane and cell wall material. Rhizobia inside the thread grow and divide, thereby keeping the tubule filled with bacteria. Infection only occurs in root hairs that are located above cortical and pericycle cells that have been pre-activated by the presence of the bacteria. These inner cortical and pericycle cells, usually opposite a protoxylem pole of the vasculature, are stimulated to undergo the cell division process forming the primordium. Between the dividing inner cells and the infection site, the outer cortical cells are also activated and form cytoplasmic bridges, which have been called pre-infection threads (van Brussel *et al.* 1992, Timmers *et al.* 1999).

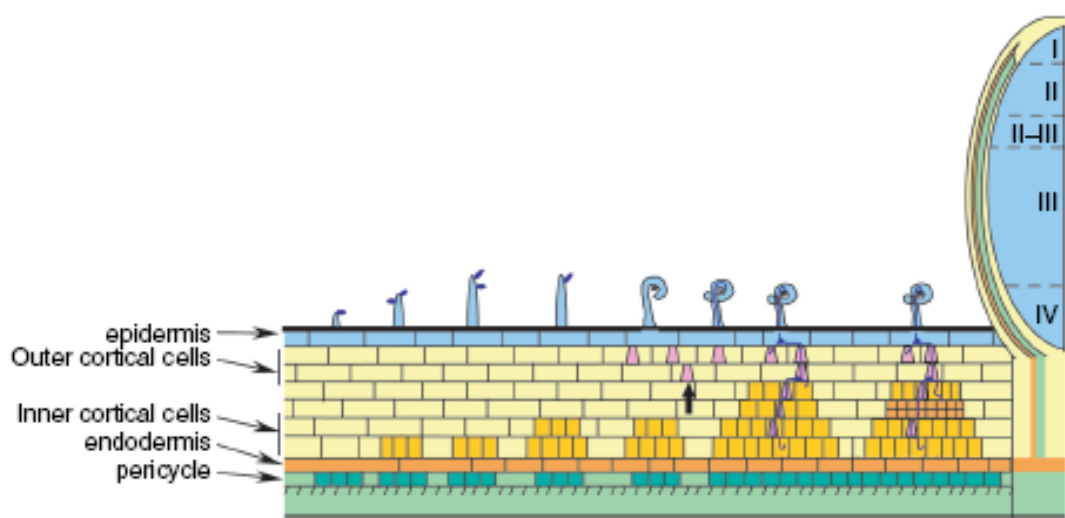


Figure I.3 – Schematic representation of the development of an indeterminate nodule. The mature indeterminate nodule consists of several zones: I, meristem; II, infection; II–III, interzone; III, nitrogen fixation; IV, senescence. Adapted and modified from Timmers *et al.*, 1999.

The infection thread grows from the epidermal cell towards the developing nodule primordium, through the pre-infection threads, by deposition of new membrane and cell-wall-like material. At the nodule primordium the infection threads ramify and infect some of the newly divided cells. Branching of the thread increases the number of sites from which bacteria can exit the thread and enter nodule cells, ensuring that a sufficient number of nodule cells are colonized. Other, non-infected cells develop into a meristem. The meristem grows outwards and the underlying and newly divided cells differentiate into different nodule tissues including a central infected region and a peripheral cortex

containing several vascular strands, which are connected to the root vasculature. In the infection zone of the nodule, just behind the meristem, the bacteria are budded-off by an endocytosis-like process such that they remain surrounded by a plant cell membrane. In these ‘symbiosomes’ the rhizobia differentiate into bacteroids and develop the capacity to fix nitrogen and to export it to the plant in exchange for carbon-rich nutrients (Lonneke *et al.*, 2005).

Other responses of rhizobia upon encountering a host root undoubtedly involve changes in the expression of genes other than those involved in Nod factor synthesis. Such genes are likely to be important for rhizobia to compete effectively with other organisms for access to growth substrates emanating from the host root, to adhere to the root surface, and to become resistant to toxic substances such as phytoalexins secreted by the root (Gage, 2004).

Polysaccharides and surface components

NodD proteins, with their ability to recognize different inducers, the complex mixtures of Nod factors as well as the various levels at which they are maintained, are not the only determinants of host specificity. Although Nod factors permit rhizobia to enter the outer door of the legume host and may play a role during nodule development (D’Haeze *et al.*, 1998, Timmer *et al.*, 1998), additional “keys” are necessary for the formation of symbiotically proficient nodules. In fact, later steps of the infection process such as infection thread formation and propagation, as well as bacterial release into the cytoplasm of infected cells, require constituents of the cell wall and in some cases secretion of specific proteins. Symbiotically relevant components of the rhizobial cell wall include extracellular polysaccharides (also known as exopolysaccharides) (EPS), lipopolysaccharides (LPS), capsular polysaccharides (CPS and KPS), and cyclic β -glucans. Surface polysaccharides (SPS) form a complex macromolecular structure at the bacterium-plant interface. They accumulate on the surface of the prokaryote as a capsular layer but are also released into the extracellular space as bacterial slime (Perret *et al.*, 2000).

The roles of rhizobial exopolysaccharides in symbiotic nodulation have been most thoroughly studied in *S. meliloti*. This species makes at least five symbiotically important polysaccharides: succinoglycan (EPS I), EPS II, K antigen, lipopolysaccharide, and cyclic β -glucans. It turns out that the first three of these are involved in the extension of infection

threads. Thus, in *R. meliloti*, succinoglycan could be regarded as a symbiotic signal (or key) required for opening the inner root hair door. Mutants that fail to produce the symbiotically active forms of these EPSs in adequate quantities are incapable of penetrating the adjacent plant cell and thus remain blocked or trapped in the infected root hair.

Strain typing: repetitive elements and insertion elements

The characterization of prokaryotes has achieved some of its most insightful tools thanks to the progressive development of molecular biology. In this respect, a major great gear shift is undoubtedly represented by the rise of PCR that, among its countless application, has led to the generation of gene- or genome-targeted strategies, which have greatly sharpened our power of studying, comparing and sorting bacteria. Within the field of DNA-based methods, originally featuring genomic restriction digests, specific probe hybridizations and plasmid profiling, the polymerase chain reaction (PCR) has added a wide array of alternatives. Some of this allow strain-level typing, such as AP-PCR (Welsh and McClelland, 1990), RAPD (Williams *et al.*, 1990), DAF (Caetano-Anollés *et al.*, 1991), AFLP (Vos *et al.*, 1995), REP, ERIC and BOX PCR techniques (Lupski and Weinstock, 1992; Versalovic *et al.*, 1991; Versalovic *et al.*, 1994; Martin *et al.*, 1992; Rademaker and Bruijn, 1997; Stern *et al.*, 1984). Polymerase-mediated approaches have also given an impulse to species-level taxonomy focused on the ribosomal operon, as exemplified by ARDRA (Vaneechoutte *et al.*, 1993) and by the PCR-mediated adaptation to microbial ecology of previously devised techniques such as DGGE (Burmeister *et al.*, 1991; Muyzer *et al.*, 1993) and SSCP (Sheffield *et al.*, 1993; Widjojoatmodjo *et al.*, 1995).

Versalovic *et al.* (Versalovic *et al.*, 1991) described a method for fingerprinting bacterial genomes by examining strain-specific patterns obtained from PCR amplification of repetitive DNA elements present within bacterial genomes. One of these repetitive elements, the BOX sequence, has been used to differentiate *Streptococcus pneumoniae* (Koeuth *et al.*, 1995, Martin *et al.*, 1992). BOX elements are located within intergenic regions and can form stem-loop structures due to their dyad symmetry. They are mosaic repetitive elements composed of various combinations of three subunit sequences referred to as boxA, boxB, and boxC. The three subunit sequences have molecular lengths of 59, 45, and 50 nucleotides, respectively.

We also devised primers, that we call *outbound*, to distinguish bacteria by a strain-level differentiating method (Muresu *et al.*, 2005, see Appendix 4) using the sequence of an insertion element (*ISRhI*) originally found in *Rhizobium sllae*, the nitrogen-fixing bacterial symbiont of the legume *Hedysarum coronarium*.

Natural populations of rhizobia in the field

Different studies have addressed the issue of genetic diversity of legume-nodulating rhizobia in natural or cultivated environments. In quantitative terms, the enumerable cells of a given species can be estimated by the Most Probable Number (MPN) technique, using incremental soil dilutions and a test plant. Levels for *R.leguminosarum* bv. *viciae* vary from zero (Chemming'wa and Vessey 2006, Ballard *et al.* 2004) to higher than 10^5 cells per gram of soil dry weight (Hirsch 1996). In the field hosting the present study the original load was assessed to be around 5.2×10^3 (Corich *et al.* 2007). Presence of the host legume can boost numbers by factors from 2- to 284-fold (Mendes and Bottomley (1998), which is conducive of a consequent increase in bacterial genotypic differentiation. In terms of effects of land management, Handley *et al.* (1998) did not observe a reduction of genotypic richness in arable soils. It appears furthermore that cultivated plants can actually promote rhizobial diversification in comparison with wild species (Mutch and Young, 2004). Other studies (Palmer and Young, 2000) find indeed a higher diversity of *R.l.* bv. *viciae* in agricultural systems compared to natural prairies. The number of types they detect from 285 isolates is however not higher than 25. In a different survey (Young *et al.* 1987) 15 electrophoretic types were delineated from the analysis of 249 isolates. Much higher levels were reported in a survey including also the bv. *trifolii* by Strain *et al.* (1995) which list 234 chromosomal types from 682 strains. Some strains appear to be dominant and recur (Corich *et al.* 2007) although their spread can be rather limited (Fagerli and Svenning, 2005). The dominant strains appear however rare, transient and not particularly persistent in their status (Handley *et al.* 1998; Corich *et al.* 2007).

The studies on *Sinorhizobium meliloti*, symbiont of alfalfa offer further information. Bromfield *et al.* (1986) analyzed 1920 nodules and distinguished 88 rhizobial variants by phage typing. Hartmann *et al.* (1998) detected 65 IS-genotypes (insertion sequence variants) in 128 *Sinorhizobium meliloti* isolates, while Bromfield *et al.* (1995) observed

only 17 IS types over 293 isolates . Higher diversity was put in evidence by Paffetti *et al.* (1996) which analyzed 96 strains from different *Medicago sativa* varieties and found each strain having a different fingerprint. In a subsequent study (Paffetti *et al.* 1998) the authors indicated the plant genotype as the prevailing factor in selecting nodulating Sinorhizobia.

Hints of rhizobium short-term microevolution have been advocated by different authors by observing a multiplication of genotypes in sites where given species and their host plants were introduced ex novo in recent times. For example in Australia only 10 strains of commercial inoculants of *R.l. bv. trifolii* for subterranean clover had been introduced since the 1950s. Nevertheless an analysis of 59 field isolates from a single site yielded over 30 different genotypic profiles (Thies *et al.* 1999). Even faster rates were indicated for exotic Bradyrhizobia introduced with soybean in the Brazilian Cerrados soils upon assays carried out seven years since their introduction (Batista *et al.* 2007). The genomic plasticity of rhizobial genomes and a frequent lateral gene transfer are among the mechanisms that can promote such sudden changes as observed for rhizobia which acquired the capability to nodulate *Biserrula pelecinus* introduced in Australia. (Nandasena *et al.* 2006).

Among the methods used to evaluate strain diversity in soils, those based on DNA amplification fingerprint comparisons have proven to be the most sensitive tools when seeking differences while RFLP-based approaches are better suited to trace similarities (Laguerre *et al.* 1996). In our study, aiming at the evaluation of differences we have opted for Random Amplified Polymorphic DNA (RAPD) fingerprinting method.

Scopes of the present study

The experimental trial described below in this chapter aimed at evaluating the extent of strain diversity occurring in a natural *Rhizobium leguminosarum* bv. *viciae* population upon isolation of bacteria from the nodules they formed on purposely-cultivated pea plants. The characterization method chosen was a DNA amplification fingerprint. The field site used was structured in several, isolated, field plots which thirteen years earlier had originally been set up by filling parcels with an allochthonous soil. Since then the cropping history had seen the cultivation of peas in four occasions. Between crops the plots had developed spontaneous vegetation and had been periodically weeded and tilled. This arrangement of the site made possible to explore whether and to which extent rhizobial genotypes had diverged from one plot to the other. The location constitutes therefore an environment in which the different plots started with an original homogeneous soil mix (containing a theoretically common bacterial community), and have had a chance to differentiate their rhizobial population through cycles of host-bacteria interaction during a period of thirteen years. Although the physical separation between plots is limited to the horizontal dimension, the set up allows to investigate for hints of different evolution of the composition of microbial assemblages who might have occurred through several years of growth.

Materials and methods

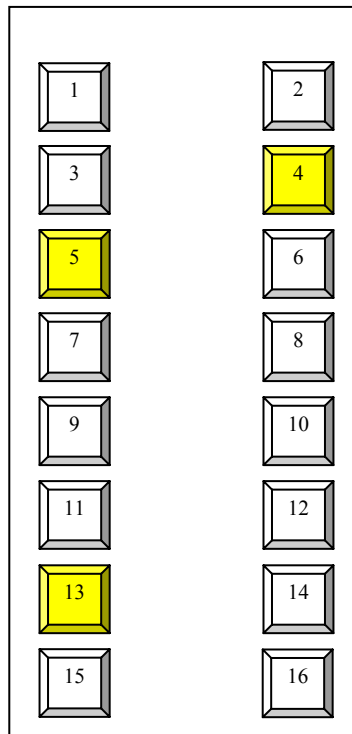
Plant cultivation

Peas (Cv. Curico) were sown on May 15th 2002 and whole plants harvested for nodules after 33 days, corresponding to their pre-flowering stage. The cultivation site consists in a series of one square meter parcels which are individually bordered by 1m-deep four-walled concrete frame, open on the bottom. The plots are located in open field and the soil thereby contained has been laid in place in year 1989 upon mixing sand, peat and clay components of allochthonous origin.



A

Figure I.3 – Cultivation site consisting in sixteen bordered parcels (A) and its schematic representation (B)



B

The subsequent cropping history prior to the present experiment has involved peas (springs of 1994, 1996, 1997, and 1999). Out of a grid of 16 available, three different plots were used for this trial, namely numbers 4, 5 and 13. A total of nine plants, three per parcel (α , β and γ), were analyzed.

Nodule referential identification and bacterial isolation

In order to referentially identify each nodule position, the whole surface-sterilized root apparatus was laid into large square sterile plastic vessels (“square petri dishes”), and its digital image acquired on a flatbed scanner, lateral roots were coded with alphabetic letters and nodules with numbers preceded by the respective root letter, in both cases proceeding from the periphery towards the crown. The nodules situated on the main root were coded directly with numbers (without letter) from the bottom to the top. Using flame-sterilized forceps and blade, the nodules were thence sequentially excised, placed into 2ml sterile cryovials, squeezed, and flooded with 1 ml YMB. A medium generally suitable to rescue and grow rhizobia from nodules, although not exclusively selective (Vincent, 1970) . The cryovials were shaken at 30°C to allow bacterial growth. The set up had the double scope of being able to store the culture at -80 C upon adding 1 ml of 80% glycerol. From these

stocks, during the following weeks, bacteria were restreaked on plates of BIII agar. Such defined medium (Dazzo, 1984) is supposedly more selective than YM for *Rhizobium* cultivation.

Characterization of the rhizobial isolates by Rep-PCR and ARDRA analysis

In order to define the different strains occupying the nodules we performed the BOX PCR, using the BOXA1R primer (Versalovic *et al.*, 1994).

Cells were lysed by resuspending a loopful of plate-grown isolated colonies in 50 μ l of lysis buffer (0.25% SDS, 0.05M NaOH) in an eppendorf tube followed by stirring for 60 sec on a vortex and heating at 95 °C for 15 min. The lysate was centrifuged for 15 min and 10 μ l of the supernatant were mixed with 90 μ l of sterile water. Lysates were stored at 4 °C prior to PCR.

One μ l of the lysate containing the total DNA of each bacterial isolate was treated in a PCR BioRad I-Cycler using the primer BOXA1R (CTACGGCAAGGCGACGCTGACG) (Versalovic *et al.*, 1994) at 2 μ M in a 25 μ l reaction volume, using the following program: initial denaturation at 95 °C for 2 min; 35 cycles at 94 °C for 3 sec, 92 °C for 30 sec, annealing at 50 °C for 1 min, elongation at 65 °C for 8 min and a final extension at 65 °C for 8 min. The PCR reaction mixture contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each dATP, dCTP, dGTP and dTTP, 2 μ M of primer and 2.5 U Taq DNA Polymerase.

One μ l of the lysate was also treated using the two 16S rDNA-targeted universal bacterial primers 63F (5'CAGGCCTAACACATGCAAGTC) (22) and 1389R (5'ACGGGCGGTGTGTACAAG) (29) at 1 μ M each in a 25 μ l reaction volume, using the following program: initial denaturation at 95 °C for 2 min; 35 cycles at 95 °C for 30 sec, 55 °C for 1 min, 72 °C for 4 min and a final extension at 72 °C for 10 min. The PCR reaction mixture contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each dATP, dCTP, dGTP and dTTP, 1 μ M of each primer and 2.5 U Taq DNA Polymerase, recombinant (InVitrogen Life Technologies). Amplicons were digested for three hours at 37 °C upon mixing 5 μ l from the 25 μ l reaction volume with 1 μ l of *Hin*6I enzyme (Pharmacia, Uppsala, Sweden) and 2 μ l of 10 x reaction buffer.

Both amplified and digested DNA were loaded on a 1.5% agarose gel, run electrophoretically for 3 h at 110 V. The ethidium bromide-stained gel was visualized over an UV transilluminator and photographed by a Kodak DC290 digital camera.

Image computer-based analysis

Electrophoretograms were compared and their relative distances assessed by computer-assisted image analysis by the software GelComparII (Applied Maths, Kortrijk, Belgium).

Results and discussion

Analysis of the nodulation patterns

A total of 831 nodules were analysed from the nine plants (a first set of results have been presented at “Accademia dei Georgofili”, Polone and Squartini, 2005 – Appendix 2). 81.2% of the nodules gave rise to OD increase in YMB, indicating microbial growth. 82% of the YMB-positive cultures yielded BIII positive cultures, indicating that some of the bacteria rescued from nodules might not have been proper *R. leguminosarum* occupants.

The majority of BIII-grown isolates did produce amplification profiles, in total 520 (examples of BOXA1R profiles are shown in Fig. I.4).

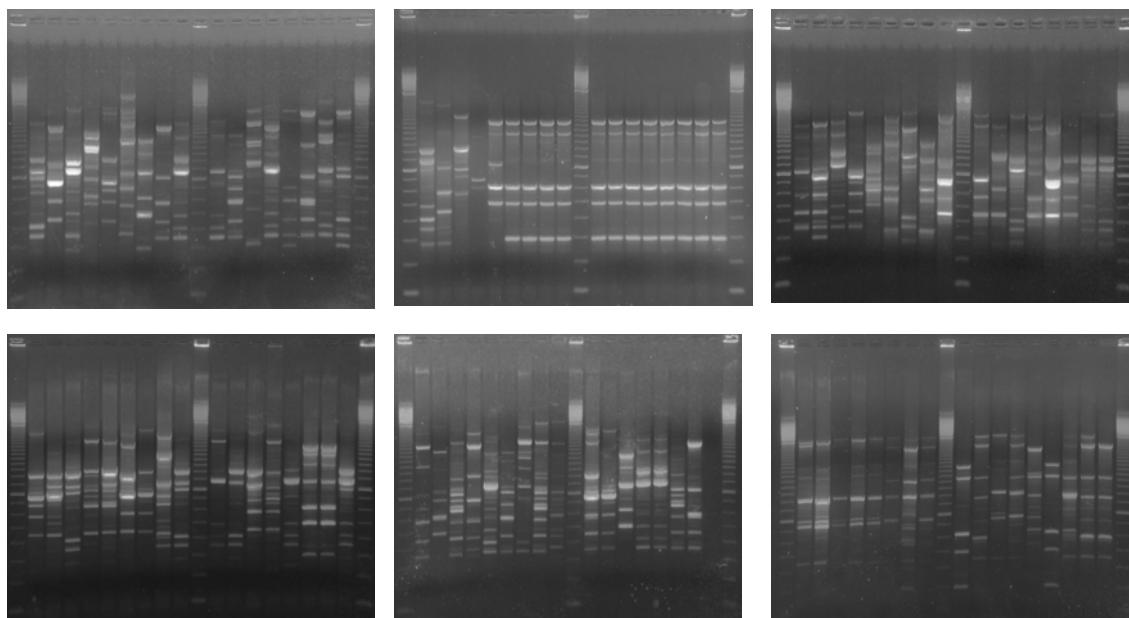


Figure I.4 – Examples of electrophoretograms of BOXA1R amplicons from rhizobia genomes isolates from *Pisum sativum*.

The digestion profiles of ARDRA-PCR products confirmed that not all nodules were occupied by rhizobial microsymbionts. 40 isolates revealed to belong to the family *Enterobacteriaceae* (Fig. I.5).

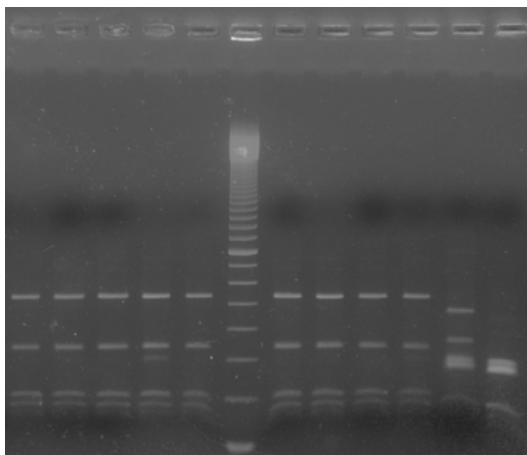


Figure I.5 – Restriction patterns of PCR-amplified 16S rDNA region digested with Hin6I.

The last lane corresponds to a rhizobial fingerprint, while the first nine lines display non-rhizobial profiles (*Entrobacteriaceae*).

The two observed facts (nodules not yielding bacteria and nodules from which bacteria other than rhizobia are rescued) are known from literature. Young *et al.* (1987) analyzed 334 pea nodules and found 7.2% “empty” nodules and 19.7% of the isolates not fitting *R. leguminosarum* phenotypes which were hypothesized as inquiline endophytes. Considering that in this study nodules were not selected, as each and every one from all the 9 root apparati were inspected, one should expect that young small white ones might be in early (not yet invaded) stages of their organogenesis or be too small to withstand surface sterilization, while others could be in senescent stages. As regards the non-rhizobial endophytes we have encountered the phenomenon in several other cases (Benhizia *et al.* 2004, Muresu *et al.*, 2008).

The compared BOXA1R profiles produced the GelComparII dendrogram (Appendix 1).

The electrophoretotypes arranged by the image analysis software were codified assigning progressive numbers starting from the top of the dendrogram, the ones resulting identical or differing by less than 10% (constituting the inherent inter-replicate variability of the system) were scored as the same profile. A further systematic criterium was adopted in order to indicate greater similarities by gathering “families” of strains (generally sharing more than 60 -70% similarity) under the same prefix number (family number) followed by a second

cardinal number separated by a dot. The non-rhizobial strains have the codification in brackets, to differentiate them from rhizobia.

Plant	4 α	4 β	4 γ	5 α	5 β	5 γ	13 α	13 β	13 γ	Total or Mean
Total nodules	136	97	90	102	45	111	99	70	81	831
Producing a BOX PCR profile	76	27	46	79	22	93	76	52	49	520
Different Profiles found	55	23	22	48	19	81	63	44	43	347
Specific profiles^a	41	12	18	40	14	67	50	39	33	314
Shared profiles^b	14	11	4	8	5	14	13	5	10	84
Single profiles^c	45	19	19	41	17	71	52	37	39	340
Recurring profiles^d	10	4	3	7	2	10	11	7	4	58
Unique profiles^e	38	12	16	36	13	60	42	36	30	283
% specific profiles./total profiles	74,55	52,17	81,82	83,33	73,68	82,72	79,37	88,64	76,74	77,01
% shared profiles./total profiles	25,45	47,83	18,18	16,67	26,32	17,28	20,63	11,36	23,26	22,99
% single profiles./total profiles	81,82	82,61	86,36	85,42	89,47	87,65	82,54	84,09	90,70	85,63
% recurring profiles./total profiles	18,18	17,39	13,64	14,58	10,53	12,35	17,46	15,91	9,30	14,37
% unique profiles/total profiles	69,09	52,17	72,73	75	68,42	74,07	66,67	81,82	69,77	70
nodules with specific profiles	45	14	40	50	15	74	61	42	36	377
nodules with shared profiles	31	13	6	29	7	19	15	10	13	143
nodules with single profiles	45	19	19	41	17	71	52	37	39	340
nodules with recurring profiles	31	8	27	38	5	22	24	15	10	180
% nodules with specific profiles	59,21	51,85	86,96	63,29	68,18	79,57	80,26	80,77	73,47	71,51
% nodules with shared profiles	40,79	48,15	13,04	36,71	31,82	20,43	19,74	19,23	26,53	28,49
% nodules with single profiles	59,21	70,37	41,30	51,90	77,27	76,34	68,42	71,15	79,59	66,17
% nodules with recurring profiles	40,79	29,63	58,70	48,10	22,73	23,66	31,58	28,85	20,41	33,83
% nodules with unique profiles	50,00	44,44	34,78	45,57	59,09	64,52	55,26	69,23	61,22	54
Different profiles/ BOXA1R obtained	0,72	0,85	0,48	0,61	0,86	0,87	0,83	0,85	0,88	0,77

Table I.1 – Quantitative nodulation scores. ^a “Specific” : profiles found only on one plant. ^b “Shared” : found on more than one plant. ^c “Single” : found once per plant. ^d “Recurring” : found twice or more per plant. ^e “Unique” : found once at all in the whole survey . In this case the number of unique profiles and the number of nodules containing unique profiles coincide.

The overall nodulation results can be seen in Tab I.1. The 520 nodules out of which a Rep-PCR profile was obtained contained a total diversity of 347 BOXA1R-distinguishable profiles.

One main consideration is that the majority of the profiles (on average 77 %) were found only on one of the plants analyzed (and hereby defined “specific”), and that most of them were found no more than once in the whole survey leading to almost 70 % of profiles defined as “unique”. Whereas cases found in two or more different pea plants are defined “shared”. Cases found once per plant are defined “singles” and amount on average to 85%, while cases found twice or more on the same plant are, for that plant, scored as “recurring”. A profile scoring as single in one plant can either be specific (and thus also unique) or be found also in another of the nine plants (shared), in which it can in turn be either single as well, or recurring.

The data can be analyzed either by the number of nodules containing a given kind of profiles, whose ratio is expressed as percent values over the total number of BOX-producing nodules, or by the number of profiles of a given kind found in each plant, and thus divided by the number of different profiles present in that plant. This measure reflects the plant’s perspective : how many nodules are allocated for single nodulators rather than recurring ones, or how many nodules are allowed to possibly specific symbionts rather than to promiscuous ones (shared). The first way of assessing results, (by profiles) is viewing the interaction more from the bacterial point of view : how do the 347 strains behave; how many could nodulate once, how many displayed specificity for a plant and how many invaded nodules in different plants.

In essence the ratio of different profiles over the total ones analyzed results rather high (0.7 %), indicating that the vast majority of the nodulating rhizobium population in the site occupies few nodules per strain and typically not more than one in an experiment of this size. The prevailing proportion of single or unique profiles suggests that, in a soil having matured a rhizobium population through at least four cropping seasons, the leading events among plant-symbiont encounters are the individual ones.

Recurring, competitive and dominant isolates

As discussed, more than 66 % of nodules in a plant are occupied by individual single cases. The remaining 33.83 % is thus home for strains which, at variable degree, are able to

nodulate repeatedly on the same root apparatus or also across those of other plants. In order to seize their outcome, table 2 shows them ranked in order of decreasing abundance listing all strains which have been found at least in two nodules. The list encompasses 63 of the 347 profiles found, the remaining 284 being each the occupants of as many single nodules. The striking evidence is that the overwhelming majority of the strains in this table (56 over 63) belong to families. This indicates that those rhizobia who can be defined competitive, proficient in lateral diffusion, able to colonize more individual plants also across different plots, and in general displaying good attitude for nodulation, are deriving from groups sharing common origins. Their BOX-detectable difference within a conserved cluster, also show that, along with the dispersion process which led to efficient legume invasion, a process of genome variation has also occurred. The trend suggests that in a soil having reached this level of strain diversity, the types who can most efficiently invade their target host niches are the ones who are most active in terms of genetic recombination.

Strain	4 α	4 β	4 γ	5 α	5 β	5 γ	13 α	13 β	13 γ	Total
162 2				18	3	1		2	1	25
145 2			22							22
163 4		1		1	1	3		3		9
22 15	5		1			1	1			8
75 4	4	2				1				7
145 3			3						4	7
162 12				7						7
22 13	5	1								6
16 1	3			1		1				5
16 3					1	2	1		1	5
16 4				3			1		1	5
18 5				3		2				5
22 6			1			1	2		1	5
75 5	3	1				1				5
46 2		2			1				1	4
80 1	3	1								4
163 2							2	2		4
22 8	2						1			3
29 1							3			3
29 2							3			3
58 3							2		1	3
75 1	1	2								3
119		2	1							3
155 2							1	2		3
162 3				3						3
166 1				1		2				3
166 4					1			1	1	3
189 4						3				3
22 1						2				2
22 5			2							2
22 7									2	2
22 16	2									2
22 19				1			1			2

22 20						1	1			2
29 4							2			2
29 7						1	1			2
30 1		1					1			2
30 3							2			2
47 1	2									2
48	1					1				2
52 2	1	1								2
55						2				2
58 6				2						2
58 7									2	2
75 6				1		1				2
79 1	1						1			2
81 1							2			2
105 1								2		2
114 1							2			2
140									2	2
145 1						2				2
155 1								2		2
155 8								2		2
155 11							2			2
155 19						2				2
163 3	1								1	2
163 8	1								1	2
168 4	1	1								2
168 6						2				2
175					2					2
176 2	2									2
185							2			2
198				2						2

Table I.2 – Strains ranked in order of decreasing abundance, listing all which have been found at least in two nodules.

Plot effect

Community similarity analysis, obtained by performing 1 to 1 comparisons between each of the 9 plants, indicated that the most similar plant pairs are 4 α with 4 β (20.3 % proportional similarity) and 5 α with 5 γ (14.9%). Both lie within a common plot, (plots 4 and 5, respectively) suggesting that the horizontal barrier to bacterial spread exerted by the concrete frame of each plot, and the distance factor itself, can have had an effect in each plot community microevolution.

Sharing plots or plants	Number of common profiles	Number of cumulated profiles *	% common	mean
Plots 4-5	4	248	1.61	1.98
Plots 4-13	5	250	2.00	
Plots 5-13	7	298	2.35	
Plots 4-5-13	4	398	1.00	-
Plants 4 β -4 γ	1	45	2.22	3.36
Plants 4 α -4 γ	1	77	1.23	
Plants 4 α -4 β	7	78	8.97	
Plants 5 α -5 γ	5	129	3.87	
Plants 5 β -5 γ	3	100	3.00	
Plants 5 α -5 β	2	67	2.98	
Plants 13 α -13 γ	4	106	3.77	
Plants 13 α -13 β	2	107	1.87	
Plants 13 β -13 γ	2	87	2.30	
Plants 5 α -5 β -5 γ	2	150	1.33	

Table I.3 – Common profiles in plants or pots.

In order to reveal the extent of such drift, we run pairwise community comparisons for each of 36 combinations ; nine of which represent comparisons within plants of the same plot (4 α vs. 4 β , 4 β vs. 4 γ , 4 γ vs. 4 α , etc.) while the remaining 27 are comparisons between plots. Besides the proportional similarity index we tested seven additional parameters devised for

ecological community comparisons, as listed in Table I.4 which summarizes the results. The mean within plots is compared with that between plots. The first five indexes are measures of similarity while the last three express distance. In agreement with the hypothesis of a plot effect, all the average values of the pairwise comparisons within plots, run with the five independent indexes, are about double than those between plots. Likewise the three indexes of distance indicate shorter values in the means within plots. As the latter measures are give more weight to the overall differences between two communities, the means of the indexes of distance within and between plots have a lesser gap, remarking that as discussed above, each of these plants tends to have a rather individual community with small overlaps with the others.

In order to evaluate whether a component of the plot effect on community diversity could be due to linear metric distance between plots, as the three plots are not equidistant, we separated from the above pairwise comparisons the partial means between plots 3 vs. 5, 5 vs. 13 and 3 vs. 13. The results did not show any distance-related trends (data not shown), indicating that the observed plot effect appears due to the physical separation itself, and that possible airborne immigration of cells from adjacent areas is not sensibly affecting similarity. This was however already evident from the very diverse community compositions in the first place. (similarity in a plot due to descent from common parental). Moreover none of the dominant isolates resulted the most abundant case in more than one plant. The score of each different plant sees a different winner.

	Prop.	Sim.	% Jaccard	Sorensen	Morisita	Horn	Sneath/S	Bray/Curt.	Chord
Mean within plots	7,62	0,04	0,07	0,32	0,10	<i>0,48</i>	<i>0,94</i>	<i>1,31</i>	
Mean between plots	3,52	0,02	0,04	0,16	0,05	<i>0,49</i>	<i>0,96</i>	<i>1,37</i>	
mean 4-5	2,63	0,02	0,03	0,11	0,04	0,47	0,97	1,39	
mean 5-13	5,08	0,03	0,06	0,28	0,08	0,52	0,94	1,34	
mean 4-13	2,85	0,02	0,03	0,08	0,04	0,48	0,97	1,39	

Table I.4 – Community similarity analysis through 8 parameters. The first five indexes are measures of similarity while the last three express distance.

***Rhizobium leguminosarum* soil community structure and dimension**

First, an ecological evaluation, applying several indexes designed for rank-abundance data in biological studies (Tab. I.5) allows to appreciate, within the species *R. leguminosarum*, the community features of the biovar *viciae* in the chosen field. A very high value of both Shannon and Simpson indexes testify the highly diverse structure of the 347 intrataxon entities identified. Low dominance and high evenness further underline how this community does not comply with frequently observed distributions in studies at species level, where few taxa with many individuals and many taxa with few individuals are recorded. In our case the absence of such characteristic low-evenness oligarchic structure is in our opinion not due to the rank level (strains instead of species), but rather to the maturity stage of the *Rhizobium leguminosarum* field population as a result of repeated cycles of symbiotic encounters with its hosts. Indeed, as the next paragraph will discuss, a previous analysis in the same field plots carried out eight years before the present study, when the soil had received only its first season of deliberate cultivation with peas, we had found a much simpler community, dominated in all plots by the same single profile.

Besides the above discussed community structure definitions, which are based on a sample of finite size, the results allow to speculate on the actual dimensions of the rhizobium population in the environment of choice in terms of phylotype richness. Several papers have addressed the task of estimating bacterial diversity from recoverable strains or clone libraries, and appropriate indexes have been devised (Hughes *et al.* 2001). In a review considering 225 of such libraries from as many published studies, Kemp and Aller (2004) discussed the most suitable indexes for estimating coverage and strain richness. The first was concluded to be best defined by the C_{ACE} estimator (Chao *et al.* 1993, Lee and Chao 1994) whereas the more classic Good's index (Good, 1953), would supposedly overestimate coverage. As regards species richness (unknown total number of phylotypes in the environment) the same authors indicate most consistent results with S_{Chao1} (Chao *et al.* 1984, Chao *et al.* 1987) estimator. It is generally recommended that more indexes be used and compared in order to trust extrapolations. In our case (347 phylotypes over 520 samples in the library, with 283 singletons and 345 cases appearing less than 10 times with a total of 473 individuals) we found the following : Good's coverage = 0.456 ; C_{ACE} = 0.402, S_{Chao1} = 1455. The value resulting from the non-parametric S_{Chao} index also agrees

with the figure which can be obtained by applying the method of Curtis *et al.* (2002), which requires two measurements : (a) the abundance of the most frequent case (which in our survey is a strain found in 25 nodules over 520), and (b) the estimated number of the total *R. leguminosarum* bv. *viciae* population of the site. In our field we know, from most probable number counts on the host plant, that about 5×10^3 cells of this species are present per gram of soil. Extending this to the total ground available to plants in our 1 square meter plots (considering an effectively explored depth of 25 cm), the total number of *R. leguminosarum* bv. *viciae* cells in the about 250 kilograms of explorable soil amounts to 1.25×10^9 . Entering the values in Curtis' plots leads to an estimated richness close to the 1000-species range curve.

Number of strains =	347
Total abundance =	520
Simpson Dominance (I) =	0,006069364
Simpson Diversity (D) =	0,9939306
Max of D =	0,9990394
Simpson evenness =	0,9948863
Inverse dominance (d) =	164,7619
Max of d =	1.041,000
d evenness =	0,1582727
Log used for H' =	10,00000
Shannon Diversity (H') =	2,393462
H'max =	2,540329
H' evenness(J%) =	0,9421856
Brillouin diversity =	2,081083
Brillouin max =	2,285088
Brillouin evenness =	0,9107232
Hill's N1 =	10,95134
Hill's N2 =	164,7619
Hill's evenness =	15,04491

Table I.5 – Ecological evaluation of the *R. leguminosarum* bv. *viciae* community.

Comparison with the *Rhizobium leguminosarum* bv. *viciae* population recorded in the same site in year 1994

The results of the present analysis, and the dynamics of rhizobium populations can be more clearly appreciated thanks to a set of available data from a previous survey that we had done eight years before in the very same plots (Corich *et al.*, 2007 – see Appendix 3). At the time, the soil (a mixture having been placed in the frames since five years), had just undergone its first event of deliberate cultivation of pea host plants, from which roots, harvesting a total of 250 nodules from plants taken in 15 different plots of the site, we isolated the bacteria and characterized them by RAPD PCR using the DNA Amplification Fingerprinting decamer primer DAF3. The analysis at the time had the scope of identifying a suitable dominant strain for further competition studies. The pea variety used was the same of the present report (cv. Curico). A second host species *Vicia faba* subsp. *minor*, was also used in pot nodulation trials using soil from the same plots. The results including details not included in Corich *et al.*, 2007 are summarized in Table I.6.

RAPD Profiles	<i>P. sativum</i> (250 nodules)	<i>V.faba minor</i> (32 nodules)
Profile #1	53.8 %	44%
Profile #2	9.7 %	13%
Profile #3	9.3 %	19%
Profile #4	10.9 %	6%
Profile #5	1.2 %	9%
Profiles from #6 to #33	<1 %	n.d.

Table I.6 – Dominant profiles in a *R. leguminosarum* bv. *viciae* community recorded in the same site eight years before ranked in order of decreasing abundance.

The community structure appears strikingly different from the one that we describe in the present report, a single strain (Profile 1) was dominating the site being able to occupy more than half of the nodules in any of the plots and confirming its competitiveness also on the alternate host plant. This was accompanied by three further profiles with a lower but

effective nodulation success (profiles 2 through 4) equally taking about 10% of the nodules in all plots. The rest of the nodules were home for a series of strains occupying in most cases less than 1% of them. Not more than a total of 33 different DAF profiles were found in the 250 nodules analyzed, and the number of unique ones was only 20. Thus it can be seen that in the following eight years, in which a total of five events of pea host cultivation have occurred (plus some occasional wild vetch weedy species between seasons) the population of *R. leguminosarum* has undergone a remarkable modification in its structure, leading to the annihilation of the dominance of a handful of strains, to be substituted by a scenario of high evenness and richness. It is also to be stressed that as DAF3 profiles have inherently more experimental variability than BOXA1R, thus leading to a possible overestimation of differences, the actual structure of the 1994 community could be even more homogeneous.

The disappearance of a leader as Profile 1 was at its time, led us to investigate its fate in the present community by amplifying and running the BOXA1R profile of the original isolate to see to which strains of the present survey it would correspond. The result found it to be identical to strain 58.7, a case found in only two nodules on plant c in plot 13. A very related although not identical profile (58.6) is found in plant a in plot 5. Thus the once dominant genotype is now found just as a relic of its pristine diffusion. Even when we consider the whole strain 58 family, encompassing seven members, these altogether occupy not more than 11 nodules of the present survey. The community appears therefore to have profoundly diverged through time. None of the strains recurring in the present one is able to dominate different plots, and the most frequently found isolate (strain 162.2) found in five plants, takes only 25 of the 520 nodules (4.8%).

Comparison of BOXA1R fingerprinting performance with that of *outbound primers*.

In order to verify the fingerprinting quality of Rep-PCR in terms of reproducibility and sensitivity, we compared (Muresu *et al.*, 2005-see Appendix 4) their performance with that of ISRh1 *outbound primers* for a set of *R. leguminosarum* bv. *viciae* strains of the present study. Eighteen strains were tested, arranged in nine pairs, giving either identical or slightly different though related ISRh1 fingerprintings. Fig. I.6 shows the comparisons. Overall, the *outbound primers* tested gave results equal to the BOXA1R. In seven out of nine cases, the

identity or the difference of the profiles were obtained by both tools; in one case the slight difference detected by BOXA1R was not evident in *outbound primers* (g), while in one other case the outbound pair was able to trace a difference not visible by BOA1R (i).

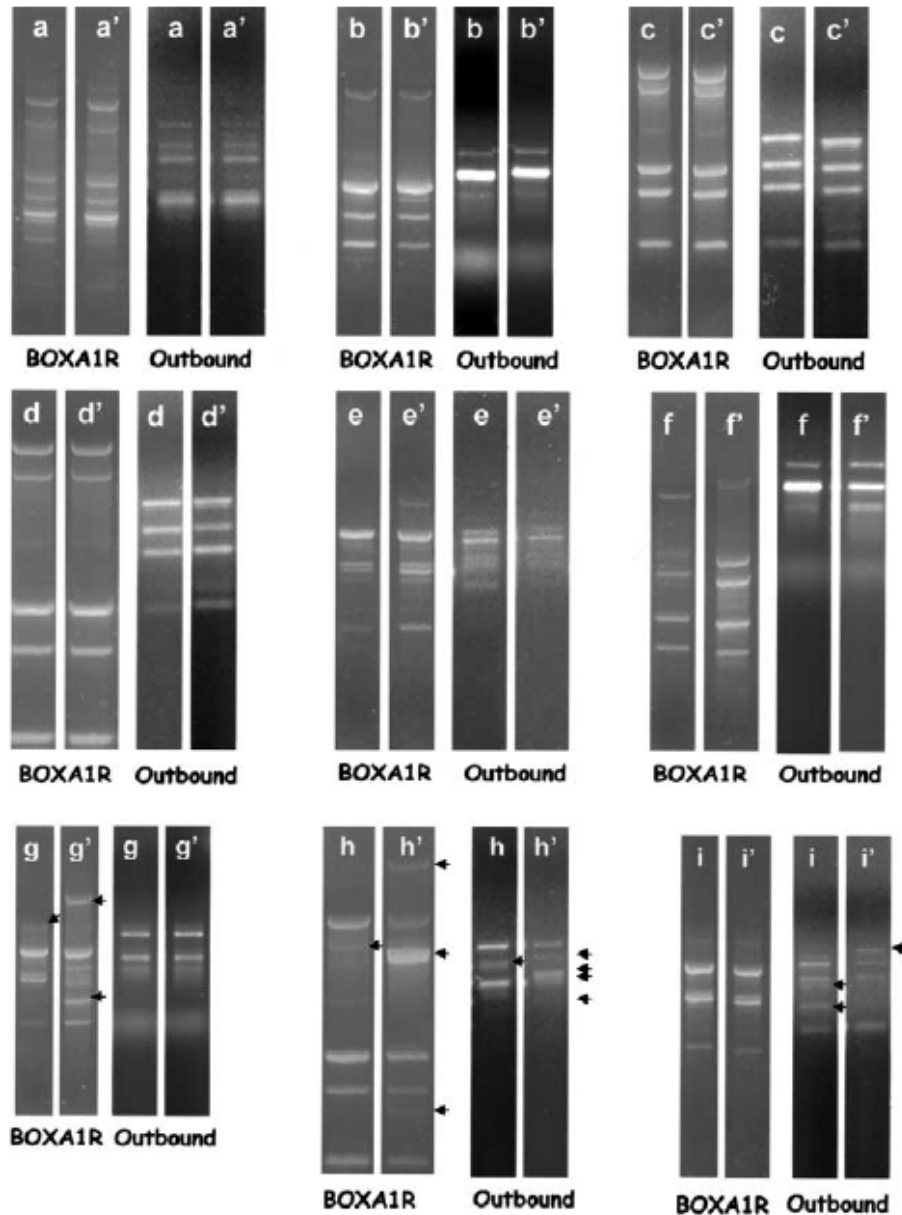


Figure I.6 – Pairwise comparison of the outbound primers fingerprinting performance with that of BOX PCR. Eighteen strains of *R. leguminosarum* bv. *viciae*, belonging to nine pairs in which the two members of each pair share either an identical (a, b, c, d, i), a highly similar (e, f) or a slightly similar (g, h) BOX PCR profile (primer BOXA1R), are shown next to their corresponding outbound-PCR profiles. For strains belonging to pairs in which

a marked difference is revealed by PCR the arrows indicate bands that differentiate the two cases.

Topological analysis of nodulation

In order to highlight possible positional patterns of invasion sites along the root host apparatus by a given rhizobial strain, the nodulation map of each plant was compiled consisting in the bidimensional image of the same showing the code of each strain next to its nodule of isolation. The nine maps are shown in Figures I.7-I.15. A coloured code points out the uniqueness or the recurrence of each strain, as described in Label I.1.

The synoptic observation of such nodulation patterns of the plant host allows to visually seize the symbiotic interaction models between plant and microsymbionts. In most plants we noticed a preferential nodule formation pattern, particularly evident in plant 4 γ , consisting in clusters alternated with nodule-free areas. Since the nodule formation is induced by molecular signals by rhizobia, this spot-distribution phenomenon is in line with a patchy rather than even distribution of rhizobia in soil. The microcolonies or the single quiescent *Rhizobium* cells can encounter a passing root. Roots can in turn attract rhizobia chemotactically with flavonoids, and undergo multiple infections during the short time of their transit. The majority of nodules develop on the primary or on the secondary roots, while nodules are found in tertiary and quaternary roots only occasionally. The peripheral nodules tend to be more abundant in roots where proximal nodules could not develop, as in plant 5 β , whose apex is absent, probably due to the herbivorous fauna. Consequently this plant shows cases of nodulation also on tertiary roots.

We can notice the presence in each apparatus of many unique cases (black font), whose arrangement appears rather random. Beyond this it is quite evident for every plant, the variably abundant presence of a characteristic strain, in most cases different for each plant specimen. Strain 145.2 is rather numerous in plant 4 γ as strain 162.2 in plant 5 α . In addition to the abundance of the strains in this images we can appreciate their topologic distribution, plant 5 α shows a cluster allocation of the dominant strain in the middle portion of the root apparatus, that is at 15-20 cm of the soil depth. Regarding rhizobia, recurring profile strains that occupy more nodules in the same plant (blue font) or in more plants of the same field parcel (green font) or of different parcels (red font) do not have a specific nodulation site as the same strain can nodulate in the same manner primary, secondary and tertiary roots.

Another important fact that arose is the possible presence of non-rhizobial bacteria (marked in brackets) in nodules. In plant 4γ the profile of isolate 145.2, displaying a 16S ribosomal gene restriction pattern typical of *Enterobacteriaceae* results the dominant strain.

Regarding “empty” nodules (codified as “n” in maps) it is possible to notice that in most cases they correspond to young small white ones that consequently might be in early (not yet invaded) stages of their organogenesis or be too small to withstand surface sterilization, while others could be in senescent stages.

Red font	Profile shared by plants of different plots
Green font	Profile shared by different plants belonging to the same plot
Blue font	Profile present in more than one nodule but only in that plant
Black font	Unique profile
()	Non-rhizobial profile (<i>Enterobacteriaceae</i>)
n	Lack of growth in YMB
*	Lack of growth in BIII
!	BOX-PCR amplification failed

Label I.1 – Codes adopted in the profile maps of figures I.7-I.15.

Figure I.7 – Map of BOXA1R profiles of plant 4α.

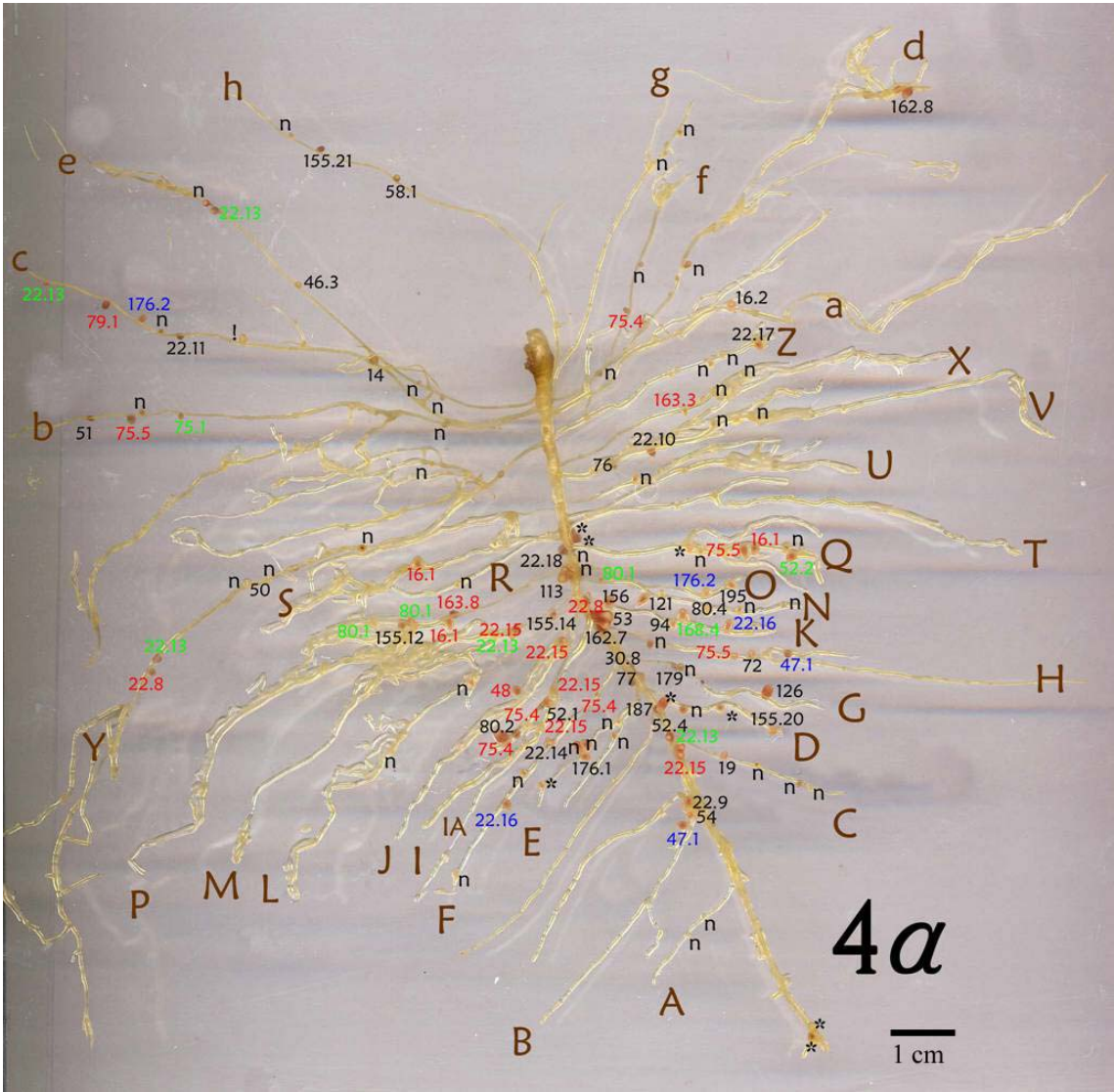


Figure I.8 – Map of BOXA1R profiles of plant 4β.

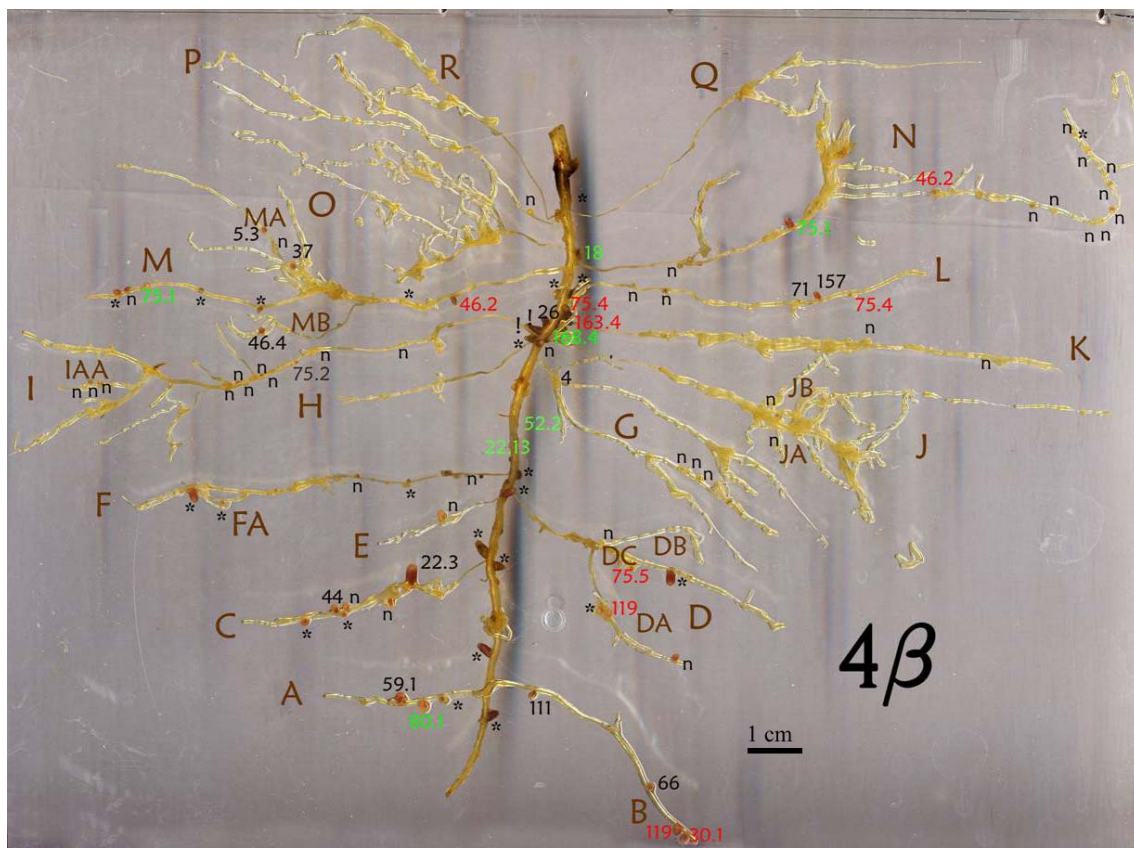


Figure I.9 – Map of BOXA1R profiles of plant 4γ

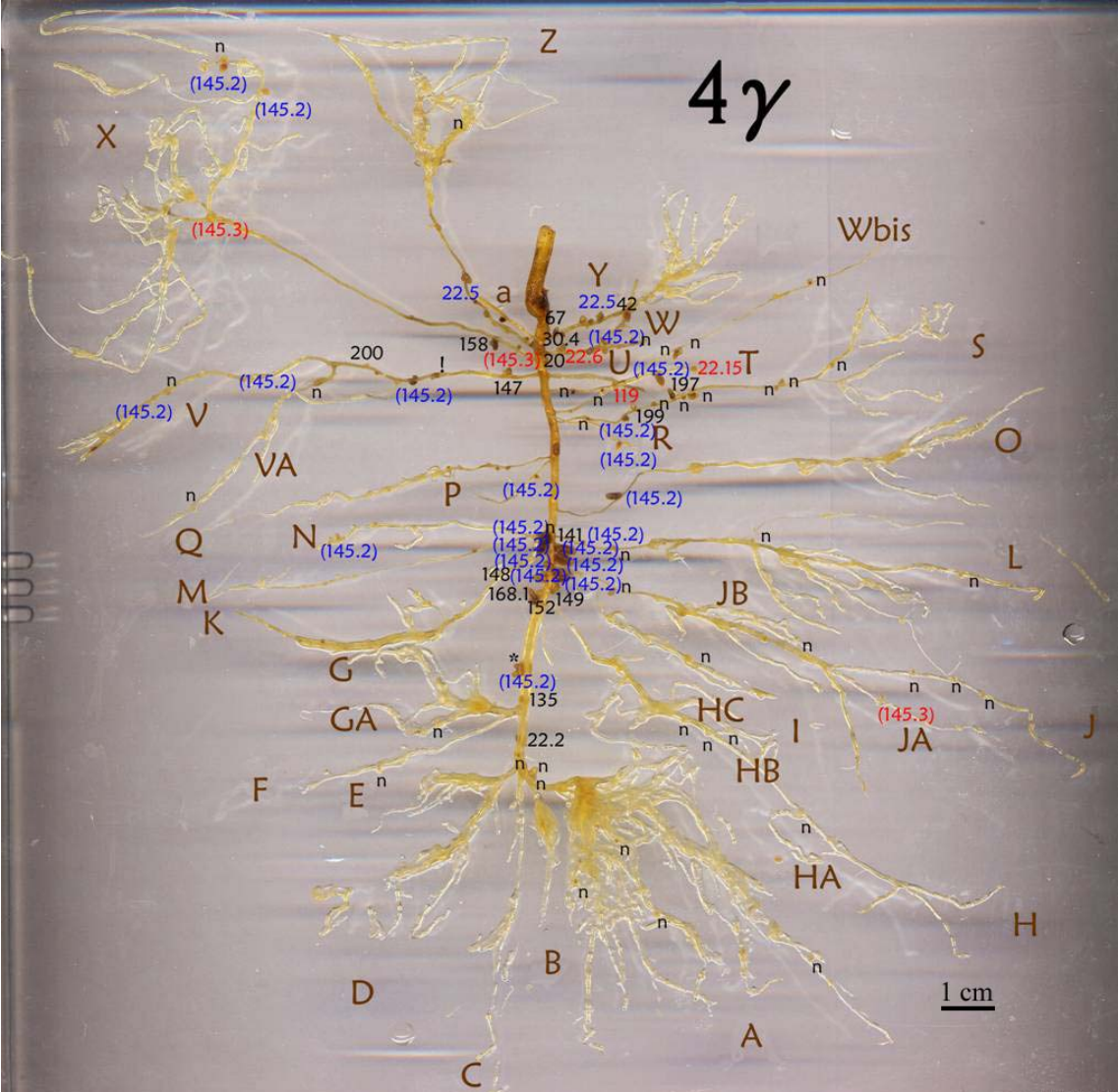


Figure I.11 – Map of BOXA1R profiles of plant 5β

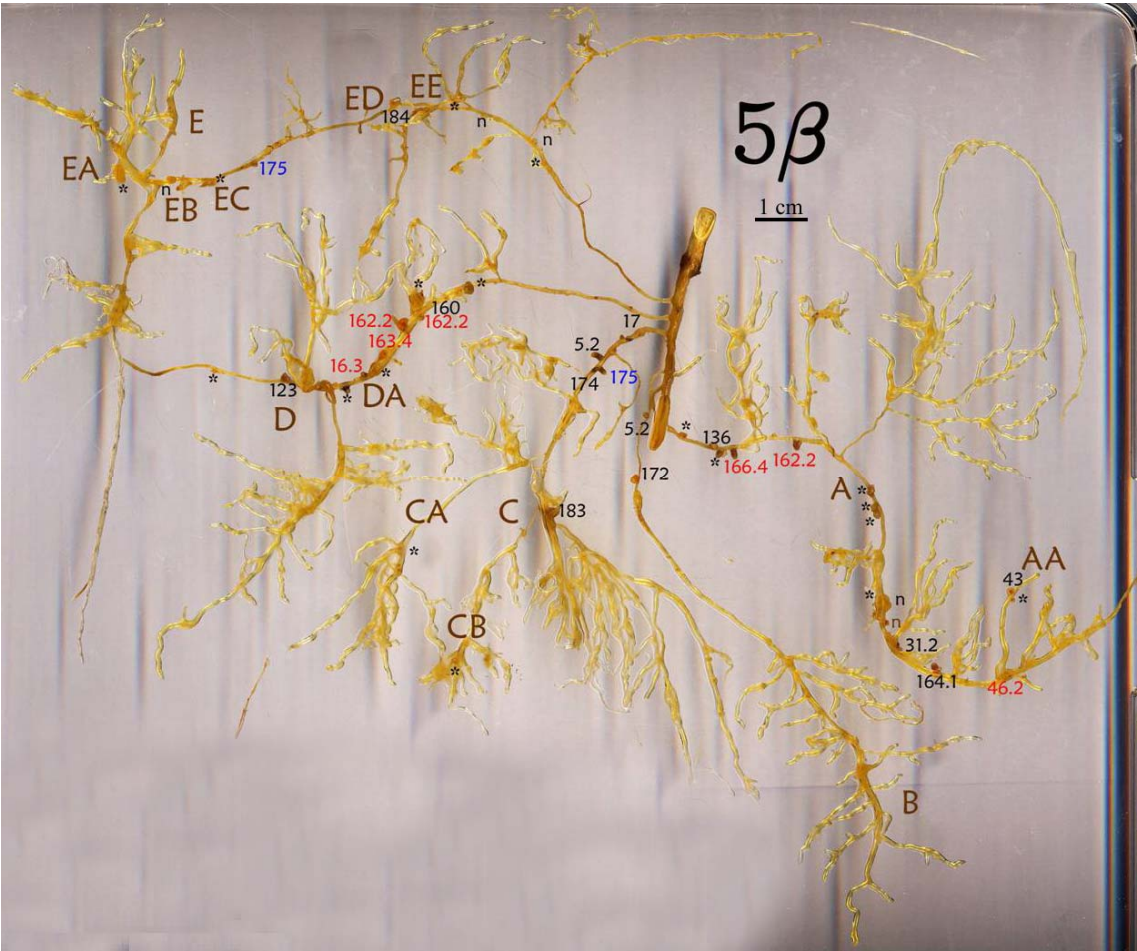


Figure I.12 – Map of BOXA1R profiles of plant 5γ

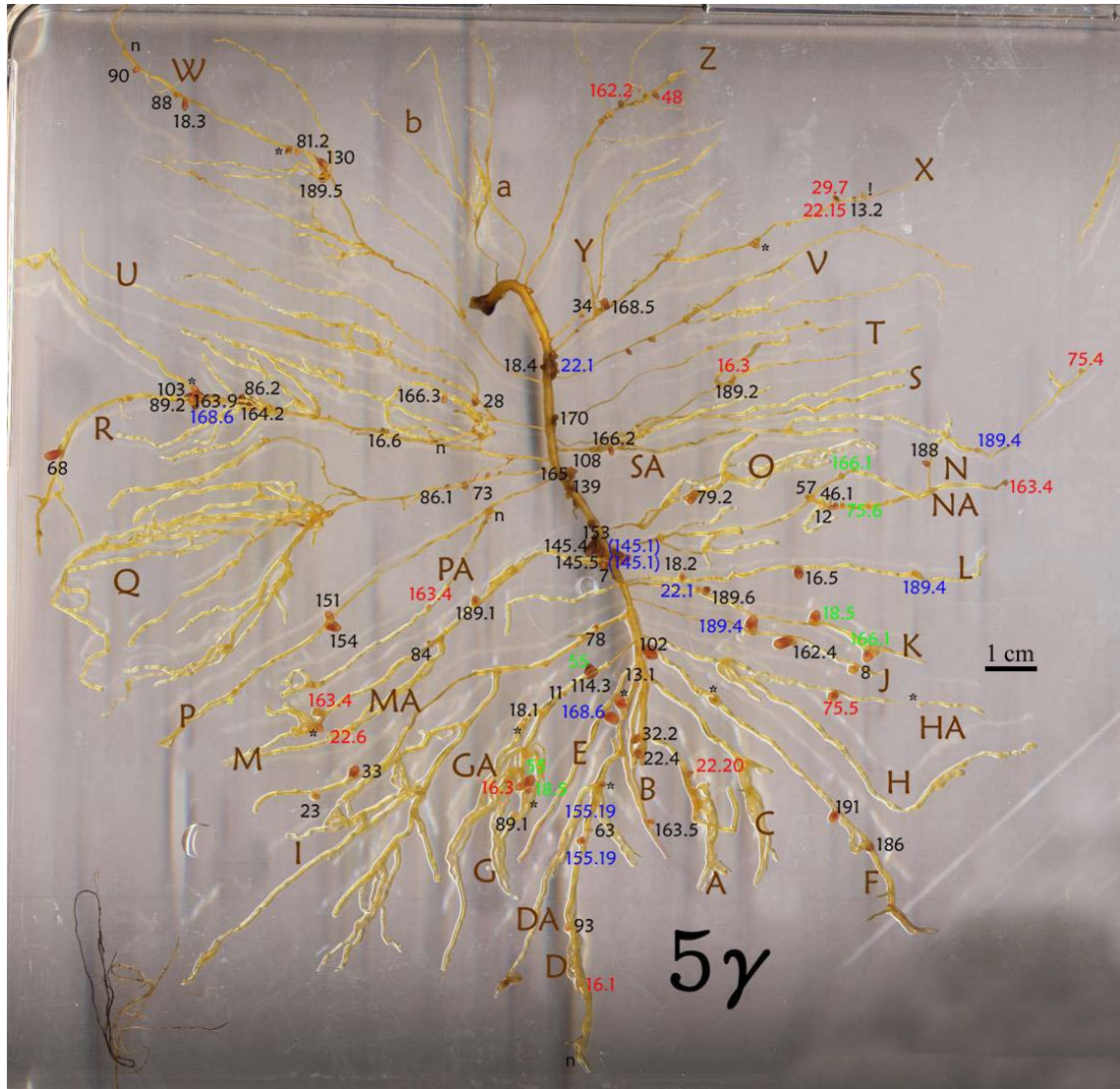


Figure I.13 – Map of BOXA1R profiles of plant 13α

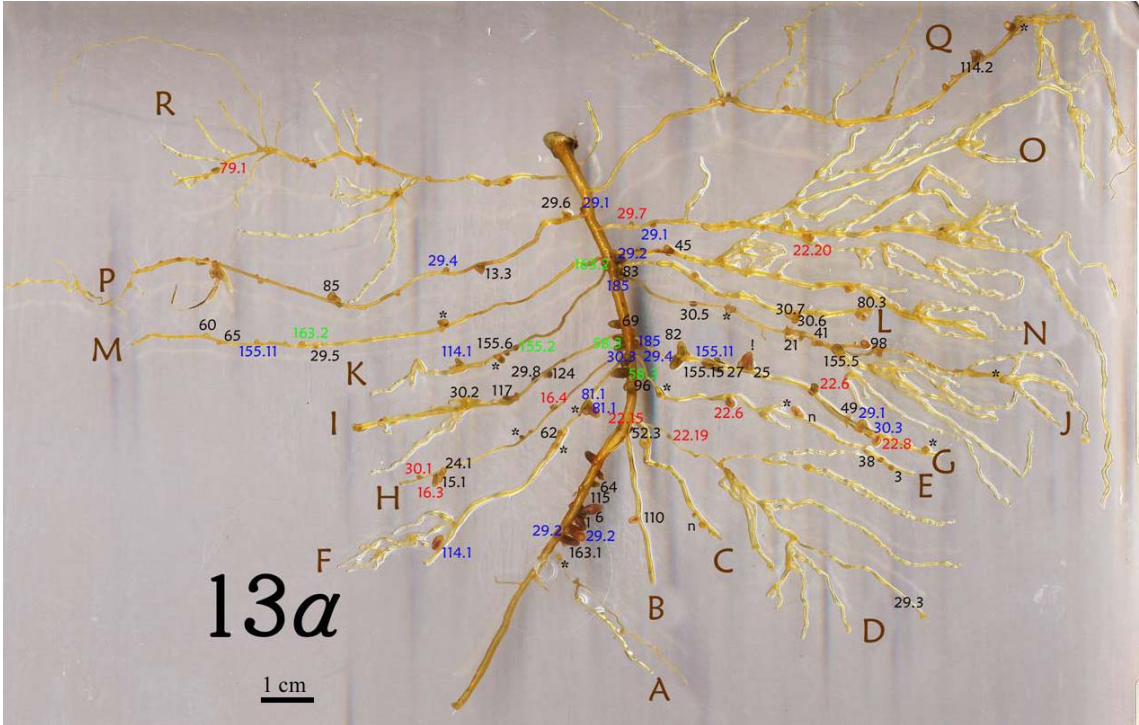


Figure I.14 – Map of BOXA1R profiles of plant 13 β

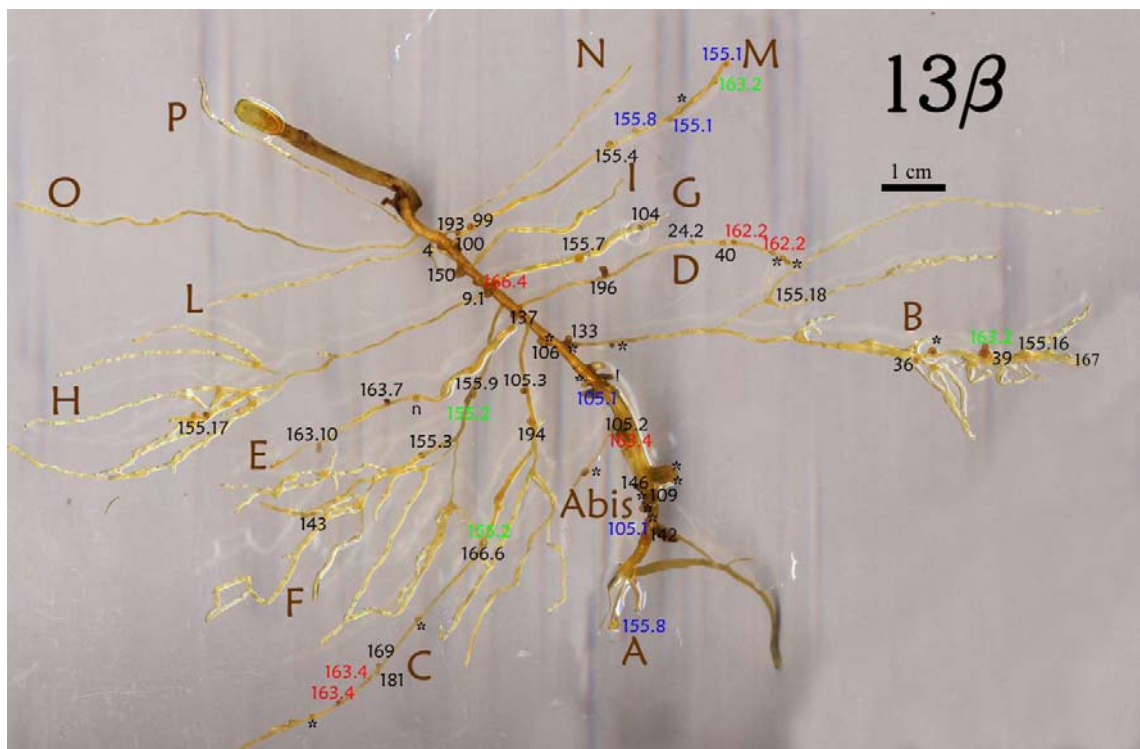
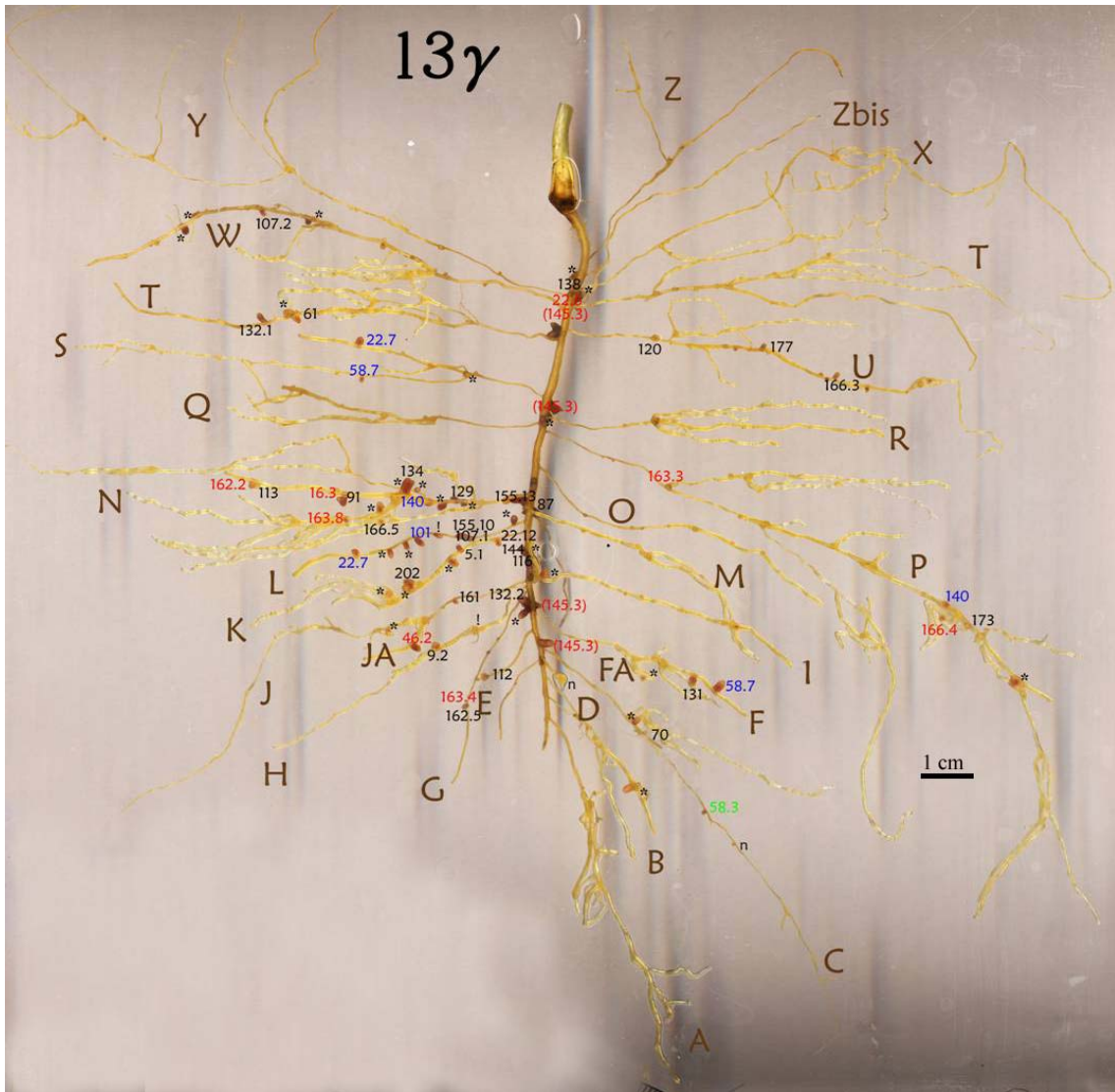


Figure I.15 – Map of BOXA1R profiles of plant 13 γ



Conclusions

Upon analyzing bacteria from 831 pea root nodules from plants cultivated in experimental field plots 520, isolates were obtained, 480 of which belonging to the biovar *viciae* of *Rhizobium leguminosarum* and 40 being identified by ARDRA analysis as members of the *Enterobacteriaceae* family. The role of non-rhizobial bacteria in the interaction with the host plant as well as with the *Rhizobium leguminosarum* bv. *viciae* strains, will be the object of further research. In the simplest hypothesis they could be simply endophytes having co-infected a nodule induced by a regular rhizobium whose cell numbers they subsequently overwhelmed. In alternative they could belong to an hitherto unassessed category of novel standalone legume symbionts. Nod gene probing/amplification and axenic plant inoculation studies will be needed to solve these questions.

Molecular typing of the 520 isolates with a PCR amplification fingerprinting method allowed to detect 347 different electrophoretic types.

As 85% of the profiles are singletons, having been found in only one nodule, the overall diversity of the site appears particularly high. Different non-parametric estimators suggest that the present analysis has revealed about one fourth of the actual strain richness for the *R. leguminosarum* species under study. High values of both Shannon and Simpson indexes testify the highly diverse structure of the intrataxon community identified. This profoundly updates the picture gathered from a prior analysis in the same field plots carried out eight years before the present study, when the soil had received only its first season of deliberate cultivation with peas. The previous community was much simpler, featuring about 30 detectable profiles and dominated in all plots by the same dominant one. Thus it can be seen that in the following eight years, in which a total of five events of pea host cultivation have occurred (plus some occasional wild vetch weedy species between seasons) the population of *R. leguminosarum* has undergone a remarkable modification in its structure with the change from a few dominant strains situation to a high evenness and richness scenario.

The hypothesis of a plot-effect on community diversity can be put forward as all the average values of the pairwise comparisons within plots, run with five independent similarity indexes are about double than those between plots. Such effect could be

contributed in part by simple metric distance between plots and in part by their different cropping history.

The majority of nodules develop on the primary or on the secondary roots, while nodules are found in tertiary and quaternary roots only occasionally.

Recurring profile strains do not have a specific nodulation site as the same strain can nodulate in the same manner primary, secondary and tertiary roots.

Some of the strains which are found more than once, show also competitive traits, They prove able to colonize more individual plants also across different plots. Such cases represent 14% of the total profiles and appear to belong in groups (“families”) sharing common origins. Their BOX-detectable difference within a conserved cluster, also show that, along with the dispersion process which led to efficient legume invasion, a process of genome variation has also occurred. The trend suggests that in a soil having reached this level of strain diversity, the types who can most efficiently invade their target host niches are the ones who are most active in terms of genetic recombination. This evidence represents a novel finding in rhizobiology.

Chapter II

Pattern of intercellular communication and detection of signal molecules in natural *Rhizobium leguminosarum* bv. *viciae* populations

Introduction

Quorum sensing, the explanation of *Vibrio fischeri* luminescence

Historically, it was thought that bacteria were solitary individuals, each growing independently of the population. However, in the early seventies Nealson *et al.* (1970) discovered that bacteria can sense and respond to the rest of the population. This phenomenon is nowadays called Quorum Sensing and is defined as the cell density-dependent regulation of gene expression (González and Marketon, 2003).

The first incidence of such a biological phenomenon came to light with the discovery of luminescence produced by certain marine bacteria such as *Vibrio fischeri* and *V. harveyi*. These bacteria, when free-living in sea water (i.e. at low cell density) are non-luminescent. However, when grown to high cell densities in the laboratory, a *V. fischeri* culture bioluminesces with a blue-green light. Interestingly, this bacterium commonly forms symbiotic relationships with different fish (such as the Japanese pinecone fish *Monocentris japonica*) and squid species such as *Euprymna scolopes* (Visick and McFall-Ngai, 2000). These marine animals carry a specialized cavity, called the light organ, in which bacteria as *V. fischeri* are housed. *E. scolopes* may express bio luminescent appearance in dark environments due to the maintenance of a high-density *V. fischeri* population (10^{10} – 10^{11} cells ml^{-1}) in the light organ. This bioluminescent phenotype is exploited by the squid in order to perform a behavioural phenomenon called counter-illumination. At night, the squid camouflages itself from predators transiting below by controlling the intensity of light that it projects downwards, thus eliminating a visible shadow created by moonlight. This is a case of perfect symbiosis, as in return *E. scolopes* provides the *V. fischeri* population with nutrients. The presence of luminescent competent *V. fischeri* cells in the light organ of juvenile squid is crucial for the correct development of this organ.

Further studies on *V. fischeri* revealed that the bacterium grows very fast, directly entering the exponential phase, but the luminescence increases only at about mid-log phase of its growth (Hastings and Greenberg, 1999). The sudden increase in luminescence was attributed to the transcriptional regulation of the enzyme, luciferase, which in turn corresponded to a threshold density of cells. This whole circuit is based on the bacterial assessment of its population density by means of release of chemical signalling molecules

or autoinducers. The autoinducer then establishes a communication between the cells, that gets reflected in the expression of a particular gene, in this case, the luciferase gene (*lux*). For a long time, bioluminescence expressed by *V. fischeri* remained a model system to study density dependent expression of a gene function.

The bioluminescence gene cluster of *V. fischeri* consists of eight *lux* genes (*luxA–E*, *luxG*, *luxI* and *luxR*), which are arranged in two bi-directionally transcribed operons.

One unit contains *luxR*, and the other unit, which is activated by the LuxR protein along with the autoinducer, contains the *luxICDABEG* operon (Engerbrecht and Silverman, 1987).

The *luxI* gene is the only *V. fischeri* gene required for synthesis of the autoinducer, 3-oxo-hexanoylhomoserine lactone (3-oxo-C6-HSL).

The initial stage of bioluminescence induction involves an interaction between the autoinducer and the transcriptional regulator protein, LuxR. *V. fischeri* cells express *luxI* at a basal level when present in low population densities, so the concentration of the molecule in the medium remains low. However, as the population density increases within the confines of a light organ, the concentration of it in the environment also increases.

As the critical concentration of OOHL is achieved (corresponding to a particular cell density or ‘quorum’ of the bacteria), OOHL diffuses back into the cell and binds to LuxR (Kaplan and Greenberg, 1985). Once the autoinducer is bound to the N-terminal regulatory domain, multimer formation by LuxR is enhanced and the C-terminal domain activates transcription from both the *lux* operons (Finney *et al.*, 2002). LuxR functions probably by the OOHL-mediated induction of a conformational change.

Induction of transcription from *luxICDABEG* operon increases the cellular levels of mRNA transcripts required both for bioluminescence and OOHL synthesis, a process referred to as autoinduction.

With increase in the concentration of OOHL molecules, more of it diffuses into the cell and is able to activate more LuxR protein within the *V. fischeri* population. Thus, autoinduction ensures that bioluminescence and signalling molecule production continues (Gera and Srivastava, 2006).

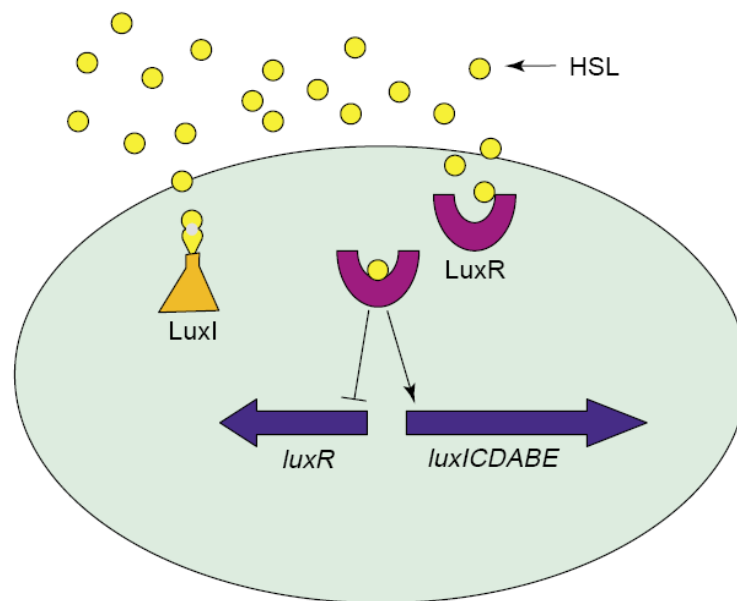


Figure II.1– Quorum sensing in *V. fischeri*. The *luxI* gene encodes for an autoinducer synthase (Lux I), which produces the autoinducer N-(3-oxohexanoyl)- homoserine lactone (HSL). HSL exits the cell and re-enters freely against a gradient when the external concentration reaches a threshold value. Upon re-entry into the cell HSL binds to the gene product of *luxR* (LuxR), a transcription factor. The HSL–LuxR complex binds upstream of the *luxICDABE* operon, facilitating the transcription of all the necessary components of the luciferase system in addition to an exponential increase in *luxI* transcription. LuxR also binds to the *luxR* promoter in a positive feedback loop (the presence of LuxR inhibits its synthesis). Adapted and modified from March and Bentley, 2004.

Quorum sensing in other organisms

In the organisms characterized so far, the quorum-sensing mechanisms are similar to those in the *P. fischeri* paradigm but the activated target genes are diverse. Examples of genes regulated by quorum sensing include the *lux* (luminescence) genes in *P. fischeri*, the *tra* (Ti plasmid transfer) genes in *A. tumefaciens*, exoenzymes and virulence factors in *Pseudomonas aeruginosa* and *Erwinia carotovora*, swarming motility in *Serratia liquefaciens*, antibiotics and violacein pigment in *Chromobacterium violaceum*, exopolysaccharide production in *Pantoea stewartii* and nodulation in *Rhizobium* (Fuqua *et*

al., 1994; Salmond *et al.*, 1995; Swift *et al.*, 1996). All of these organisms have one or more LuxR and LuxI homologues. In addition to sharing similar quorum-sensing mechanisms, most of the organisms establish symbiotic or pathogenic relationships with eukaryotic hosts. Therefore, it is not surprising that symbiosis, pathogenesis, and quorum sensing are intertwined in a complex story of gene regulation. Furthermore, the possibility has been raised that in natural habitats, different bacterial species communicate with one another to coordinate their behavior (Bassler, 1999; Bassler, 2000). An example of this is the bacterial community that naturally colonizes the roots of tomato plants (Steidle *et al.*, 2001). It has been suggested that the AHLs act as signals for coordination of the functions of the different populations within this rhizosphere community.

AHL characteristics

AHLs consist of an HSL head group attached to a variable acyl side chain (R_2 in figure II.2). The amphipathy of the AHL molecule seems to be a balance between the hydrophobic side chain and the hydrophilic HSL ring. These characteristics presumably allow the AHLs to traverse the phospholipid bilayer of the cell membrane and to navigate the aqueous intracellular and extracellular environments (Fuqua *et al.*, 2001). The acyl chain varies in length, from 4 to 18 carbons in those AHLs identified so far. Variability also exists in the third carbon position (R_1 in Fig. II.2) of the acyl chain, where there can be a hydrogen, hydroxyl, or oxo substitution.

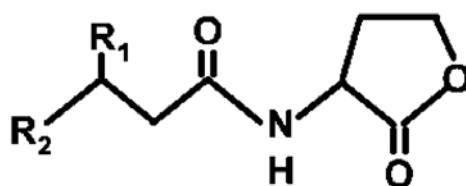


Figure II.2 – The general structure of AHLs is shown in bold. R_1 , $-H$, $-OH$ or $=O$; R_2 , $-CH_3$, $-(CH_2)_2-14CH_3$ or $-(CH_2)_5CH=CH(CH_2)_5CH_3$. Modified from Soto *et al.*, 2006

The overall length of the side chain and the chemical modification at the third carbon position provide the specificity to quorum-sensing signals. To add complexity, most

organisms produce more than one type of AHL and different organisms can produce the same AHL. Therefore, there is some overlap in the production and recognition of AHLs by different organisms (González and Marketon , 2003).

Quorum sensing in *R. leguminosarum* bv. *viciae*

Of the nitrogen-fixing rhizobia, quorum sensing is best characterized in *R. leguminosarum* bv. *viciae*. Several quorum-sensing systems (*rai*, *rhi*, *cin*, and *tra*) have been identified and are intertwined in a complex regulatory network (Fig. II.3) (Cubo *et al.*, 1992; Lithgow *et al.*, 2000; Rodelas *et al.*, 1999; Wilkinson *et al.*, 2002; Wisniewski-Dye *et al.*, 2002). *cinIR* locus appears to be the master control for three other AHL-dependent quorum-sensing control systems. The *cinRI* system resides on the chromosome and produces 3-OH-C14:1-HSL, a long-chain AHL, originally termed *small* bacteriocin for its bacteriocin-like activity and positively influences the *tra* and *rai* systems. *cinI*, the AHL synthase gene, is positively autoregulated by CinR and 3-OH-C14:1-HSL. The mechanism of the regulation of *cinR* is unclear, since mutations in *cinR* or *cinI* or even the lack of the *rhi* and *tra* systems do not seem to affect *cinR* expression (Lithgow *et al.*, 2000). However, *cinR* expression is cell density dependent. More important, though, is the observation that mutations in *cinR* and *cinI* led to decreased levels of all of the short-chain AHLs, suggesting that the *cin* system is situated at the top of the quorum-sensing network. pRL1JI harbors both the *tra* and *rhi* systems, as well as the genes that confer growth sensitivity in response to 3-OH-C14:1-HSL.

The *tra* system is responsible for the production of 3-oxo-C8-HSL and controls conjugal plasmid transfer.

The *rhi* system is composed of *rhiR* (a *luxR* homolog), *rhiI* (a *luxI* homolog), and the *rhiABC* operon. The biochemical role of the *rhiABC* genes has not been established, since there are currently no similar genes products of defined functions in the databases and mutations of these genes have no observed biochemical effects. It was demonstrated that *rhiABC* was controlled by RhiR and that flavonoids repressed the expression of both *rhiR* and *rhiABC* decreasing by about 50% the level of expression of the *rhiABC* operon (Cubo *et al.*, 1992).

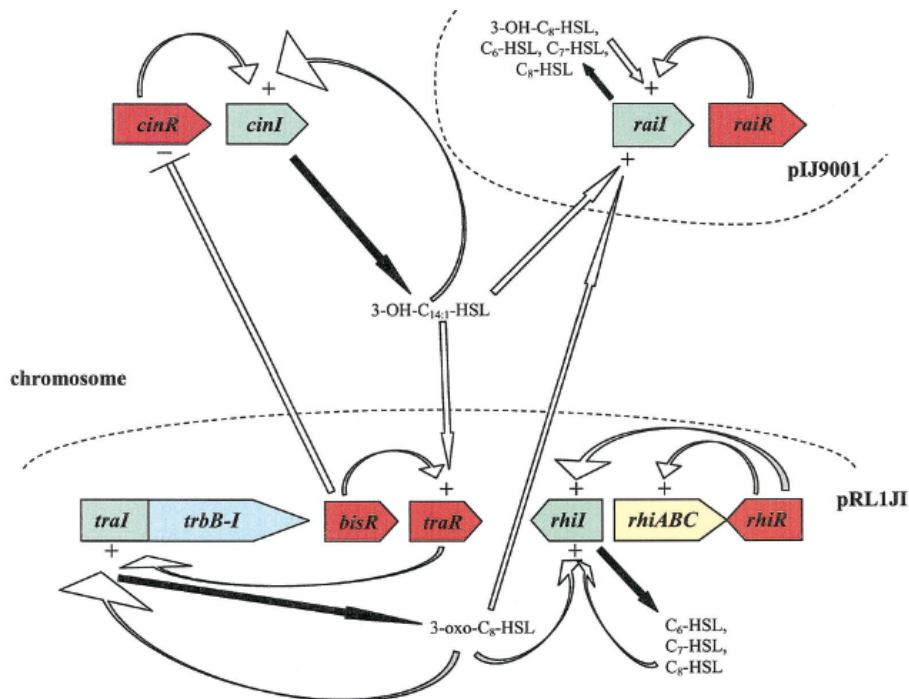


Figure II.3 – *R. leguminosarum* bv. *viciae* quorum-sensing network. *R. leguminosarum* harbors four known quorum-sensing systems. The *cinRI* system resides on the chromosome and produces 3-OH-C14:1-HSL, which positively influences the *tra* and *rai* systems. BisR plays a dual role in activating *traR* and repressing *cinR* in response to 3-OH-C14:1-HSL, thereby linking the *cin* and *tra* systems. pRL1JI harbors both the *tra* and *rhi* systems, as well as the genes that confer growth sensitivity in response to 3-OH-C14:1-HSL. The *tra* system is responsible for the production of 3-oxo-C8-HSL and controls conjugal plasmid transfer, while the *rhi* system produces several short-chain AHLs and influences nodulation efficiency by an unknown mechanism. The *raiRI* locus resides on pIJ9001 and also produces several short-chain AHLs; however, little is known about the role of this quorum-sensing system. Modified from Gonzalez and Marketon, 2003.

Although the function of *rhiABC* is unknown, *rhiA* was shown to be highly expressed in the rhizosphere but not in bacteroids, since its product, the protein RhiA, is found in large amounts around the legume roots but not within the legume nodules and seems to be specific to *R. leguminosarum* bv *viciae*, indicating that it may play some host-specific role in the interaction between this biovar and its symbiotic partners (Dibb *et al.*, 1984). Further investigation identified *rhiI* (Rodelas *et al.*, 1999) and showed that it was responsible for

the synthesis of several short-chain AHLs, including C6-HSL, C8- HSL, and another compound comigrating with C7-HSL.

The *raiRI* locus resides on pIJ9001 and also produces several short-chain AHLs; however, little is known about the role of this quorum-sensing system.

Although much work has gone into characterizing the quorum-sensing network of *R. leguminosarum*, little is known about the role of these systems in the life cycle of the organism. Mutations in the *rai*, *cin*, and *tra* systems do not have any apparent defects in nodulation. The *rhi* system seems to play a role in nodulation efficiency, but no dramatic defect has been observed for *rhi* mutants that might suggest a possible mechanism. The only system with a defined role is the *tra* system, since it was clearly shown to regulate the conjugal transfer of pRL1JI, a symbiotic plasmid. However, the advantage of having plasmid transfer under the control of the *cin* system is not apparent. Lastly, the growth inhibitory role of OH-C14:1-HSL and *cinRI* is still a mystery. This AHL-mediated growth inhibition was shown by Gray *et al.* (1996) to result from an early induction of the stationary phase, but only strains carrying pRL1JI are sensitive to the growth inhibition. Furthermore, addition of OH-C14:1-HSL has been shown to promote starvation survival of *R. leguminosarum* cultures that enter stationary phase at low cell density.

Detection of AHL

Screening for AHL production from bacterial strains has typically relied on bacteriological monitor systems (Swift *et al.*, 1993; Cha *et al.*, 1998; Gram *et al.*, 1999). Numerous bioassays and sensor systems have been developed to allow facile detection, characterization and quantitation of microbial acyl HSLs^{34–40}. These monitor systems consists of a phenotypic response (e.g. bioluminescence, violacein production, β -galactosidase activity or growth inhibition) activated through an AHL-receptor protein. Such strains contain an easily assayable reporter gene and lack all AHL synthases, such that reporter activity requires exogenous AHLs. Each receptor protein responds to a different range of AHLs (McClellan *et al.*, 1997; Cha *et al.*, 1998; Winson *et al.*, 1998).

Two strains used as such biosensors are *Chromobacterium violaceum* CV026 and *R. leguminosarum* bv. *viciae* A34.

Chromobacterium violaceum, a Gram-negative bacterium commonly found in soil and water, produces the characteristic dye violacein, a water insoluble purple pigment with

antibacterial activity. Pigment production in *C. violaceum* is regulated via quorum sensing. McClean *et al.* (1997) subjected *C. violaceum* to mini-Tn5 transposon mutagenesis obtaining a double Tn5 insertion, violacein negative, white mutant (CV026). The transposon insertion sites have been mapped to a putative repressor locus and to a luxI homologue (*cviI*) respectively. This makes CV026 a simple biosensor for the detection of AHLs. The length of the N-linked acyl chain is a key structural feature determining the relative agonistic activities of the series AHL and AHT compounds evaluated in the CV026 violacein induction assay. The CviR of *C. violaceum* responds mainly to short chained unsubstituted AHLs (AHLs with acyl chain ranging from 4 to 8 carbons). HHL, the natural *C. violaceum* AHL, is the most active agonist. Then OHHL, BHL and OHL can also be detected, but their activity is much more weaker than HHL.

The growth of some strains of *Rhizobium leguminosarum* bv. *viciae* is inhibited by N-(3-hydroxy-7-cis tetradenoyl)-L-homoserine lactone (3OH-C_{14:1}-HSL), which was previously known as the small bacteriocin before its characterization as an N-acyl homoserine lactone (Wilkinson *et al.*, 2002). *R. leguminosarum* bv. *viciae* strain A34 behaves in such manner. The growth-inhibitory effect was found to be bacteriostatic rather than due to cell death and it was concluded that 3OH-C_{14:1}-HSL could switch the bacteria into the stationary-growth phase. The A34 sensitivity to 3OH-C_{14:1}-HSL is used in growth inhibition tests to detect the presence of this quorum sensing molecule

Scopes of the present study

The aim of our experimental tests on quorum sensing described below, was first to evaluate the spatial diffusion range of HSL molecules by a microscopy-based approach, using a producer and a sensor of quorum signals whose inducible genes were fused to the green fluorescent protein reporter gene. In second instance we analyzed the natural populations of *Rhizobium leguminosarum* bv. *viciae* described in the previous chapter by screening their production of AHL signal molecules using microbial biosensors in plate assays.

Materials and methods


Quantification of Quorum sensing calling distances by fluorescence microscopy and CMEIAS image analysis

Bacterial strains. In this work, we used two genetically tagged strains: one of *Pseudomonas aeruginosa*, namely strain MH211, that constitutively makes AHL and expresses a red fluorescent protein (RFP) enabling its detection by fluorescence microscopy. The second is an *E. coli*, strain JB357, that does not synthesize AHL, but, due to a gene fusion luxR PluxI-gfp (ASV), produces a green fluorescent protein (GFP) in response to sufficient external AHL secreted by the *P. aeruginosa* strain and can be detected by its cellular green fluorescence. The *gfp* gene in the *E. coli* biosensor strain is the ASV variant, which when activated, makes the cell retain green fluorescence transiently for only a few hours before it switches back to darkness.

The 3-dimensional system. Cell suspensions of both strains were mixed briefly in molten LB agar cooled down to 43°C and 300 µl of this mixed suspension were pipetted onto a sterile glass slide. A coverslip was applied to flatten the layer down. Then the glass slide was placed into an empty Petri dish, sealed with parafilm and incubated for 24 h at 37°C. This experimental design had the advantage of allowing to study cell-to-cell communication in 3-dimensions, more closely approximating the real world situation that microbes face in biofilms. During the incubation in such covered slide culture, aeration and cell division were very limited, so cells rarely formed colonies but nevertheless emitted cell communication signals that form gradients. However oxygen diffusion did occur near the edge of the coverslip, creating the opportunity for microcolonies to occasionally develop in this area and allowing to analyze also whole-colony behaviour.


Image editing and CMEIAS analysis. The low cell density in this experimental design was such that allowed to select microscope fields from which each image acquired would contain one green cell (or one microcolony), and one red cell. Surrounding fields and


deeper planes of focus were carefully inspected to rule out the presence of other red cells at distances closer than the one appearing in the image.




CMEIAS®
*Center for Microbial Ecology
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About CMEIAS



 **The MSU Center for
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CMEIAS® 

New generation image analysis software for computer-assisted microscopy of microorganisms and their ecology.
Development team: Frank Dazzo, Jinhui Liu, Bin Yu, Olga Glagoleva, Anil Jain, Dom Trione, Jaime Maya, Ed Marshall, Feng-I Liu, Chandan Reddy, Amar Prabhu, Madhu Wadekar, Ronen Peretz, Jose Zurdo, Amit Gore, John Urbance, Ramesh Bollempalli, Donna McGarrell, Hass Hammoud, Kirsten Kulek, Mingfei Li, Jun Wang, Clay Dowling, Lei Gao, Nisha Hollingsworth, Gang Tang, Stephan Gantner, Youssef Yanni, Guoyu Zhu, Colin Gross, Elisa Polone, Deena Nasr, Andrea Squartini, Abu-Bakr Gomaa, Crystal Passmore, Chris Monosmith, Jen Baric, Lei Shan, Shin-ichi Nakano, Chris Meyers, Shiva Zamani, Nithin Phillips, Ashley Hankinson

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**Strengthen microscopy-based approaches for understanding
microbial ecology at spatial scales relevant to the microbes...**

Figure II.4 – CMEIAS is a free downloadable (<http://www.cme.msu.edu/CME/index.html>) image analysis program which uses various measurement features and two object classifiers to extract size and shape measurements of segmented, digital images of microorganisms and classify them into their appropriate morphotype.

Each digital picture was acquired in two steps, with the first image focused on the green cell using a WIB filter and the second picture focused on the red cell using an SWG filter. These barrier filters would maximally allow the green and red emitted fluorescence light to transfer, respectively. Then each pair of colored images was merged into one to retain the X, Y spatial coordinates of the fluorescent cells using Adobe Photoshop.

By calibrating the degrees of turning of the micrometric fine focusing knob, it was possible to determine how many micrometers apart were the two cells in the vertical (Z) dimension. The Center for Microbial Ecology Image Analysis System (CMEIAS) software (Fig II.4) operating in UTHSCSA ImageTool was used to spatially calibrate these composite images and then to measure the linear distance separating the object centroid of each activated green fluorescent AHL-sensor cell and its nearest red fluorescent AHL-source cell neighbor. By knowing this 2-dimensional distance plus the vertical distance measured separately by using the calibrated focusing knob, it was possible to calculate the real, 3-dimensional distance between the cells, representing the "effective calling distance" of AHL-mediated cell-to-cell communication. This triangulation calculation is based on the Pythagora's theorem (the square root of the sum of each cathetus's square).

Screening for the production of AHLs

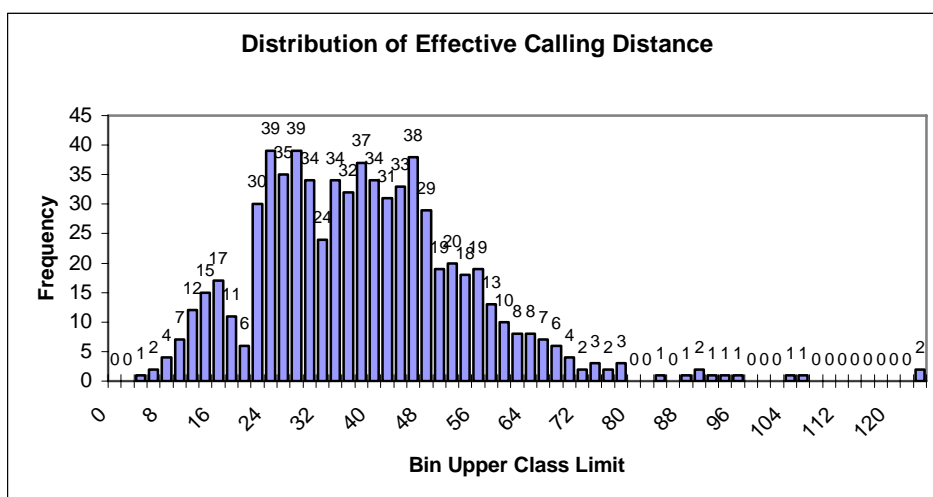
***Chromobacterium violaceum* bioassay.** *Chromobacterium violaceum* CV026 was streaked on an LB agar plate with kanamycin 25µg/ml and incubated at 28°C. A loopful was resuspended in 20 ml of LB broth with kanamycin 25µg/ml and grown at 28°C overnight. After 24h ,10 ml of the *C. violaceum* culture were transferred in 90 ml of LB broth and grown at 28°C for 18 hours. The resulting culture was mixed in proportion 1:2 with Salt Top Agar (10g/l tryptone, 5g/l NaCl, 6.5 g/l agar) previously melted and cooled down at 43°C. A volume of 180µl was pipetted in each well of a 96-well microtiter plate. The *Rhizobium leguminosarum* bv. *viciae* strains were then streaked, one in each well and the microtiter plate was incubated at 28°C The microtiter plates were incubated overnight at 28°C and examined for the stimulation of violacein synthesis, indicated by purple pigmentation of the wells.

***R. leguminosarum* A34 bioassay.** Strains to be tested for the production of 3OH-C_{14:1}-HSL were streaked in the centre of a TY agar plate. *Rhizobium leguminosarum* strain A34 was grown in TY broth at 28°C overnight. An aliquot of the culture was suspended in TY broth to give an OD₆₀₀ of 0.01-0.05, and the latter was added to three volumes of TY soft-agar (10g/l agar). The previously streaked petri dishes were overlaid with a thin layer of the *R. leguminosarum* A34 mix and inoculated at 28°C. The production of 3OH-C_{14:1}-HSL was assessed by evaluating and measuring the size of growth inhibition halos following 2 days of growth at 28°C.

Results and discussion

Quantification of Quorum sensing calling distances by fluorescence microscopy and CMEIAS image analysis

By using computer-assisted microscopy, we analyzed the effective calling distance between AHL-source and AHL-sensor strains of bacteria at single-cell resolution. The frequency distribution of computed effective calling distances is shown in Fig. II.5. The majority of the activated sensor cells were in close proximity to signal-producing cells, with the mode of effective calling distances being 46.8 μm . The full range of effective calling distances measured in this study extended to a distance of 123.7 μm . This maximal effective calling distance is very long-range for bacteria considering that they are approximately 1-2 μm in length, equivalent to approximately the length of a European soccer field.



effective cell-to-cell communication. This finding is fully consistent with recent results obtained using a similar biosensor system of AHL-mediated bacterial communication during colonization of plant roots (Gantner *et al.* 2006).

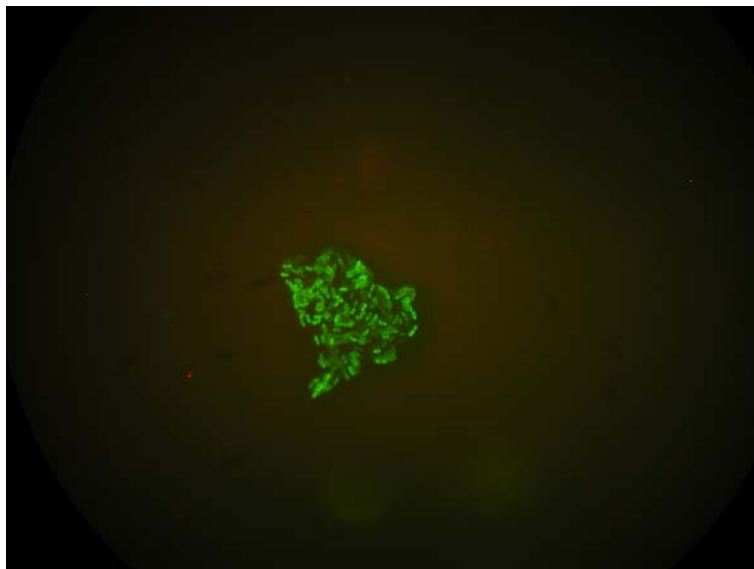


Figure II.6 – *E. coli* microcolony activated by a single *P. aeruginosa* red cell.

Detection of AHLs in natural *R. leguminosarum* bv. *viciae* populations

The object of the present study stems from the previous field trial (see Chapter I). The microbial inhabitants of pea root nodules formed on 9 plants purposely sown in a soil within the University's experimental agricultural station had been isolated and characterized upon DNA-based PCR-electrophoretic fingerprinting. They were then stored at -80°C as glycerol stocks.

The plants come from 3 different delimited parcels of a soil: 4, 5 and 13, as shown in Figure I.3B. Three plants were considered from each parcel, coded α , β and γ .

In this study strains isolated from the plants of the three plots were submitted to two assays to detect their production of quorum sensing molecules.

A total of 520 strains has been examined and in 436 cases both the bioassays could be performed, 402 of which represented by *R. leguminosarum* bv. *viciae* strains and 34 characterized as *Enterobacteriaceae*.

Examples of the results are shown in Figg. II.7-8.

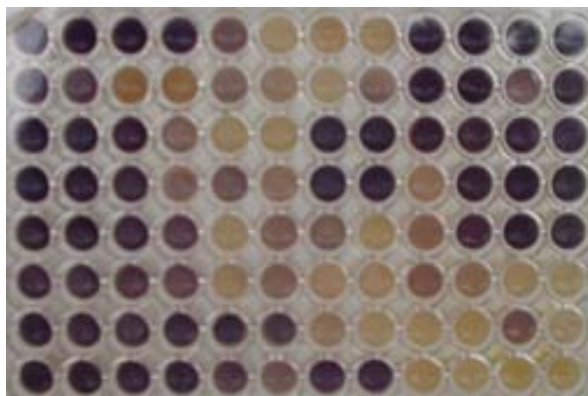


Figure II.7 – Example of induction of violacein synthesis in *C. violaceum* CV026 by *R. leguminosarum* bv. *viciae* AHLs.



Figure II.8 – Example of growth inhibition of *R. leguminosarum* bv. *viciae* strain A34 by strains of the same species producing C14 AHL. Left: negative isolate, Right: positive isolate.

The data are summarized in Table II.1. The following observations can be made. (a) The production of medium-short chain AHL quorum sensing signals is a prevailing phenotype in the *R. leguminosarum* bv. *viciae* population examined, being the frequency of positive cases in the overall CV026 experiment 77.1 %. The trait is however not indispensable for pea nodulation as negative strains are encountered in this nodule-selected population sample. (b) The production of long chain (C14) AHL revealed by the growth inhibition assay also scores as a numerically dominant feature, the positive cases counted are 82.3% of the total *R. leguminosarum* bv. *viciae* strains. (c) The occurrence of quorum sensing signalling phenotypes is different in the three plots, being more represented in plot 4 and 5. This suggests a possible independent microevolution of the rhizobial communities.

An important fact to point out is that the totality of *Enterobacteriaceae* strains (34 cases) were negative to both phenotypes proving that they do not produce short medium-short chain nor long chain AHLs detectable by these assays.

Plant	Plot 4			Plot 5			Plot 13		
	4 α	4 β	4 γ	5 α	5 β	5 γ	13 α	13 β	13 γ
Total strains examined	75	26	13 (29)	70	17	79 (2)	50	38	34 (3)
Total strains examined in plot	114 (29)			166 (2)			122 (3)		
positive violacein assay	71	26	7	62	8	61	29	27	19
negative violacein assay	4	0	6 (29)	8	9	18 (2)	21	11	15 (3)
% positive violacein assay	94%	100%	53%	88%	47%	77%	58%	71%	55%
% positive violacein assay in plot	91%			78%			61%		
positive growth inhibition	71	23	10	63	12	64	40	25	23
negative growth inhibition	4	3	3 (29)	7	5	15 (2)	10	13	11 (3)
% positive growth inhibition	94%	88%	76%	90%	70%	81%	80%	65%	67%
% positive growth inhibition in plot	91%			83%			72%		
Positive to both above phenotypes	68	23	7	58	7	53	27	21	13
% Positive to both above phenotypes	85%			71%			50%		
Violacein + , Growth inhib. -	3	3	0	4	1	8	2	6	5
% violacein + , Growth inhib. -	5%			7%			10%		
Violacein - , Growth inhib. +	3	0	3	5	5	9	13	4	10
% violacein - , Growth inhib. +	5%			11%			22%		
Negative to both above phenotypes	1	0	3 (29)	3	4	9 (2)	8	7	6 (3)
% negative to both above phenotypes	3%			9%			17%		

Table II.1 – Quantitative summary of the results from the two assays, violacein induction in *C. violaceum* CV026 and growth inhibition of cospecific indicator strain *R. leguminosarum* A34. The numbers in brackets indicate strains of *Enterobacteriaceae*.

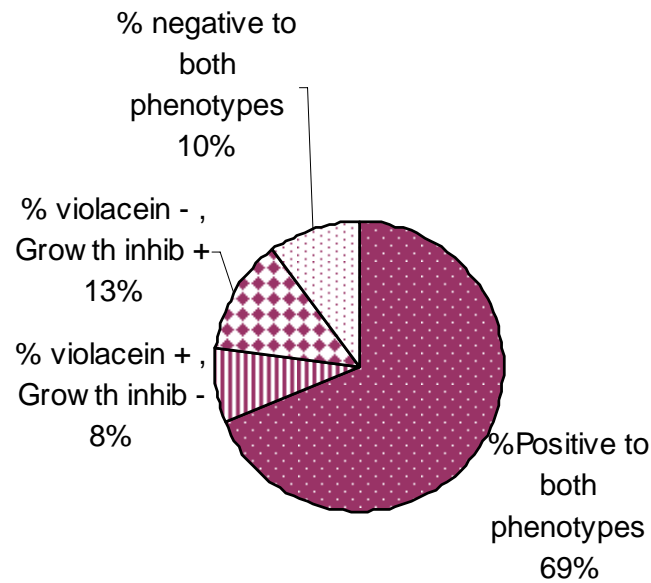


Figure II.8 – Frequency of *R. leguminosarum* bv. *viciae* strains positive or negative to one or both bioassays.

In terms of coincidence of phenotypes this is not strictly correlated. 68.9 % of the cases are found positive for both, 10.2% of the cases resulted negative in any of the two bioassays and the remaining 20.9% of the strains showed to produce only one or the other kind of AHLs (Fig. II.9).

In Chapter I the same strains were characterized by a fingerprinting PCR technique allowing to assess the similarity degree among the microsymbionts in terms of genetic relatedness. Consequently different patterns of nodulation could be detected, as several strains were able to colonize more nodules (recurring) while others, the majority, were found in only one nodule in the whole survey (520 nodules collected in 9 plants) and thus considered “unique”. We also considered if the profile was found in only a certain plant (specific) or in different plants (shared). Moreover the profile can be part of a group (family) of profiles joint by a high degree of similarity (see Chapter I for details).

In order to evaluate any correlation between the strain behaviour in terms of signal molecule production and their nodulation pattern these data were matched (Table II.2) with those obtained in the BOXA1R characterization analysis.

The strains have been subdivided in the resulting 8 profile-types as listed in Table II.2.

The data do not include the *Enterobacteriaceae* strains, and consider rhizobia only.

plot	plot 4				plot 5				plot 13			
	CV026		A34		CV026		A34		CV026		A34	
result	+	-	+	-	+	-	+	-	+	-	+	-
Total cases	114				166				122			
unique^a (specific)^b	30				46				40			
	25	5	26	4	27	19	30	16	21	19	21	19
unique (specific)-family^c	27				42				33			
	26	1	25	2	35	7	35	7	22	11	25	8
recurring^d (specific)	0				5				2			
	0	0	0	0	4	1	5	0	0	2	0	2
recurring (specific)-family	2				19				18			
	0	2	2	0	16	3	17	2	12	6	16	2
single^e (shared)^f	2				1				0			
	2	0	0	0	1	0	1	0	0	0	0	0
single (shared)-family	14				16				15			
	13	1	13	1	13	3	14	2	9	6	14	1
recurring (shared)	2				0				0			
	2	0	2	0	0	0	0	0	0	0	0	0
recurring (shared)-family	37				38				14			
	36	1	34	3	36	2	36	2	10	4	11	3

Table II.2 – AHLs production behaviour and nodulation pattern of *R. leguminosarum* bv. *viciae* populations nodulating plants of *P. sativum*. ^a “Unique” : found once at all in the whole survey. ^b “Specific” : profiles found only on one plant. ^c “Family” : belonged to a group sharing great similarity. ^d “Recurring” : found twice or more per plant. ^e “Single” : found once per plant. ^f “Shared” found on more than one plant.

The lack of production of C4-8 and C14 AHLs seems more frequent in unique profile strains, especially for rhizobia collected in plot 5, indeed 26.1% of unique profiles of this plot are negative to A34 bioassay, while only 7.6% of all the other profiles do not produce an halo in the test. Likewise 29.5% unique profiles of plot 5 are negative to CV026 assay, and the negative cases for the remaining profiles are 11.3%. In the other two plots, 4 and 13, still the frequency of negative cases is higher for unique strains than for non-unique ones, even though the gap is smaller (Table II.3).

plot	plot 4		plot 5		plot 13	
bioassay	CV026	A34	CV026	A34	CV026	A34
Unique profiles	10.5 %	10.5 %	29.5 %	26.1 %	41.0 %	36.9 %
Non-unique profiles	7.0 %	7.0 %	11.3 %	7.6 %	36.7 %	16.3 %

Table II.2 – Frequency of negative cases for the two bioassays, violacein induction in *C. violaceum* CV026 and growth inhibition of cospecific indicator strain *R. leguminosarum* A34, for strains scoring as “unique” or “non-unique”.

Conclusions

Quantification of Quorum sensing calling distances

This study of AHL mediated cell-to-cell communication quantified at single cell resolution through AHL-source and AHL-sensor strains by using computer-assisted microscopy indicates that the phenomenon can occur between individual bacteria as even one single cell can produce sufficient AHL signal molecules to communicate with a neighbour cell within calling distance. Moreover a whole microcolony of sensor cells can be activated by the AHL produced by a single producer cell. Thus, an individual cell can produce an AHL diffusion profile sufficient for the activation of nearby bacteria, suggesting that cell-to-cell communication is governed more by the spatial proximity of cells within AHL gradients than by a quorum group requirement of high population density. The maximum spatial distance was found to be 123.7 μm , a very long distance if compared to a bacterium size (1-2 μm). As the contribute of farther-away producers outside the microscope field range can not be ruled out, these value are to be considered only indicative. Biophysical studies on AHL diffusion are in progress to verify the compliance of the observed results in terms of distance and output range.

Detection of signal molecules in natural *Rhizobium leguminosarum* bv. *viciae* populations

Natural bacterial populations isolated from peas nodules were screened for short-medium and long chained AHL production using two reporter systems, respectively *Chromobacterium violaceum* CV026 and *Rhizobium leguminosarum* A34. Most of the natural *rhizobium* strains were found to be quorum-signalling positive. The production of C₄-C₈ chain length molecules was observed in 77% of the 402 *R. leguminosarum* bv *viciae* screened strains and 82% of the latter showed to produce also C₁₄ AHLs. None of the reporter systems detected the production of AHLs by any of the 36 *Enterobacteriaceae* strains tested.

The quorum-positive trait is not indispensable for pea nodulation by *rhizobium* as strains testing negative to one or both phenotypic assays are also encountered in this nodule-selected population sample.

The lack of production of C₄₋₈ and C₁₄ AHLs seems more frequent in non-dominant strains, intended as those found in only one nodule per plant or in only one nodule in the whole experiment.

Chapter III

Root nodule bacteria of wild legumes from Sardinia

Introduction

Nodule-associated bacteria in wild legumes

In Chapter I the nitrogen-fixing symbiosis between plants of the family Leguminosae and their specific microsymbionts, collectively named rhizobia, was widely discussed. A great deal of rhizobium research has been traditionally devoted to cultivated species, for which the rhizobial microsymbionts have been thoroughly characterized and described (Thies *et al.*, 2001). In contrast, little attention has been dedicated to the root-nodule symbionts of truly wild legumes, intended as those whose ecology is for the most part independent of human action or carry-over. The *Leguminosae* family includes over 18,000 species, (<http://www.ildis.org/Leguminosae/>) but only a minor portion, mostly representing by crops of agricultural interest, has been examined, thus the knowledge available on the biological diversity of interactions between legumes and microbes is still very small. The traditional strategy to investigate nodule-associated microbial symbionts involves their isolation and cultivation from internal tissues of surface-sterilized nodules (Vincent, 1970). The description of symbiotic partnerships for the different legumes has therefore traditionally relied, as a startpoint, upon the generally regular culturability of the bacterial occupant within the nodule when streaked on yeast-mannitol-based agar plates. Despite the physiological transformation of vegetative bacteria into non-dividing bacteroids, it is normally observed that rhizobia are regularly cultured from surface-disinfected crushed nodules. This implies that either some vegetative rods (still confined or recently released from infection threads) have not undergone the bacteroid conversion (Timmers *et al.* 2000), or that some bacteroids can be resuscitated back to the culturable state, or both. Numerous studies confirmed that nodules can be colonized internally by several bacterial genera, unrelated to rhizobial symbiotic nitrogen fixation. Philipson and Blair (1957) found diverse species, including Gram positives, in roots and nodules of healthy red and subterranean clover plants. Sturz *et al.* (1997) showed that rhizobia recovery from red clover nodule tissue could yield up to 4.3×10^9 cfu/g fresh weight, but that, at the same time, 3.0×10^5 cfu/g of non-rhizobial endophytes, belonging to 12 different species, could be cultured from the same nodules. *Agrobacterium* sp. was signalled in nodules of tropical legumes (De Lajudie *et al.*, 1999). In bean nodules Mhamdi and coworkers (2005) found, along with *Rhizobium*,

Agrobacterium-like bacteria and proved that these could invade new nodules upon co-inoculation with rhizobia and affect their nodulation performance (Mrabet *et al.*, 2006). Actinobacteria as *Streptomyces lydicus* have been reported to colonize pea nodules (Tokala *et al.*, 2002).

Materials and methods

Bacterial strain isolation and culture conditions

Root segments bearing nodules from the different plants were washed free of soil under running water, then encaged in a fine-mesh steel holder and surface-sterilized by immersion in 95% ethanol for 20 seconds followed by 5% sodium hypochlorite for 3 minutes; and finally washed 7 times with sterile distilled water. As alternative surface sterilization procedure, in place of the NaClO step, after the ethanol treatment, nodules were immersed in 0.1 % HgCl₂ for 2 minutes, and the H₂O washes were extended to 10. All treatments were performed under microbiologically controlled conditions. Tests to validate surface-sterilization of plant tissues were performed by touching them several times on the surface of plate count agar (PCA, Difco) plates prior to isolation of the interior microbiota.

Surface-sterilized root nodules were transferred into empty sterile plastic dishes and squashed in 50-150 µl of sterile physiological saline solution, the volume varying in proportion to the nodule size. About one fifth of the resulting suspension volume was withdrawn for lysis and direct 16S rDNA PCR, aimed at verifying the identity of the prevailing bacterial species by a culture-independent approach. The remaining portion from the squashed nodule suspension was streaked on yeast-mannitol agar (YMA) plates containing Congo-red (Vincent, 1970), on Plate Count Agar (PCA), and on defined BIII-agar (Dazzo, 1982). As alternative rhizobial media with pH-buffering capability, YMA containing 4g/l calcium carbonate (Jordan 1984) and medium I (Hovieson *et al.* 1988, also known as 1/2 Lupin Agar), were also tested. The latter was modified by using 0.17 g/l K₂HPO₄ and 0.13 g/l KH₂PO₄. Plates were incubated at room temperature for up to two weeks.

Plant cultivation and nodulation tests

Mature pods were collected from wild plants in their natural habitat and kept dry at room temperature until used. Seeds were removed manually from pods, surface-sterilized by immersion in 70% ethanol for 30 sec followed by stirring in 0.1% HgCl₂ for 7 min, and rinsed in 7 changes of sterile deionized water. Seeds were pre-imbibed for 3 hours in the

final wash. Dormancy was broken by mechanic scarification with autoclaved material as follows; seeds were transferred over a ribbed rubber sole fitted in a polypropylene box and gently streaked for five seconds with bodywork-grade medium grain sandpaper. Germination and concomitant verification of surface sterility were obtained by spreading seeds on YMA plates wetted with 10 drops of sterile water, and incubated inverted for three days in the dark. Germinated seedlings were transferred aseptically to sterilized plastic Leonard jars (Fig. III.1) containing a water-washed, oven dried, quartziferous sand-vermiculite 1/3 mixture, fed from the bottom with nitrogen-free Fåhræus solution.

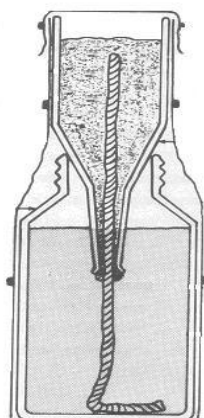


Figure III.1 – Schematic representation of a Leonard jar system.

The rooting mixture was re-humidified with 1/10 vol of sterile nitrogen-free Fåhræus solution and autoclaved in plastic biohazard type bags prior to transfer to the top portion of the Leonard jar assembly. Bacteria were inoculated by dispensing a 1 ml suspension of an overnight-grown liquid culture in YMB medium (approximately 10^8 cells/ml). Seeds were covered with a layer of autoclaved gravel and the entire Leonard jar assemblies were transferred to a growth cabinet (Angelantoni, Sas. Massa Martana, Italy) programmed for a 16 h daylight photoperiod at 23 °C, night temperature of 18 °C and 60% constant relative humidity. Plants were inspected as early as after 40 days for nodule formation and grown for up to three months. For nodulation by natural symbionts, uninoculated seedlings were transplanted to jars containing field-collected soil instead of the sand-vermiculite mix.

DNA extraction

Cells were lysed by resuspending a loopful of plate-grown isolated colonies in 50 µl of lysis buffer (0.25% SDS, 0.05M NaOH) in an eppendorf tube followed by stirring for 60 sec on a vortex and heating at 95 °C for 15 min. The lysate was centrifuged for 15 min and 10 µl of the supernatant were mixed with 90 µl of sterile water. Lysates were stored at 4 °C prior to PCR. For direct PCR analysis, nodules were squashed in 50 µl of sterile physiological saline solution and 10 µl of the suspension were transferred to 50 µl of lysis buffer, and treated according to the same protocol described above for DNA isolation.

16S rDNA and ARDRA analysis

One µl of the lysate containing the total DNA of each bacterial isolate was treated in a PCR BioRad I-Cycler using the two 16S rDNA-targeted universal bacterial primers 63F (5'CAGGCCTAACACATGCAAGTC) (Marchesi *et al.* 1998) and 1389R (5'ACGGGCGGTGTGTACAAG) (Osborn *et al.* 2000) at 1 µM each in a 25µl reaction volume, using the following program: initial denaturation at 95 °C for 2 min; 35 cycles at 95 °C for 30 sec, 55 °C for 1 min, 72 °C for 4 min and a final extension at 72 °C for 10 min. The PCR reaction mixture contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each dATP, dCTP, dGTP and dTTP, 1 µM of each primer and 2.5 U Taq DNA Polymerase, recombinant (InVitrogen Life Technologies). Amplicons were digested overnight at 37 °C upon mixing 5 µl from the 25 µl reaction volume with 1 µl of *Cfo*I enzyme (Pharmacia, Uppsala, Sweden) and 2 µl of 10 x reaction buffer. Digested DNA was loaded on a 1.5% agarose gel, run electrophoretically for 3 h at 110 V. The ethidium bromide-stained gel was visualized over an UV transilluminator and photographed by a Kodak DC290 digital camera.

DNA Sequencing

One µl of the amplicon resulting from the above described PCR amplification was mixed with 1 µl containing 6.4 picomoles of the above described forward primer 63F in a 0.2 ml polypropylene tube and then dried by incubating the tube open for 15 min at 65 °C in an I-Cycler thermal cycler. The template and primer mix was directly used for di-deoxy-cycle

DNA sequencing with fluorescent terminators (Big Dye, Perkin-Elmer/Applied Biosystems, Foster City CA) and run in an Applied Biosystems ABI Prism 3730XL automated DNA sequencer. Chromatograms were analyzed by Chromas 2.23 software (Technelysium Pty Ltd, Tewantin Australia).

Results and discussion

Plant collection and nodule examination

An extensive search was conducted to locate plants of interest in different suitable biotypes within Sardinia during springs of 2004 and 2005. The legumes sampled, the nearest urban settlement, the geographical coordinates of the sampling site, and the number of nodules collected and analyzed were as follows: *Hedysarum spinosissimum* (Giòscari, 40°42' N, 8°33' E; and Castelsardo 40°54' N, 8°41' E, 15 nodules), *H. glomeratum* (Pimentel, 39°29' N, 9°04' E, and Segariu, 39°34' N, 8°57' E, 13 nodules) *Hippocrepis unisiliquosa* (Castelsardo, 25 nodules), *Scorpiurus muricatus* (Castelsardo, 25 nodules), *Tetragonolobus purpureus* (Nurèci 39°50' N, 9°01' E, 34 nodules), *Ornithopus compressus*, (Bolòtana, 40°19' N, 8° 57' E, 24 nodules) *Ornithopus pinnatus* (Bolòtana, 21 nodules), and *Psoralea bituminosa* (Castelsardo, 5 nodules).



Figure III.2 – *Tetragonolobus purpureus*

The root systems of all field-collected species bore tubercular structures that varied in number and shape (Fig. III.3). Root nodules on *T. purpureus* and *P. bituminosa* were typically round and determinate, and very large in the case of the latter species, whereas the root nodules on the other legumes had elongated-indeterminate to irregular shapes. Multi-

lobed nodules were present on roots of *H. spinosissimum* often in relation with the site of isolation.

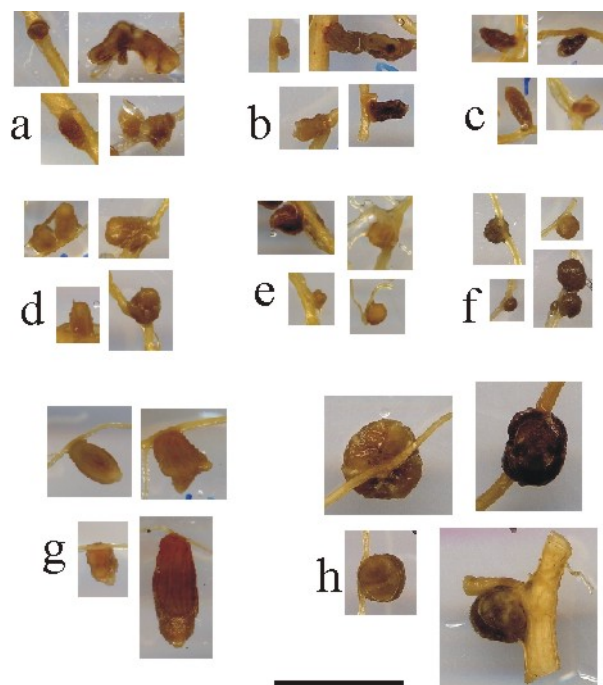


Figure III.3 – Examples of excised root nodules of the different plants included in this study. a: *H. glomeratum*; b: *H. spinosissimum*; c: *H. unisiliquosa*; d: *O. compressus* ; e: *O. pinnatus*; f: *T. purpureus*; g: *S. muricatus*; h: *P. bituminosa*. Scale bar equals 1 cm.

Bacteria isolation from nodules

In total, 79 nodules from the 2004 campaign and 25 nodules from the 2005 campaign were evaluated. The surface-sterilization treatment was generally efficient, since in most cases, no colonies developed on PCA plates upon which nodules were rolled prior to sectioning. In about 10% of the cases where surface sterilization was not achieved, the squashed nodule samples were not further considered. A total of 161 culturable bacterial isolates were isolated from inside the surface-sterilized nodules from the eight plant species harvested in Sardinia. Sequencing of the corresponding amplified 16S rDNA revealed at least 12 broad lineages, encompassing a diversity represented by several taxa as defined by

GenBank database homologies. The ranked abundance of different bacteria in the nodules is summarized in Table III.1.

Host Plant	H. spin.	H. glom.	T. purp.	H. unis.	S. mur.	P. bit.	O. com.	O. pin.	TOT.
NO GROWTH	0	3	0	9	5	4	1	2	24
<i>Enterobacteriaceae</i> ¹	8	1	1	0	0	0	8	4	22
<i>Bacillus</i> ²	2	2	2	1	5	0	0	1	13
<i>Pseudomonas</i> ³	6	0	0	0	0	0	6	0	12
<i>Curtobacterium</i> ⁴	0	0	0	0	0	0	0	4	4
<i>Rhizobium</i> ⁵	2	0	0	0	0	0	0	1	3
<i>Staphylococcus</i> ⁶	0	1	2	0	0	0	0	0	3
<i>Xanthomonas</i> ⁷	2	0	0	0	0	0	0	0	2
<i>Microbacterium</i> ⁸	0	0	0	0	0	0	0	1	1
<i>Arthrobacter</i> ⁹	0	0	0	0	0	1	0	0	1
<i>Mesorhizobium</i> ¹⁰	0	0	1	0	0	0	0	0	1
<i>Agrobacterium</i> ¹¹	1	0	0	0	0	0	0	0	1
<i>Ralstonia</i> ¹²	0	1	0	0	0	0	0	0	1
<i>Thiobacillus</i> ¹³	0	0	0	0	1	0	0	0	1

Table III.1 – Number of nodules in which members of the phylogenetic groups were found in the different plants.

Quite unexpectedly, rhizobial lineages were rarely found, amounting to only single-colony occurrences in four nodule squashes that also yielded various other taxa. The most common result (27%) from plating nodule squashes was no development of microbial growth on YMA BIII nor PCA plates. When growth occurred, less than ten colonies developed. This contrasts with typical results using cultivated legumes, where the rhizobial occupants rescued by such techniques normally form a profuse lawn along most of the streak length on these plating media, indicating their abundance and culturability. In the present study,

only nodules from *Hedysarum spinosissimum* and *Tetragonolobus purpureus* yielded a dense lawn of confluent bacterial colonies, but none of these were rhizobia. As controls in our experiments we often included nodules from the cultivated legume *Hedysarum coronarium* which, on the contrary, always yielded a fully culturable load of *Rhizobium sullae*, ruling out the possibility that the results observed with the other legumes could be due to a general fault of the surface sterilization procedure.

Nodulation tests

The standard procedure used for the isolation of legume nodule symbionts revealed the absence of a consistent culturable rhizobial occupant. We next checked whether the different bacterial isolates could induce nodule formation on their hosts under gnotobiotic conditions. A series of nodulation tests were performed in growth cabinets using sterilized sand/vermiculite in Leonard jars and extending the cultivation for up to two months. Five plant species, propagated from surface-sterilized seeds were tested, including all *Hedysarum* species used here and in our previous studies, plus *Ornithopus compressus* tested for the first time. A total of 24 different purified isolates, encompassing all the diverse taxa in Table III.1 were tested either alone or in mixed inocula containing up to 10 strains. These inocula included also the strains whose 16S rDNA had high homology to *Mesorhizobium* and *Rhizobium*. No nodules were produced in any of such gnotobiotic plant tests, including the above isolates related to rhizobia. Occasionally shovel-like swellings similar to those that develop in sulla, *H. coronarium* (Squartini *et al.* 1993), developed on *H. spinosissimum* roots, but these are known to be modified short lateral roots that form independently of bacteria (A. Squartini and F. Dazzo, unpublished).

Direct 16S PCR from nodules

Since the negative nodulation results indicated that the various bacterial occupants did not induce the nodules from which they were isolated, we performed direct PCR from the squashed nodule samples, targeting 16S rDNA with eubacterial primers to test the hypothesis that the true rhizobial occupants may have lost their culturability within the nodules. The results clearly showed that indeed most of these nodules actually contain a

dominant amount of rhizobial DNA, sufficient in most cases to overwhelm and outcompete in the PCR amplification the heterologous DNA of the other non-rhizobial occupants and produce a clean sequence chromatogram upon nucleotide sequencing of the amplicon. The eubacterial DNA from some of the nodules gave a mixed but still readable sequence, with a major template series of peaks, superimposed over rather high background (data not shown), further confirming that rhizobia are dominant but not alone within the nodule. In nodule cases of *H. spinosissimum*, *H. purpureum* where the non-rhizobial occupants produced the most profuse growth on plates (rather than sparse colonies), also the direct PCR resulted in the prevailing amplification of the gamma-proteobacterial occupant. The only case where the 16S rDNA sequence from the whole nodule squash matched the same taxon as the corresponding culturable isolate was the *Mesorhizobium* sporadically found in *Tetragonolobus purpureus*.

Cultivation of wild plant species from seed in their natural soil

The above studies were performed on flowering plants collected during April 2004 and 2005. To test whether the rhizobia eventually lose culturability within the nodules or during a possible late-seasonal physiological stress of the plants, a growth chamber test was set up using seedlings of *H. spinosissimum* and *H. glomeratum* (Fig. III.4) derived from natural seeds collected in July-August. Seeds were surface sterilized, germinated on PCA plates and *transplanted* into Leonard jars filled with Sardinian soil from two sites (one was a compact soil from Giòscari, and the other was a sandy soil in an erosive hill near the coastal city of Castelsardo) where the two species naturally occur. Plants were harvested as early as 40 days after germination (early third-leaf stage) to obtain young nodules induced and invaded by their natural microbial partners before exposure to the stress of mature plant senescence.



Figure III.4 – Example of nodulation test in Leonard jars at the time of nodule collection. From the left, the first line of bottles are *H. glomeratum* plants cultivated in Castelsardo soil, in the second line Leonard jars contain the same host plant but in Giòscari soil. In the fourth line *H. glomeratum* was cultivated in sterile quartziferous sand-vermiculite and inoculated with a strain isolated in a previous nodulation test sharing 99% homology with *Mesorhizobium sp.* strain H-4 (AF279889). The other lines are represented by positive and negative controls.

Both hosts formed two to five nodules in both soils. Ten nodules were analyzed, but even in this case the same situation observed with all the nature-borne plants was confirmed. That is, three nodule squashes did not yield colonies, the other seven produced one to five colonies per nodule. The identity of these culturable taxa revealed 99% homology to *Bacillus simplex* (DQ457600) from five nodules, 98% homology to *Bacillus megaterium* (DQ457599) from two nodules, and one single colony with 99 % homology to *Thiobacillus sp.* (DQ457598). Nevertheless the direct PCR of eubacterial 16S rDNA from the nodule squashes gave the same sequence with 99% identity to *Mesorhizobium sp.* strain H-4 (AF279889). In terms of endophyte dynamics, considering that those examined in this case represent early phases of their ontogenesis and confirm that endophytic invasion of the nodules has already started at this stage.

Some of the *H. glomeratum* plants grown in soil from Giòscari were kept for up to three months in the growth cabinet. An analysis of seven nodules from these plants yielded culturable colonies with 100 % homology to *Staphylococcus aureus* (DQ457597), 100% to *Rothia mucillaginosa* (DQ457602), and, in the case of one nodule, an isolate (DQ457601) with 99% homology to *Mesorhizobium sp.* strain H-4 (AF279889), whose identity completely matches the one consistently obtained by direct PCR from nodule squashes of this legume species, indicating that in this case a sporadic event of culturability of the rhizobial occupant was possible. A subsequent nodulation test (Fig. III.4), this time, in sterile conditions, on the same host plant *H. glomeratum* was run with this cultured strain, which proved able to re-induce the abundant formation of nodules and regularly sustain plant growth as indicated by the green foliage and healthy aspect. However attempts at re-isolating the inoculated strain led again to the usual scenario: most nodules, from different individual plants, did not yield any growth.

Variations tested in the isolation procedure

The hypothesis of a general problem resulting in a too harsh sterilization method was investigated. Using nodules either collected in nature or developed in the growth cabinet, we tested different alternatives among the standard methods used for rhizobia (Vincent, 1970, Jordan 1984, Somasegaran and Hoben, 1994). However, using either hypochlorite or mercuric chloride-based procedures on different nodules from *H. glomeratum* or *H. spinosissimum*, did not alter the outcome. A lower concentration of NaClO (3%) was also tested yielding the usual results. While the same protocols used on positive control nodules of sulla (*H. coronarium*) always enabled full recovery of *Rhizobium sullae* as ascertained by ARDRA and 16S sequencing. In light of possible osmotic damage or salinity impact we checked both saline solution and distilled water as alternatives to resuspend bacteria from nodule squashes and, to relief from possible toxic compounds, we performed serial dilutions prior to plating. None of these measures did succeed in solving the issue. We also considered the possible sensitivity to acidic pH for the rhizobia of our wild legumes as some literature reports indicate for certain strains of *Sinorhizobium meliloti* (Howieson *et al.* 1988) or *Bradyrhizobia* from *Arachis* (Macciò *et al.* 2002). In those species colony development of rhizobia is halted by the acidification resulting from their own metabolism.

The problem is often coupled to calcium requirements (Howieson *et al.* 1992) and we addressed it with media reported to circumvent the inhibition such as YMA containing calcium carbonate (Jordan, 1984) or media featuring the phosphate buffer (Howieson *et al.* 1988; Nanadasena *et al.* 2001). But none of these could relieve the non-culturability phenomenon.

Exploring the possibility of a strict seasonal dependence of rhizobium viability, we collected *Hedysarum spinosissimum*, in early february 2006 (in the Algerian site of Constantine), while still in its youngest recognizable stage, consisting in the newly emerged 3-4 cm tall plantlet displaying the first composite leaf and an average of 2-3 root nodules. The results (no culturable rhizobia) were not different from the ones observed in spring isolations. We again included, as a positive control, nodules from a spontaneous stand of *Hedysarum coronarium* collected on the same day in a nearby location, which produced, as expected, regularly growing streaks of *Rhizobium sllae*.

Parallel studies of nodules from cultivated legumes in the same area, such as clover (Mateos *et al.* 1992), pea (Corich *et al.* 2001), or sulla (*Hedysarum coronarium*), routinely yielded fully culturable rhizobia, implying that physiological hindrances imposed by climatic or habitat factors could not explain why rhizobia lost their culturability within nodules of the legumes investigated in the present study. A question that arose was whether rhizobia could ever be cultured from nodules of these legumes. This was the case for *H. spinosissimum*, from which in Israel, Kishiniewsky *et al.* (2003) using the standard methods, isolated strains whose 16S rDNA sequence clusters in the *Mesorhizobium* branch with 99% identity to the one we obtain by direct PCR from the unculturable occupant of nodules of the same species. To this we can add the single isolate from one of our *T. purpureus* nodules. Thus, culturability appears to be an exception rather than the normal situation, and it may often pass unnoticed or simply unpublished.

Conclusions

The data presented here reveal two novel aspects of rhizobiology, namely that root nodules of some Mediterranean legumes harbour prevailingly non-culturable rhizobia, and these same nodules are colonized internally by an array of culturable non-rhizobial endophytes. By introducing a direct PCR analysis of nodule endophytes, the problem of non-culturability is circumvented, and in most cases this approach reveals the rhizobial aetiological agent that resides in each nodule. Recovery of the rhizobial occupant to the culturable state could not be achieved under the conditions used. This phenomenon of non-culturable rhizobia within nodules appears to be commonplace as we tested different legume species.

Our data show that the diverse nodule endophytes are most often represented by a few colony forming units, allowing identification of the unculturable rhizobium by direct nodule PCR. But in nodules of some host species (*H. spinosissimum*, *T. purpureus*), the non-rhizobial occupant is sometimes abundant yielding a dominant 16S sequence.

The inability of pure cultures of the non-rhizobial occupants to nodulate the legume under microbiologically controlled conditions precludes the hypothesis of their involvement in that type of symbiosis.

However, the abundance of these bacterial endophytes within perfectly healthy plants suggests that other beneficial interactions may be operative.

Besides the early demonstration of endophytes within legume nodules, the main standing issue is the unexpected non-recoverable state that afflicts rhizobia in these plants. Having observed the phenomenon also when reisolating from nodules originated in sterile vermiculite (after inoculating *H. glomeratum* seedlings with the only culturable strain that we obtained), one can point out that the fact is caused by the plant itself and not by other possible environmental factors existing in the soils of origin.

Chapter IV

VBNC (Viable But Not Culturable) state in microbial plant-interacting species

Introduction

VBNC

It had long been assumed that a bacterial cell was dead when it was no longer able to grow on routine culture media. We now know that there are many situations where a cell loses culturability but remains viable and potentially able to regrow. Cells in this state (viable but not culturable” VBNC) typically demonstrate very low levels of metabolic activity, but on resuscitation they are again culturable.

A typical VBNC response is shown in Fig. IV.1. As shown by the “culturable” curve, exposure to one or more environmental stresses results in a regular decline in colony forming units (CFU). However during this period of decline, “total cell counts” generally remain fairly constant. The key test that determines whether such cells are dead, or alive but in a VBNC state, is the “viability count”. Several such assays can be used to demonstrate this trait, but in all cases these characterize some aspect of metabolic activity or of cellular integrity which indicates that the cells are alive, even if they are unable to develop into colonies on culture media.

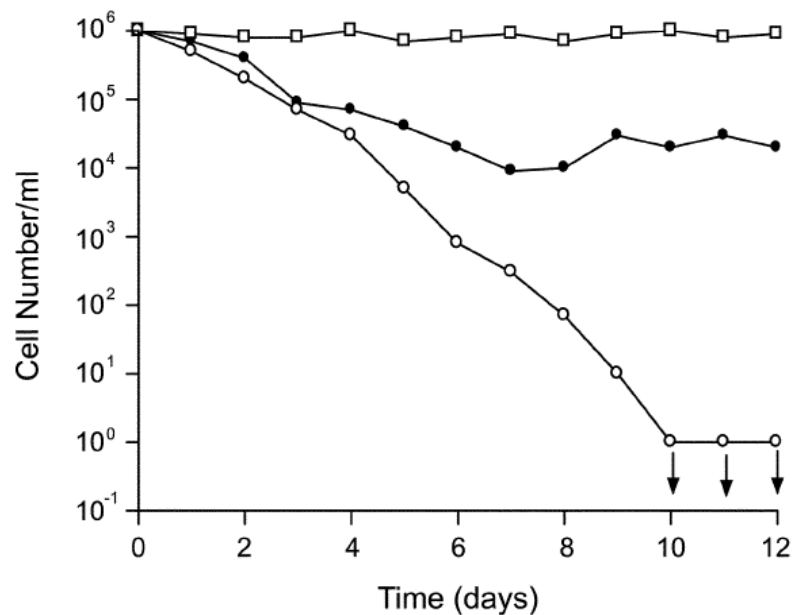


Figure IV.1 – Typical VBNC response to a stressful condition. Shown are total cell counts (□), culturable counts (○), and viable counts (●). Modified from Oliver, 2005.

Cells enter the VBNC state as a response to some form of natural stress, such as starvation, incubation outside the temperature range of growth, oxygen limitation, pH variations, salt concentration (e.g. elevated osmotic concentrations in seawater), nutrient deficiencies or exposure to white light (Oliver, 2000b). These typically are environmental stresses that might be lethal if the cells did not enter this dormancy state. Whereas a determination of the total number of cells present in a population may be obtained using DAPI or acridine orange staining, viable cell counts are typically determined using the substrate responsive assay of Kogure *et al.* (1979), by examining intracellular hydrolysis of CTC or reduction of INT as an indication of metabolic activity (Zimmerman *et al.*, 1978; Rodriguez *et al.*, 1992), or by establishing the presence of an intact cytoplasmic membrane (*BacLight*® or propidium iodide).

Cells entering the VBNC state often exhibit dwarfing, and during this period a number of major metabolic changes occur, including reductions in nutrient transport, respiration rates, and macromolecular synthesis (Porter *et al.*, 1995; Oliver, 2000a).

ATP levels, which decline rapidly in dead and moribund cells, have been found to remain high in VBNC cells (Beumer *et al.*, 1992; Federighi *et al.*, 1998). Further, recent studies have demonstrated continued gene expression by cells in the VBNC state (Lleò *et al.*, 2000, 2001; Yaron and Matthews, 2002). Extensive modifications in cytoplasmic membrane fatty acid composition that appear to be essential for entry into this state have been described (Day and Oliver, 2004). Biochemical changes in the cell walls of VBNC cells, have also been documented.

The VBNC state can only be a significant means of survival if they are able to again become metabolically active. A secondary consequence of this process of “resuscitation” is that they again become culturable.

A major and long-standing problem regarding resuscitation has been the difficulty in showing conclusively that the culturable cells that result after resuscitation of a VBNC population are a result of true resuscitation of the dormant cells, as opposed to re-growth of a few culturable cells which were not detected in the otherwise totally VBNC population.

This problem developed largely from the lack of stringent controls in many early studies on resuscitation (Kell *et al.*, 1998).

In the present study our approach was aimed at verifying whether quorum sensing AHL signal molecules could to some extent contribute to restoring culturability in VBNC cells of different bacterial species including rhizobia and other gram-negative species.

Materials and methods

Bacterial strains and growth conditions

Strains used in this work were: *Burkholderia ambifaria* MCI7, *B. ambifaria* PHP7, *B. cenocepacia* MCII35, *B. cenocepacia* MVPC ½, *Pseudomonas tolasii* P12, *B. plantarii* (ATCC 43733), *B. glumae* (ATCC 33617), *B. cepacia* (ATCC 25416), *Rhizobium leguminosarum* bv. *viciae* 248 and *R. leguminosarum* bv. *viciae* 300.

Batch cultures of all test strains were grown aerobically under shaking in 250-ml Erlenmeyer flasks containing 100 ml of nutritive medium. Medium and temperature differed depending on the strain: *B. ambifaria* MCI7, *B. ambifaria* PHP7, *B. cenocepacia* MCII35, *B. cenocepacia* MVPC ½, *P. tolasii* P12 were cultivated in NB (nutrient broth powder [Merck], 8 g; water to 1 liter) at 30 °C, *B. plantarii* (ATCC 43733), *B. glumae* (ATCC 33617) in KB (proteose peptone 20 g, MgSO₄·7H₂O 1,5g, KH₂PO₄ 1,2 g, glycerol 10 g, water to 1 liter) at 37 °C, *B. cepacia* (ATCC 25416) in LB (tryptone 10 g; yeast extract 5 g; NaCl 5 g; water to 1 liter) at 30°C, *R. leguminosarum* bv. *viciae* 248 and *R. leguminosarum* bv. *viciae* 300 in YMB (K₂HPO₄ 0.5 g; MgSO₄·7H₂O 0.2 g; NaCl, 0.1 g; yeast extract 0.4 g; and mannitol 10 g, water to 1 liter) at 30 °C.

Microcosms

To determine if stressful conditions could induce the VBNC state in these strains, several microcosms were established.

For anaerobic incubation, 30 ml late-log-phase grown cells were transferred to 400 ml bottles containing 120 ml of the appropriate nutritive medium (NB, KB, LB, YMB), then they were tightly sealed with a rubber stopper and incubated at the optimal growth temperature (30 °C or 37°C) under shaking.

To impose a nutrient limitation stress, 50 ml of late-log-phase grown cells were centrifuged at 5000 g for 15 min and washed twice with 0.9 % saline solution. The washed cell pellets were then resuspended in 5 ml 0.9 % saline solution. A specified volume of this suspension was transferred to 400 ml bottles containing 100 ml of 0.9% saline solution in order to produce a final density of 10⁷-10⁹ cells/ml. This same operation was done to produce a

second set of cell suspensions using water instead of 0.9% saline solution. Twenty mls of each suspension were transferred to 50 ml Falcon tubes.

To create both an oxygen and nutrients limiting condition 5 ml of the latter suspensions (cells in 0.9% saline solution or in sterile water) were transferred to 10 ml vacuum tubes to which was later added 5 ml of N₂ gas in the headspace.

Both sets of tubes were incubated at room temperature under darkness.

At various intervals over a 8-week period, samples were analysed for culturability by plating decimal dilutions on the same media as grown earlier, but solidified with agar. Viability, total count and tetrazolium salt reduction activity were determined as described below.

Determination of viability by fluorescence microscopy

Samples (10 ml) were centrifuged at 5500 rpm for 15 min and resuspended in phosphate buffer solution.

For total direct count (TDC), bacteria were stained using acridine orange, a toxic, fluorescing dye that stains DNA and RNA. When it binds to double-stranded DNA, it fluoresces green, when it binds with the phosphate groups of single-stranded DNA or RNA, it fluoresces orange (Bloem, 1995).

Qualitative analysis of the presence of culturable cells was performed with the fluorescent LIVE/DEAD *BacLight*® bacterial viability kit. *BacLight* is composed of two nucleic acid-binding stains: SYTO 9 and propidium iodide. SYTO 9 penetrates all bacterial membranes and stains the cells green, while propidium iodide only penetrates cells with damaged membranes, and the combination of the two stains produces red fluorescing cells. Thus bacteria with intact membranes (i.e., viable bacteria) absorb the stain that fluoresces green while excluding the red fluorescent stain, whereas bacteria with damaged membranes (i.e., dead bacteria) absorb the fluorescent red stain. Optimal incubation conditions were found to be 15 to 20 min, at room temperature in the dark.

In addition, the same samples were stained further with CTC (5-cyano-2,3-ditolyltetrazolium chloride) to detect respiratory activity, this assay uses of a redox sensitive compound that fluoresces red when reduced by an active electron transport system .

Stained bacteria were captured by microfiltration through a pore size black polycarbonate membrane filter (Millipore). Filters were air dried and mounted with a low fluorescence immersion oil (Molecular Probes) on glass microscope slides.

TDC, BacLight and CTC-reduction positive reactions were observed by fluorescence microscopy (Olympus fluorescent microscope BX60) equipped with a blue 420-nm exciter filter (Olympus BP 490).

Attempts of cell resuscitation with AHLs

The rescuing experiment was performed to check the potential recovery of culturable cells after exposure to AHL molecules. The molecules used are listed below:

-
1. N-(β -Ketocaproyl)-DL-Homoserine lactone;
 2. L-Homoserine Lactone Hydrochloride;
 3. N-Z-L Homoserine Lactone ;
 4. N-Hexanoyl-DL-Homoserine Lactone ;
 5. N-Dodecanoyl-DL-Homoserine Lactone;
 6. N-Butyryl-DL-Homoserine Lactone;
 7. N-Tetradecanoyl-DL-Homoserine Lactone;
 8. N-Decanoyl-DL-Homoserine Lactone;
 9. N-Octanoyl-DL-Homoserine Lactone;
 10. N-(β -Ketocaproyl)-L-Homoserine Lactone.
-

The molecules were dissolved in ethyl acetate and added with sterile deionized water to a final concentration of 1mM.

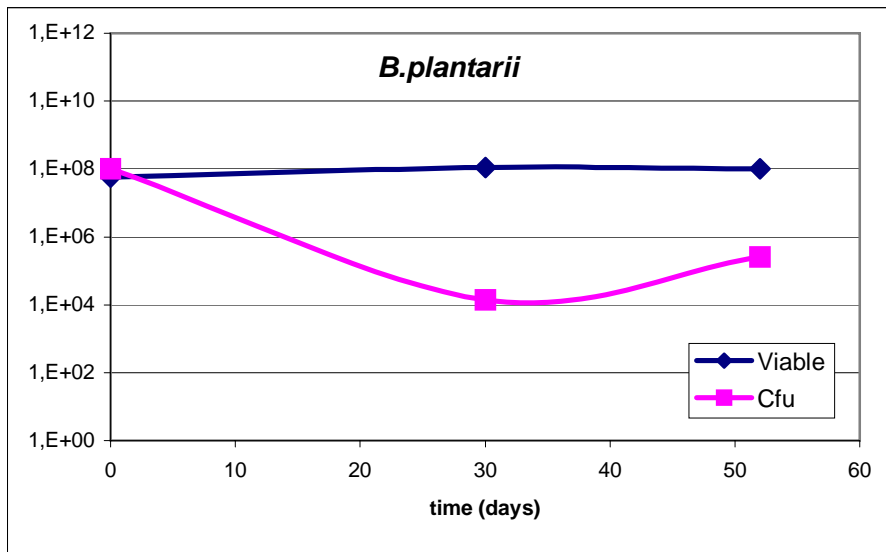
Bacterial suspensions of cells considered VBNC and AHL solution were mixed to the appropriate nutritive agar medium to a final concentration in the plates of $4 \cdot 10^{-5}$ mM. Plates were incubated at the optimal growth temperature for 3-7 days.

Results and discussion

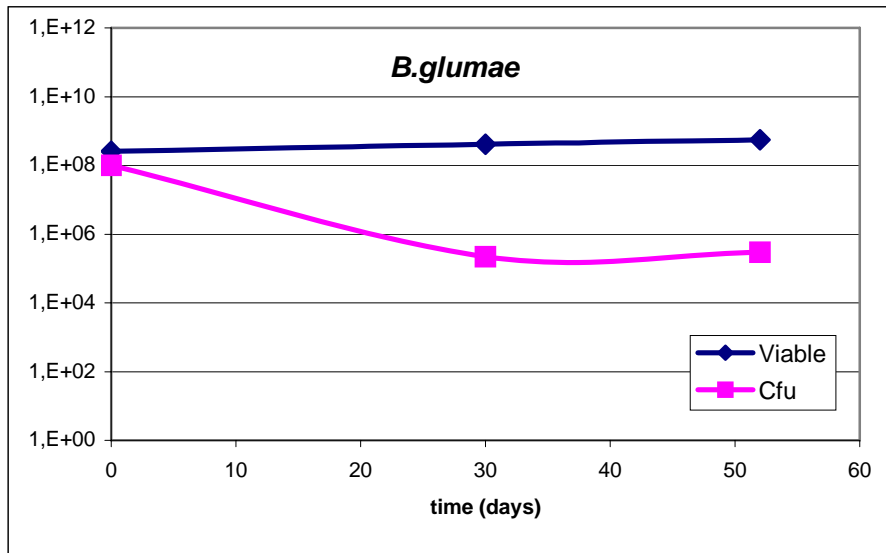
The above tests indicated that *B. ambifaria* MCI7, *B. ambifaria* PHP7, *B. cenocepacia* MCII35, *B. cenocepacia* MVPC ½, *B. cepacia* (ATCC 25416) did not enter into a VBNC state in any of the microcosms, but some test strains exhibited a decrease in population size based on viable plate counts and direct microscope counts of green fluorescent cell density using the BacLight system. Further, all cells of *B. cenocepacia* MVPC ½ lost viability when exposed to nutrient-limiting conditions while suspended in 0.9% saline solution (but not while suspended in water), and also when exposed to both O₂ and nutrients-limiting condition while suspended in saline or water. *P. tolasii* P12 entered into a VBNC state while suspended in 0.9% saline solution and exposed to both O₂ and nutrients-limiting conditions (Fig. IV.4 G). *B. plantarii* (ATCC 43733), *B. glumae* (ATCC 33617) behaved similarly, i.e., they entered into a VBNC state under anaerobic incubation (Fig. IV.2 A-B), and both without nutrients and oxygen (Fig. IV.4 C-D-E-F). *R. leguminosarum* 248 lost its viability when exposed to both O₂ and nutrients-limiting conditions, but it entered into the VBNC state in the others microcosms (Fig. IV.2 C, Fig IV.3 A). *R. leguminosarum* 300 entered into the VBNC state under all imposed stress conditions except anaerobiosis (Fig IV.3 B, Fig IV.4 A-B).

Under the experimental conditions no effect of AHL in rescuing cell culturability could be observed. The AHL molecules had no effect either in enhancing the entrance in the VBNC cells.

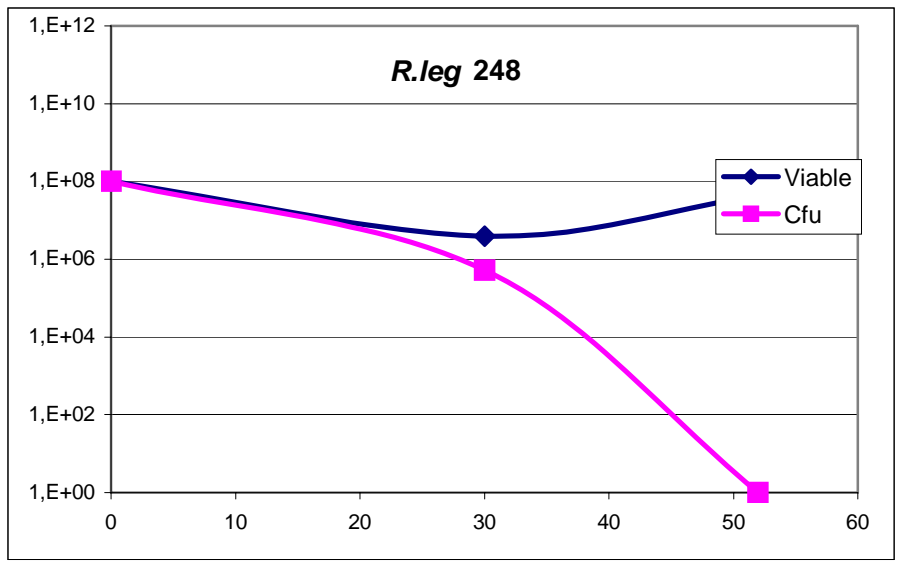
Results suggest the absence of a direct link between quorum sensing and VBNC.



A

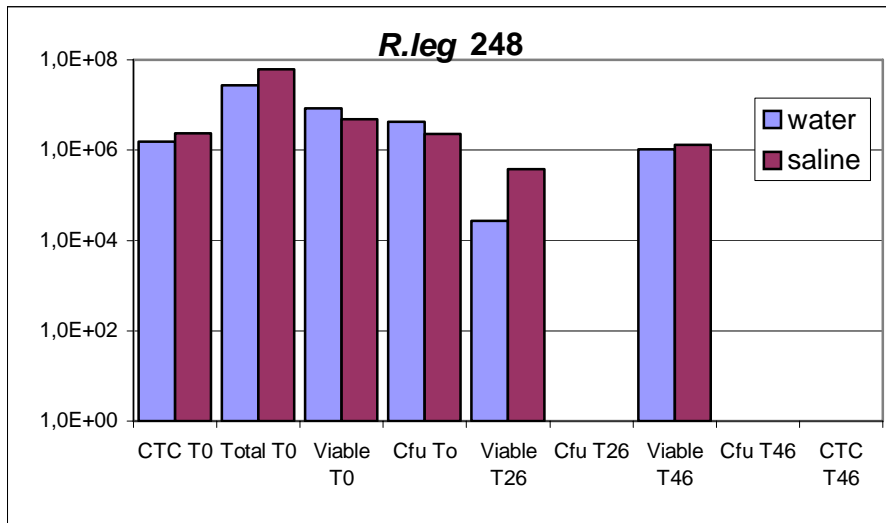


B

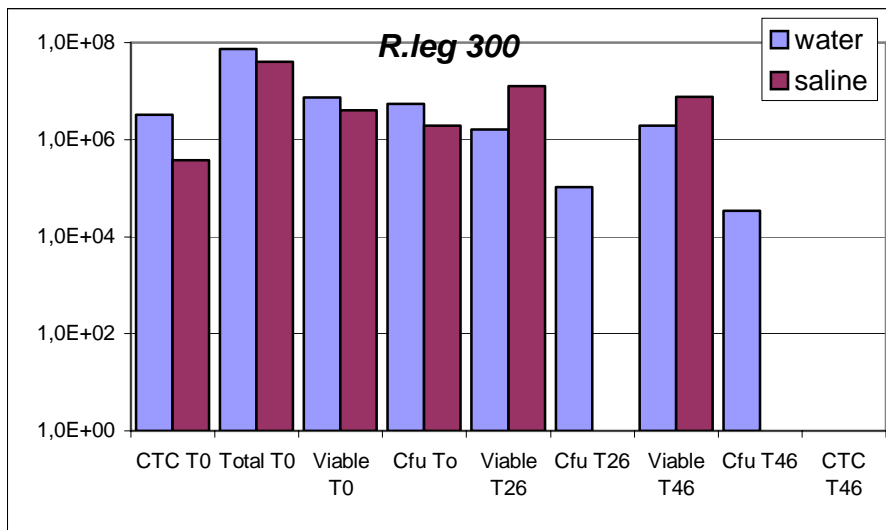


C

Figure IV.2 – Anaerobic incubation

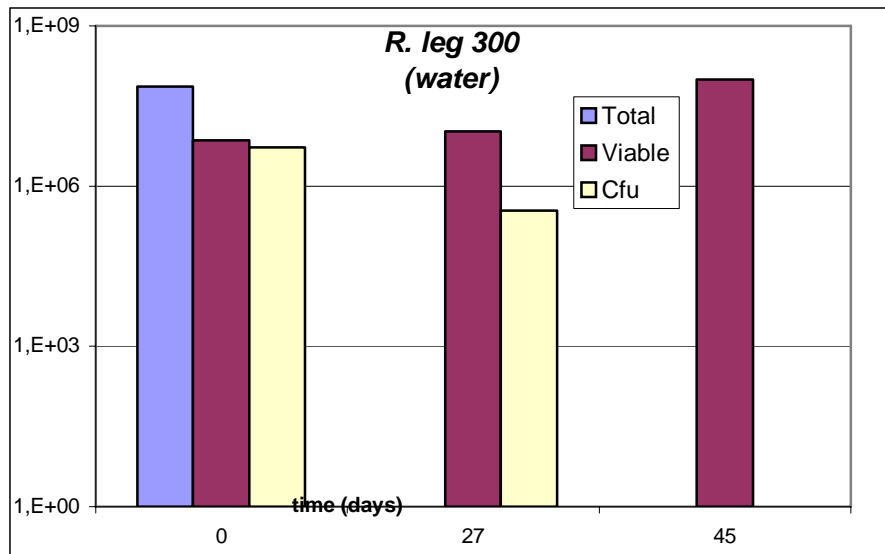


A

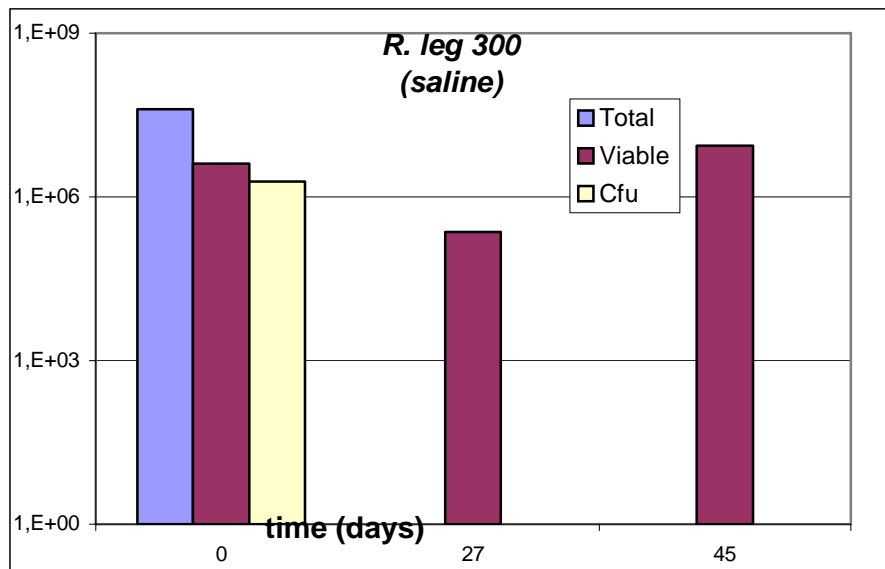


B

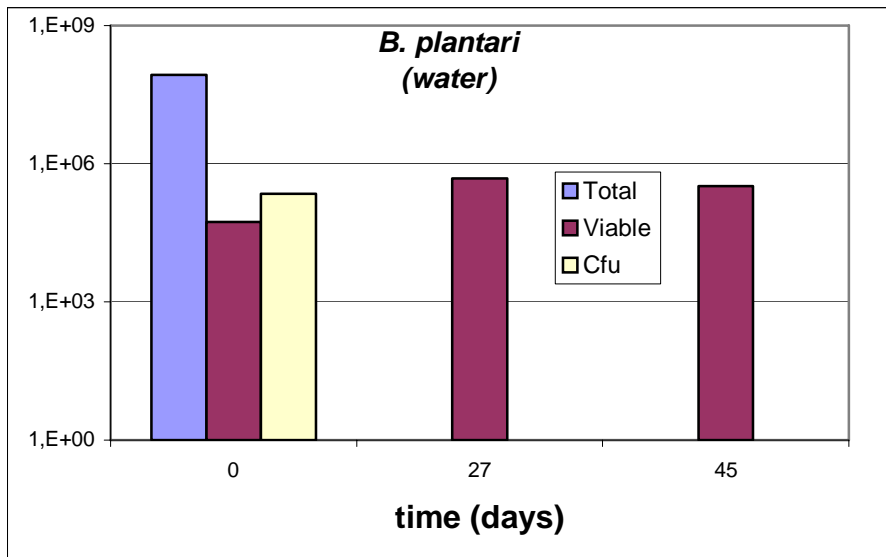
Figure IV.3 – Nutrient-limiting incubation



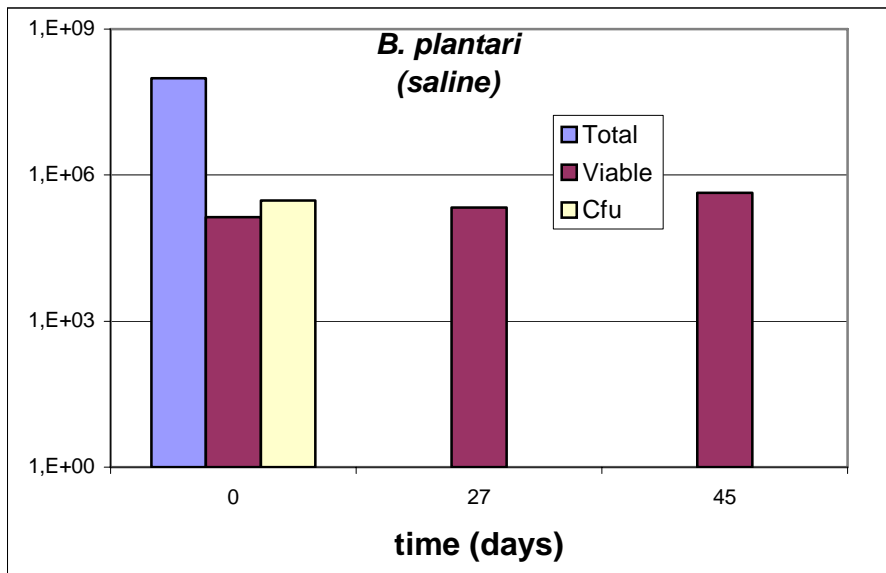
A



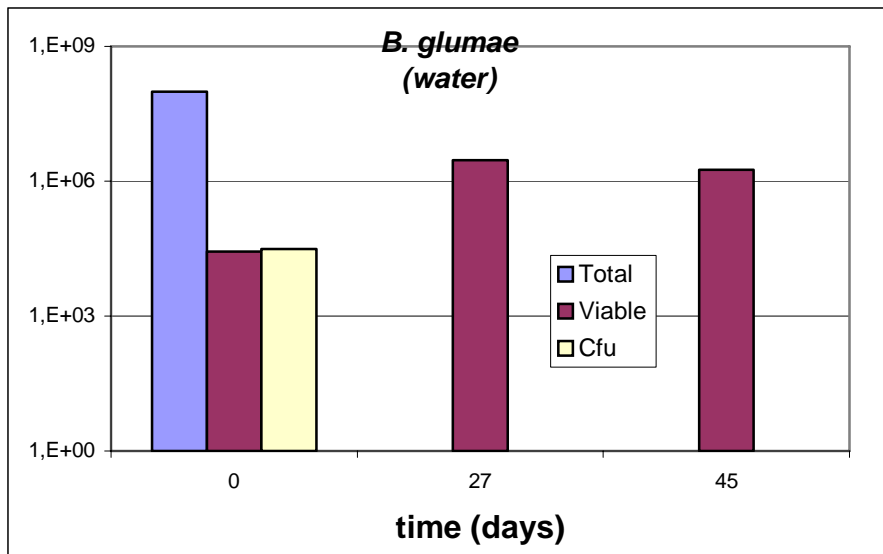
B



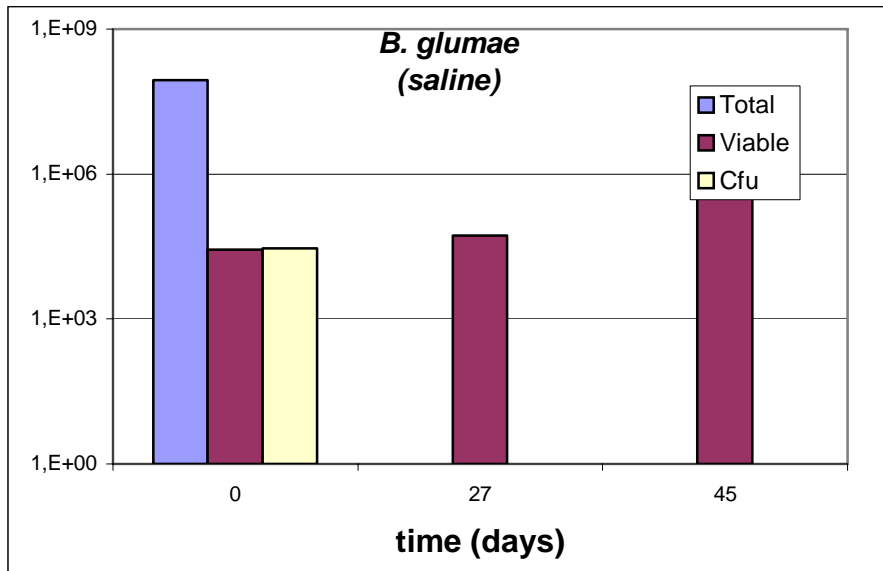
C



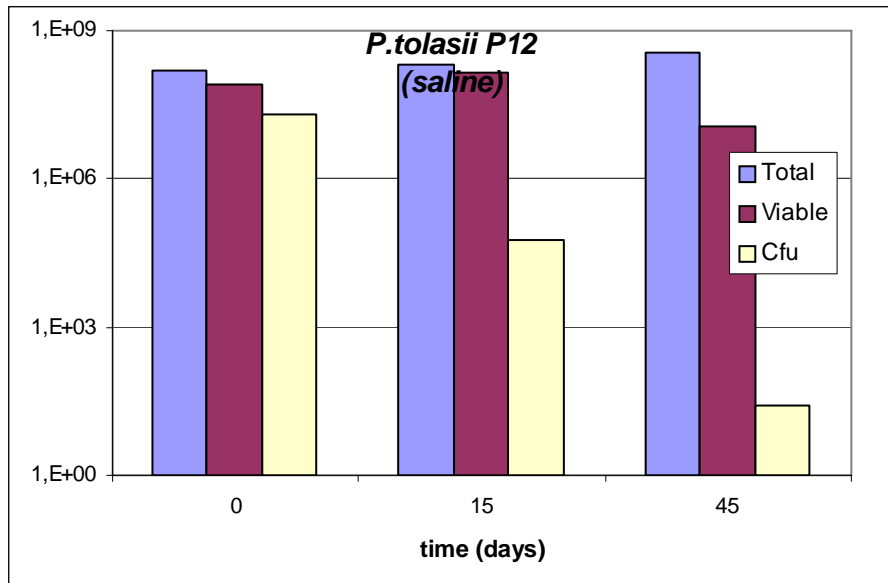
D



E



F



G

Figure IV.4 – Oxygen and nutrients-limiting incubation

Conclusions

Stressful conditions as nutrient deficiencies and oxygen limitations are conducive of a VBNC state for some of the tested bacteria. The specific condition required can vary even at strain level, as indicated in Table IV.1.

As regards the physiological status of the VBNC microorganisms, they showed integrity and functionality of the cytoplasmic membrane for the whole experimental time (55 days). Indeed they kept a certain selective permeability for propidium iodide.

Regarding their metabolism, the CTC-reduction activity was positive only in the earlier phase of the tests and only for rhizobial strains. The activity was no longer detectable in VBNC state, probably due to the deep dormancy status.

The Quorum Sensing-related AHLs tested did not show any efficiency in rescuing cells from the VBNC status to a culturable one, suggesting that, at least under the conditions used in the experiment, these signal are not involved in the process of recovery from viable but non culturable state. Their possible involvement in the entrance into such state was equally undetectable.

BACTERIAL STRAIN	MICROCOSM
<i>Burkholderia plantarii</i>	Anaerobiosis
<i>Burkholderia glumae</i>	Anaerobiosis
<i>Burkholderia plantarii</i>	Oxygen and nutrient limitation, in water
<i>Burkholderia plantarii</i>	Oxygen and nutrient limitation, in saline solution
<i>Burkholderia glumae</i>	Oxygen and nutrient limitation, in water
<i>Burkholderia glumae</i>	Oxygen and nutrient limitation, in saline solution
<i>Pseudomonas tolasii</i> P12	Oxygen and nutrient limitation, in saline solution
<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> 248	Anaerobiosis
<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> 248	Nutrient limitation, in saline solution
<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> 248	Nutrient limitation, in water
<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> 300	Oxygen and nutrient limitation, in saline solution
<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> 300	Oxygen and nutrient limitation, in water
<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> 300	Nutrient limitation, in saline solution
<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> 300	Nutrient limitation, in water

Table IV.1 – Bacterial species and strains tested and factors inducing their entry into VBNC state.

References

- Ballard N., McInnes C. A. and Davidson J. A. (2004) Size, symbiotic effectiveness and genetic diversity of field pea rhizobia *Rhizobium leguminosarum* bv. *viciae*) populations in South Australian soils. *Soil Biology and Biochemistry* 36: 1347-1355.
- Bassler B. L. (1999) How bacteria talk to each other: regulation of gene expression by quorum sensing. *Curr. Opin. Microbiol.* 2:582–587.
- Bassler B. L. (2002) Small talk. Cell-to-cell communication in bacteria. *Cell* 109:421–424.
- Batista J. S., Hungria M., Barcellos F. G., Ferreira, M. C. and Mendes I. C. (2006) Variability in *Bradyrhizobium japonicum* and *B. elkanii* Seven Years after Introduction of both the Exotic Microsymbiont and the Soybean Host in a Cerrados Soil. *Microbial Ecology*, 53: 270-284.
- Benhizia Y., Benhizia H., Benguedouar A., Muresu R., Giacomini A. and Squartini A. (2004) Gammaproteobacteria can nodulate legumes of the genus *Hedysarum*. *Syst Appl Microbiol* 27: 462–468.
- Benson D. R. and W. B. Silvester (1993) Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. *Microbiol. Rev.* 57:293–319
- Beumer R. R., de Vries J. and F. M. Rombouts (1992) *Campylobacter jejuni* non-culturable coccoid cells. *Intern. J. Food Microbiol.* 15, 153-163.
- Bolem J. (1995) Fluorescent staining of microbes for total direct count. *Molecular Microbiol Ecology Manual*. Akkerman A.D.L., van Elsas D.J., and De Bruijin F.J.(eds) 4.1.8: 1-12, Kluwer Academic Publisher, London

- Bromfield E. S. P., Barran L. R. and Wheatcroft R. (1995) Relative genetic structure of a population of *Rhizobium meliloti* isolated directly from soil and from nodules of alfalfa (*Medicago sativa*) and sweet clover. *Mol Ecol.* 4:183–188.
- Bromfield S. P., Sinha Indu B. and Wolynetz M. S. (1986) Influence of Location, Host Cultivar, and Inoculation on the Composition of Naturalized Populations of *Rhizobium meliloti* in *Medicago sativa* Nodules. *Applied and Environmental Microbiology* 51: 1077-1084.
- Burmeister M., diSibio G., Cox D. R. and Myers R. M. (1991) Identification of polymorphisms by genomic denaturing gradient gel electrophoresis: application to the proximal region of human chromosome 21. *Nucleic Acids Res.* 19, 1475–1481.
- Caetano-Anollés G., Bassam B. J. and Gresshoff P. M. (1991) DNA amplification fingerprinting using very short arbitrary oligonucleotides. *Biotechnology* 9, 553–557.
- Carlson R. W., Price N. P. and Stacey G. (1994) The biosynthesis of rhizobial lipooligosaccharide nodulation signal molecules. *Mol. Plant-Microbe Interact.* 7:684–695.
- Cha C., Gao P., Chen Y.-C., Shaw P. D. and Farrand S. K. (1998) Production of acyl-homoserine lactone quorum-sensing signals by Gram-negative plant-associated bacteria. *Mol. Plant-Microbe Interact.* 11:1119–1129.
- Chao A. (1984) Non-parametric estimation of the number of classes in a population. *Scand. J. Statist.* 11, 265-270.
- Chao A. (1987) Estimating the population size for capture-recapture data with unequal catchability. *Biometrics* 43, 783-791.
- Chao A., Ma M. C. and Yang M. C. K. (1993) Stopping rules and estimation for recapture debugging with unequal failure rates. *Biometrika* 80, 193-201.

- Chemming'wa G. and Vessey J. K. (2006) The abundance and efficacy of *Rhizobium leguminosarum* bv. *viciae* in cultivated soils of the eastern Canadian prairie. *Soil Biology and Biochemistry* 38:294-302
- Corich V., Giacomini A., Carlot M., Simon R., Tichy H., Squartini A. and Nuti M. P. (2001) Comparative strain-typing of *Rhizobium leguminosarum* bv. *viciae* natural populations. *Can J Microbiol* 47:580-584.
- Corich V., Giacomini A., Vendramin E., Vian P., Carlot M., Concheri G., Polone E., Casella S., Nuti M. P. and Squartini A. (2007) Long term evaluation of field-released genetically modified rhizobia. *Environmental Biosafety Research*. 6:167-181.
- Cubo M. T., Economou A., Murphy G., Johnston A. W. and Downie J. A. (1992) Molecular characterization and regulation of the rhizosphere-expressed genes *rhiABCR* that can influence nodulation by *Rhizobium leguminosarum* biovar *viciae*. *J. Bacteriol.* 174:4026–4035.
- Curtis T. P., Sloan W. T. and Scannell J. W. (2002) Estimating prokaryotic diversity and its limits. *Proc. Natl. Acad. Sci. USA* 99, 10494-10499.
- D’Haeze W., Gao M., De Rycke R., van Montagu M., Engler G. and Holsters M. (1998) Roles for azorhizobial Nod factors and surface polysaccharides in intercellular invasion and nodule penetration, respectively. *Mol. Plant-Microbe Interact.* 11:999–1008.
- Day A. P. and Oliver J.D. (2004) Changes in membrane fatty acid composition during entry of *Vibrio vulnificus* into the viable but nonculturable state. *J. Microbiol.* 42, 69-73.
- Dazzo F. (1984) Leguminous root nodules In: BURNS R., SLATER J., Eds., “Experimental Microbial Ecology” Blackwell Scientific Publishers, Oxford, pp. 431-446

- Dazzo F. B. (1982) Leguminous root nodules, pp. 431-446. In: *Experimental Microbial Ecology* (R. Burns, J. Slater, eds.) Oxford, Blackwell Scientific Publications.
- De Lajudie P., Willems A., Nick G., Mohamed T. S., Torck U., Filai-Maltouf A., Kersters K., Dreyfus B., Lindström K. and Gillis M. (1999) *Agrobacterium* bv. 1 strains isolated from nodules of tropical legumes. *Syst Appl Microbiol* 22: 119-132.
- de Ruijter N. C. A., Rook M. B., Bisseling T. and Emons A. M. C.. (1998) Lipochito-oligosaccharides re-initiate root hair tip growth in *Vicia sativa* with high calcium and spectrin-like antigen at the tip. *Plant J.* 13:341–350.
- Denarie J., Debelle F. and Rosenberg C. (1992) Signaling and host range variation in nodulation. *Annu. Rev. Microbiol.* 46:497–531.
- Diaz C. L., Logman T., Stam H. C. and Kijne J. W. (1995) Sugar-binding activity of pea lectin expressed in white clover hairy roots. *Plant Physiol.* 109:1167–1177.
- Diaz C. L., Van Spronsen P. C., Bakhuizen R., Logman G. J. J., Lugtenberg E. J. J. and Kijne J. W. (1986) Correlation between infection by *Rhizobium leguminosarum* and lectin on the surface of *Pisum sativum* roots. *Planta* 168:350–359.
- Dibb N. J., Downie J. A. and Brewin N. J. (1984). Identification of a rhizosphere protein encoded by the symbiotic plasmid of *Rhizobium leguminosarum*. *J. Bacteriol.* 158:621–627.
- Engerbrecht J. and Silverman M. (1987) Nucleotide sequence of the regulatory locus controlling expression of bacterial genes for bioluminescence. *Nucleic Acids Res.* 15, 10455–10467.

- Esseling J. J., Lhuissier F. G. and Emons A. M. (2003) Nod factor-induced root hair curling: continuous polar growth towards the point of Nod factor application. *Plant Physiol.* 132:1982–1988.
- Fagerli I. L. and Svenning M. M.(2005) Arctic and subarctic soil populations of *Rhizobium leguminosarum* biovar trifolii nodulating three different clover species: Characterisation by diversity at chromosomal and symbiosis loci. *Plant and Soil* 275: 371-381.
- Federighi M., Tholozan J. L., Cappelier J. M., Tissier J. P. and Jouve J. L. (1998) Evidence of non-cocoid viable but non-culturable *Campylobacter jejuni* cells in microcosm water by direct viable count, CTC-DAPI double staining, and scanning electron microscopy. *Food Microbiol.* 15, 539-550.
- Finney A. H., Blick R. J., Murakami M., Ishihama A. and Stevens A. M. (2002) Role of the C-terminal domain of the alpha subunit of RNA polymerase in LuxR-dependent transcriptional activation of the lux operon during quorum sensing. *J. Bacteriol.*, , 184, 4520–4528.
- Fuqua C., Parsek M. R. and Greenberg E. P. (2001) Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu. Rev. Genet.* 35:439–468.
- Fuqua W. C., Winans S. C. and Greenberg E. P. (1994) Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriol.* 176:269–275.
- Gage D. J.(2004) Infection and invasion of roots by symbiotic, Nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol Mol Biol Rev* 68, 280-300.
- Gantner S., Schmid M., Durr C., Schuegger R., Steidle A., Hutzler P., Langebartels C., Eberl L., Hartmann A. and Dazzo F. B. (2006) In situ quantitation of the spatial scale of

calling distances and population density-independent *N*-acylhomoserine lactone-mediated communication by rhizobacteria colonized on plant roots. *FEMS Microbiol. Ecol.* 56:188–194.

Gera C. and Srivastava S. (2006) Quorum-sensing: The phenomenon of microbial communication. *Current Science* 90 (5) 666-677

González J. E. and Marketon M. M. (2003). Quorum sensing in nitrogen-fixing bacteria. *Microbiol Mol Biol Rev* 67, 574–592.

Good I.J. (1953) The population frequencies of species and the estimation of population parameters. *Biometrika* 40, 237-264.

Gram L., Christensen A. B., Ravn L., Molin S. and Givskov M. (1999) Production of acylated homoserine lactones by psychrotrophic members of the *Enterobacteriaceae* isolated from foods. *Appl. Environ. Microbiol.* 65: 3458–3463.

Gray K. M., Pearson J. P., Downie J. A., Boboye B. E. and Greenberg E. P. (1996) Cell-to-cell signaling in the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum*: autoinduction of a stationary phase and rhizosphere- expressed genes. *J. Bacteriol.* 178:372–376.

Handley B. A., Hedges A. J., and Beringer J. E. (1998) Importance of host plants for detecting the population diversity of *Rhizobium leguminosarum* biovar *viciae* in soil. *Soil Biology and Biochemistry.* 30: 241-249

Hartmann A., Giraud J. J., and Catroux G. (1998) Genotypic diversity of *Sinorhizobium* (formerly *Rhizobium*) *meliloti* strains isolated directly from a soil and from nodules of alfalfa (*Medicago sativa*) grown in the same soil. *FEMS Microbiology Ecology* 25:107-116.

- Hastings J. W. and Greenberg E. P. (1999) Quorum sensing: the explanation of a curious phenomenon reveals a common characteristic of bacteria. *J. Bacteriol.* 181, 2667–2668.
- Heidstra R., Geurts R., Franssen H., Spaik H., van Kammen A. and T. Bisseling. (1994) Root hair deformation activity of nodulation factors and their fate on *Vicia sativa*. *Plant Physiol.* 105:787.
- Hill T.C.J., Walsh K.A., Harris J.A. and Mo, B.F. (2003) Using ecological diversity measures with bacterial communities. *FEMS Microbiol. Ecol.* 43, 1-11.
- Hirsch A. M. (1999) Role of lectins (and rhizobial exopolysaccharides) in legume nodulation. *Curr. Opin. Plant Biol.* 2:320–326.
- Hirsch P. R. (1996), Population dynamics of indigenous and genetically modified rhizobia in the field. *New Phytologist* 133: 159–171.
- Howieson J. G., Ewing M. A. and D’Antuono M. F. (1988) Selection for acid tolerance in *Rhizobium meliloti*. *Plant and Soil* 105: 179-188.
- Howieson J. G., Robson A. D. and Abbott L. K. (1992) Calcium modifies pH effects on the growth of acid-tolerant and acid-sensitive *Rhizobium meliloti*. *Aust J Agric Res* 43: 765-72.
- Hughes J. B., Hellmann J. J., Ricketts T. H. and Bohannan B. J. M. (2001) Counting the uncountable: statistical approaches to estimating microbial diversity. *Appl. Environ. Microbiol.* 67:4399–4406.
- Jansson J. D. van Elsas and Bailey M.J. (eds.), *Tracking Genetically-Engineered Microorganisms*. Landes Biosciences, Georgetown, TX.

- Jordan D. C. (1984). Family III. *Rhizobiaceae*. In: *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 234-256. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Kaplan H. B. and Greenberg E. P. (1985) Diffusion of autoinducer is involved in the regulation of the *Vibrio fischeri* luminescence system. *J. Bacteriol.* 163, 1210–1214.
- Kell D. B., Kapreylants A. S., Weichart D. H., Harwood C.L. and Barer M.R. (1998) Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Ant. van Leeuwenhoek* 73,169-187.
- Kemp P. F. and. Aller J. Y. (2004) Bacterial diversity in aquatic and other environments: what 16S rDNA libraries can tell us. *FEMS Microbiol. Ecol.* 47:161–177.
- Kishinevsky B. D., Nandasena K. G., Yates R. J., Nemas C. and Howieson J. G. (2003) Phenotypic and genetic diversity among rhizobia isolated from three *Hedysarum* species: *H. spinosissimum*, *H. coronarium* and *H. flexuosum* *Plant and Soil* 251: 143-153.
- Ko euth T., Versalovic J., Lupski J. R. (1995) Differential subsequence conservation of interspersed repetitive *Streptococcus pneumoniae* BOX elements in diverse bacteria. *Genome Res.*;5:408–418.
- Kogure K., Simidu U. and Taga N. (1979) A tentative direct microscopic method for counting living marine bacteria. *Can. J. Microbiol.* 25,415-420.
- Laguerre G., Mavingui P., Allard M., Charnay M., Louvrier P., Mazurier S.I., Rigottier-Gois L., and Amarger N. (1996) Typing of Rhizobia by PCR DNA fingerprinting and PCR-Restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: Application to *Rhizobium leguminosarum* and its different biovars. *Applied and Environmental Microbiology* 62: 2029-2036.

- Lee S.-M. and Chao A. (1994) Estimating population size via sample coverage for closed capture-recapture models. *Biometrics* 50, 88-97.
- Lhuissier F. G. P., De Ruijter N. C. A., Sieberer B. J., Esseling J. J. and Emons A. M. C. (2001) Time course of cell biological events evoked in legume root hairs by *Rhizobium* Nod factors: state of the art. *Ann. Bot.* 87: 289–302
- Lithgow J. K., Wilkinson A., Hardman A., Rodelas B., Wisniewski-Dye F., Williams P. and Downie J. A. (2000) The regulatory locus *cinRI* in *Rhizobium leguminosarum* controls a network of quorum-sensing loci. *Mol. Microbiol.* 37:81–97.
- Lleò M. M., Bonato B., Tafi M. C., Signoretto C., Boaretti M. and Canepari P.. (2001) Resuscitation rate in different enterococcal species in the viable but non-culturable state. *J. Appl. Microbiol.* 91, 1095-1102.
- Lleò M. M., Pierobon S., Tafi M. C., Signoreto C. and P. Canepari, (2000) mRNA detection by reverse transcription-PCR for monitoring viability over time in an *Enterococcus faecalis* viable but nonculturable population maintained in a laboratory microcosm. *Appl. Environ. Microbiol.* 66, 4564-4567.
- Long S. R. (1996) *Rhizobium* symbiosis: nod factors in perspective. *Plant Cell* 8:1885–1898.
- Lonneke M., Hogg B., Bersoult A. and Cullimore J. V. (2005) Integration of signalling pathways in the establishment of the legume-rhizobia symbiosis. *Physiologia Plantarum* 123: 207–218. 2005
- Lupski J.R. and Weinstock G.M. (1992) Short, interspersed repetitive DNA sequences in prokariotic genomes. *J. Bacteriol.* 174, 4525–4529.

- Macciò D., Fabra A., and Castro S. (2002) Activity and calcium interaction affect the growth of Bradyrhizobium sp. and the attachment to peanut roots. *Soil Biol Biochem* 34: 201-208.
- Marchesi J. R., Sato T., Weightman A. J., Martin T. A., Fry J. C., Hiom S. J. and Wade W. G. (1998) Design and Evaluation of Useful Bacterium-Specific PCR Primers That Amplify Genes Coding for Bacterial 16S rRNA. *Appl Environ Microbiol* 64: 795-799.
- Martin B., Humbert O., Camara M., Guenzi E., Walker J., Mitchell T., Andrew P., Prudhomme M., Alloing G., Hakenbeck R. (1992) A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. *Nucleic Acids Res.*;20:3479–3483.
- Mateos P., Jimenez-Zurdo J., Chen J. W., Squartini A., Haack S., Martinez-Molina E., Hubbell D. and Dazzo F. (1992) Cell-associated pectinolytic and cellulolytic enzymes in *Rhizobium leguminosarum* bv. *trifolii*. *Appl Environ Microbiol* 58: 1816-1822.
- McClellan K.H., Winson M.K., Fish L., Taylor A., Chhabra S.R., Camara, M., Daykin M., Lamb J. H., Swift S., Bycroft B. W., Stewart G. S. and Williams P. (1997) Quorum-sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology* 143: 3703–3711.
- Mendes I. C., and Bottomley P. J. (1998) Distribution of a population of *Rhizobium leguminosarum* bv. *trifolii* among different size classes of soil aggregates. *Applied and Environmental Microbiology* 64: 970-975.
- Mhamdi R., Mrabet M., Laguerre G., Tiwari R. and Aouani M. E. (2005) Colonization of *Phaseolus vulgaris* nodules by Agrobacterium-like strains. *Can J Microbiol* 51:105–111.

- Miller D. D., de Ruijter N. C. A., Bisseling T. and Emons A. M. C.. (1999) The role of actin in root hair morphogenesis: studies with lipochito-oligosaccharide as a growth stimulator and cytochalasin as an actin perturbing drug. *Plant J.* 17:141–154.
- Mrabet M., Mnasri B., Romdhane S. B., Laguerre G., Aouani M. E and Mhamdi R. (2006). *Agrobacterium* strains isolated from root nodules of common bean specifically reduce nodulation by *Rhizobium gallicum*. *FEMS Microbiol Ecol* 56:304-309
- Muresu R., Polone E., Sulas L., Baldan B., Tondello A., Delogu G., Cappuccinelli P., Alberghini S., Benhizia Y., Benhizia H., Benguedouar A., Mori B., Calamassi R., Dazzo F. B. and Squartini A. (2008) Coexistence of predominantly nonculturable rhizobia with diverse, endophytic bacterial taxa within nodules of wild legumes. *FEMS Microbiology Ecology* DOI:10.1111/j.1574-6941.2007.00424.x
- Muresu R., Sulas L., Polone E. And Squartini A. (2005) PCR primers based on different portions of insertion elements can assist genetic relatedness studies, strain fingerprinting and species identification in rhizobia. *FEMS Microbiology Ecology* 54, 445-453.
- Mutch L. A. and Young J. P. W. (2004) Diversity and specificity of *Rhizobium leguminosarum* biovar *viciae* on wild and cultivated legumes. *Molecular Ecology* 13: 2435-2444.
- Muyzer G., Dewaal E.C. and Uitterlinden A.G. (1993) Profiling of complex microbialpopulations by denaturing gradient gelelectrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16S ribosomal-RNA. *Appl. Environ. Microbiol.* 59, 695–700.
- Nandasena K. G., O'Hara G. W., Tiwari R. P., and Howieson J. G. (2006). Rapid In Situ Evolution of Nodulating Strains for *Biserrula pelecinus* L. through Lateral Transfer of a Symbiosis Island from the Original Mesorhizobial Inoculant. *Applied and Environmental Microbiology* 72: 7365-7367.

- Nandasena K. G.; O'Hara G. W., Tiwari R. P., Yates R. J. and Howieson J. G. (2001) Phylogenetic relationships of three bacterial strains isolated from the pasture legume *Biserrula pelecinus* L. *Int J Syst Evol Microbiol*, 51: 1983-1986
- Nealson K. H., Platt T. and Hastings J. W. (1970) Cellular control of the synthesis and activity of the bacterial luminescent system. *J. Bacteriol.* 104:313–322.
- Oldroyd G. E. D., Engstrom E. M., and Long S. R. (2001). Ethylene inhibits the Nod factor signal transduction pathway of *Medicago truncatula*. *Plant Cell* 13:1835–1849.
- Oliver J. D. (2000a). Problems in detecting dormant (VBNC) cells and the role of DNA elements in this response, p. 1-15. *In* J.K.
- Oliver J. D. (2000b) The viable but nonculturable state and cellular resuscitation, p. 723-730. *In* C.R. Bell, M. Brylinsky, and P. Johnson-Green (eds.), *Microbial Biosystems: New Frontiers*. Atlantic Canada Soc. Microb. Ecol., Halifax, Canada.
- Oliver J. D. (2005) The viable but nonculturable state in bacteria. *The Journal of Microbiology* 43: 93-100
- Osborn A. M., Moore E. R. B. and Timmis K. N. (2000) An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environ Microbiol* 2:39-50.
- Paffetti D., Daguin F., Fancelli S., Gnocchi S., Lippi F., Scotti C. and Bazzicalupo M. (1998) Influence of plant genotype on the selection of nodulating *Sinorhizobium meliloti* by *Medicago sativa*. *Antonie van Leeuwenhoek*. 73: 3-8

- Paffetti D., Scotti C., Gnocchi S., Fancelli S., Bazzicalupo M. (1996) Genetic diversity of an Italian *Rhizobium meliloti* Population from Different *Medicago sativa* Varieties. *Applied and Environmental Microbiology* 62: 2279-2285.
- Palmer K. M., and Young J. P. W. (2000) Higher Diversity of *Rhizobium leguminosarum* biovar *viciae* Populations in Arable Soils than in Grass Soils. *Applied and Environmental Microbiology* 66: 2445-2450.
- Patriarca E. J., Tatè R. and Iaccarino M. (2002) Key Role of Bacterial NH₄⁺Metabolism in Rhizobium-Plant Symbiosis. *Microbiol Mol Biol Rev* 66, 203-222.
- Perret X., Staehelin C. and Broughton W. J. (2000) Molecular basis of symbiotic promiscuity. *Microbiol. Mol. Biol. Rev.* 64:180–201.
- Philipson M. N. and Blair I. D. (1957) Bacteria in clover root tissue. *Can J Microbiol* 3:125–129.
- Pignatti S (1982) *Flora d'Italia*. Edagricole, Bologna
- Polone E. and Squartini A. (2005) La radice e i microrganismi della rizosfera: analisi dei pattern di nodulazione di *Pisum sativum* in popolazioni naturali di *Rhizobium leguminosarum* bv. *viciae*. In: “Le radici delle piante coltivate: acquisizioni scientifiche ed innovazioni tecniche” Atti dell’Accademia dei Georgofili Collana: Quaderni
- Porter J., Edwards C. and Pickup R. W. (1995) Rapid assessment of physiological status in *Escherichia coli* using fluorescent probes. *J. Appl. Bacteriol.* 4, 399-408.
- Rademaker J.L.W. and de Bruijn F.J. (1997) Characterization and classification of microbes by rep-PCR genomic fingerprinting and computer-assisted pattern analysis In: DNA markers: protocols, applications and overviews (Caetano-Anollés G. and Gresshoff P.M., Eds.), pp. 151–171. John Wiley and Sons, Inc., New York, NY.

- Ridge R. W. (1992) A model of legume root hair growth and *Rhizobium* infection. *Symbiosis* 14:349–373.
- Rodelas B., Lithgow J. K., Wisniewski-Dye F., Hardman A., Wilkinson A., Economou A., Williams P. and Downie J. A. (1999) Analysis of quorum-sensing-dependent control of rhizosphere-expressed (*rhi*) genes in *Rhizobium leguminosarum* bv. *viciae*. *J. Bacteriol.* 181:3816–3823.
- Rodriguez G. G., Phipps D., Ishiguro K. and Ridgway H. F. (1992) Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. *Appl. Environ. Microbiol.* 58, 1801-1808.
- Salmond G. P., Bycroft B. W., Stewart G. S. and Williams P. (1995) The bacterial “enigma”: cracking the code of cell-cell communication. *Mol. Microbiol.* 16:615–624.
- Sheffield V.C., Beck J.S., Kwitek A.E., Sandstrom D.W. and Stone E.M. (1993) The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions. *Genomics* 16, 325–332.
- Sieberer B. and Emons A. M. C. (2000) Cytoarchitecture and pattern of cytoplasmic streaming in root hairs of *Medicago truncatula* during development and deformation by nodulation factors. *Protoplasma* 214:118–127.
- Smit G., Kijne J. W. and Lugtenberg B. J. J. (1987) Involvement of both cellulose fibrils and a Ca²⁺-dependent adhesin in the attachment of *Rhizobium leguminosarum* to pea root hair tips. *J. Bacteriol.* 169:4294–4301.
- Smit G., Kijne J. W. and Lugtenberg B. J. J. (1986) Correlation between extracellular fibrils and attachment of *Rhizobium leguminosarum* to pea root hair tips. *J. Bacteriol.* 168:821–827.

- Smit G., Kijne J. W. and Lugtenberg B. J.. (1989) Roles of flagella, lipopolysaccharide, and a Ca²⁺-dependent cell surface protein in attachment of *Rhizobium leguminosarum* biovar *viciae* to pea root hair tips. *J. Bacteriol.* 171:569–572.
- Somasegaran P. and H. J. Hoben (1985) *Methods Legume-Rhizobium Technology*. Paia, Hawaii. NifTAL Center, 367 pp.
- Soto M. J., Sanjuán J. And Olivares J. (2006) Rhizobia and plant pathogenic bacteria: common infection weapons. *Microbiology* 152, 3167–3174
- Squartini A., Dazzo F., Casella S. and Nuti M. P. (1993). The root nodule Symbiosis between *Rhizobium 'hedysari'* and its drought-tolerant host *Hedysarum coronarium*. *Symbiosis* 15: 227-238.
- Steidle A., Sigl K., Schuegger R., Ihring A., Schmid M., Gantner S., Stoffels M., Riedel K., Givskov M., Hartmann A., Langebartels C. and Eberl L. (2001) Visualization of *N*-acylhomoserine lactone-mediated cell-cell communication between bacteria colonizing the tomato rhizosphere. *Appl. Environ. Microbiol.* 67:5761–5770.
- Stern M.J., Ames G.F.L., Smith N.H., Robinson E.C. and Higgins, C.F. (1984) Repetitive extragenic palindromic sequences: a major component of the bacterial genome. *Cell* 37, 1015–1026.
- Strain S. R., Whittam T. S. and Bottomley P. J. (1995) Analysis of genetic structure in soil populations of *Rhizobium leguminosarum* recovered from the USA and the UK. *Molecular Ecology*. 4: 105–114.
- Sturz A. V., Christie B. R. and Matheson B. G. (1998) Associations of bacterial endophyte populations from red clover and potato crops with potential for beneficial allelopathy. *Can J Microbiol* 44: 162-167.

- Swift S., Throup J. P., Williams P., Salmond G. P. and Stewart G. S. (1996) Quorum sensing: a population-density component in the determination of bacterial phenotype. *Trends Biochem. Sci.* 21:214–219.
- Swift S., Winson M. K., Chan P. F., Bainton N. J., Birdsall M., Reeves P. J., Rees C. E. D., Chhabra S. R., Hill P. H., Throup J. P., Bycroft B. W., Salmond G. P. C., Williams P. and Stewart G. S. A. B. (1993) A novel strategy for the isolation of *luxI* homologues: evidence for the widespread distribution of a LuxR:LuxI superfamily in enteric bacteria. *Mol. Microbiol.* 10:511–520.
- Thies J. E., Holmes E. M. and Vachot A. (2001) Application of molecular techniques to studies in Rhizobium ecology: a review. *Australian Journal of Experimental Agriculture* 41: 299-319.
- Thies J. E., Wijkstra G. and Ronson C. (1999) What does strain persistence really mean? In: Martinez-Romero *et al.* (eds). Highlights of Nitrogen Fixation. Kluwer Academic/Plenum Publishers, New York.
- Timmers A. C. J., Auriac M. C., Truchet G. (1999) Refined analysis of early symbiotic steps of the Rhizobium–Medicago interaction in relationship with microtubular cytoskeleton rearrangements. *Development* 126: 3617–3628
- Timmers A. C. J., Soupène E., Auriac M. C., de Billy F., Vasse J., Boistard P. and Truchet G. (2000) Saprophytic intracellular rhizobia in alfalfa nodules. *Molecular Plant–Microbe Interactions* 13: 1204–1213.
- Timmers A. C., Auriac M. C., de Billy F. and Truchet G. (1998) Nod factor internalization and microtubular cytoskeleton changes occur concomitantly during nodule differentiation in alfalfa. *Development* 125:339–349.

- Tokala R. K., Strap J. L., Jung C. M., Crawford D. L., Hamby Salove M., Deobald L. A., Bailey F. and Morra M. J. (2002) Novel plant-microbe rhizosphere interaction involving *Streptomyces lydicus* WYEC108 and the pea plant (*Pisum sativum*). *Appl Environ Microbiol* 68: 2161–2171.
- Van Batenburg F. D. H., Jonker R. and Kijne J. W.. (1986) *Rhizobium* induces marked root hair curling by redirection of tip growth, a computer simulation. *Physiol. Plant* 66:476–480.
- van Brussel A. A. N., Bakhuizen R., van Spronsen P. C., Spaink H. P., Tak T., Lugtenberg B. J. J. , Kijne J. W. (1992) Induction of preinfection thread structures in the leguminous host plant by mitogenic lipooligosaccharides of rhizobium. *Science* 257: 70–72
- van Rhijn P., Fujishige N. A., Lim P. O. and Hirsch A. M.. (2001) Sugarbinding activity of pea lectin enhances heterologous infection of transgenic alfalfa plants by *Rhizobium leguminosarum* biovar *viciae*. *Plant Physiol.* 126: 133–144.
- Vaneechoutte M., De Beenhouwer H., Claeys G., Verschraegen G., De Rouck A., Paepe N., Elaichouni A. and Portaels F. (1993) Identification of Mycobacterium species by using amplified ribosomal DNA restriction analysis. *J. Clin. Microbiol.* 31, 2061–2065.
- Versalovic J., Koeuth T., Lupski J. R. (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res.*;19:6823–6831.
- Versalovic J., Schneider M., de Bruijn F.J. and Lupski J.R. (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol. Cell. Biol.* 5, 25–40.
- Vincent J. M. (1970) A manual for the practical study of the rootnodule bacteria. IBP Handbook no. 15, Blackwell Scientific Publications, Oxford

- Visick K. L. and McFall-Ngai M. J. (2000) An exclusive contract: specificity in the *Vibrio fischeri*–*Euprymna scolopes* partnership. *J. Bacteriol.* 182, 1779–1787.
- Vitousek P. M., Aber J. D., Howarth R. W., Likens G. E., Matson P. A., Schindler D. W., Schlesinger W. H. and Tilman D. G. (1997) Human alteration of the global nitrogen cycle: sources and consequences. *Ecol. Appl.* 7:737–750
- VonBodman, S. B., Bauer, W.D. & Coplin, D. L. (2003). Quorum sensing in plant-pathogenic bacteria. *Annu Rev Phytopathol* 41, 455–482.
- Vos P., Hogers R., Bleeker M., Reijans M., van de Lee T., Hornes M., Frijters A., Pot J., Peleman J., Muiper M. and Zabeau M. (1995) AFLP: a new concept for DNA fingerprinting. *Nucleic Acids Res.* 21, 4407–4414.
- Welsh J. and McClelland M. (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* 18, 7213–7218.
- Widjoatmodjo M.N., Fluit A.C. and Verhoef J. (1995) Molecular identification of bacteria by fluorescence-based PCR single-strand conformation polymorphism analysis of the 16S rRNA gene. *J. Clin. Microbiol.* 33, 2601–2606.
- Wilkinson A., Danino V., Wisniewski-Dye F., Lithgow J. K. and Downie J. A. (2002) *N*-Acyl-homoserine lactone inhibition of rhizobial growth is mediated by two quorum-sensing genes that regulate plasmid transfer. *J. Bacteriol.* 184:4510–4519.
- Williams J.G., Kubelik A.R., Livak K.J., Rafalsky J.A. and Tingey S.V. (1990) DNA polymorphisms amplified by arbitrary primers are useful genetic markers. *Nucleic Acids Res.* 18, 6531–6535.

- Winson M. K., Swift S., Hill P. J., Sims C. M, Griesmayr G., Bycroft B. W., Williams P. and Stewart G. S. A. B. (1998) Engineering the *luxCDABE* genes from *Photobacterium luminescens* to provide a bioluminescent reporter for constitutive and promoter probe plasmids and mini-Tn5 constructs. *FEMS Microbiol. Lett.* 16:193–202.
- Wisniewski-Dye F., Jones J., Chhabra S. R. and Downie J. A. (2002) *railR* genes are part of a quorum-sensing network controlled by *cinI* and *cinR* in *Rhizobium leguminosarum*. *J. Bacteriol.* 184:1597–1606.
- Yaron S. and Matthews K. (2002) A reverse transcriptase-polymerase chain reaction assay for detection of viable *Escherichia coli* O157:H7: investigation of specific target genes. *J. Appl. Microbiol.* 92, 633-640.
- Young L. Demetriou and Apte R. G. (1987) *Rhizobium* population genetics: enzyme polymorphism in *Rhizobium leguminosarum* from plants and soil in a pea crop. *Applied and Environmental Microbiology* 53, 397-402.
- Zehr J. P., Jenkins B. D., Short S. M. and Stewart G. F. (2003) Nitrogenase gene diversity and microbial community structure: a cross-system comparison. *Environ. Microbiol.* 5:539–554
- Zimmerman R., Iturriaga R. and Becker-Birck J. (1978) Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. *Appl. Environ. Microbiol.* 36, 926-935.

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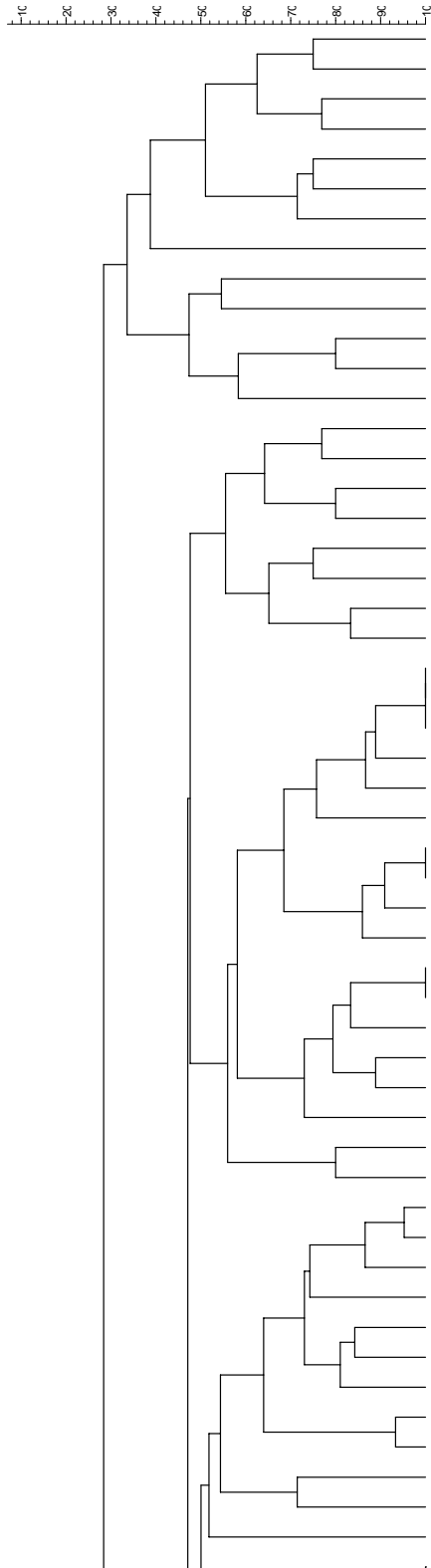
Infine la mia gratitudine va alla mia famiglia, ringrazio mia mamma per essermi stata vicina.

Appendix 1

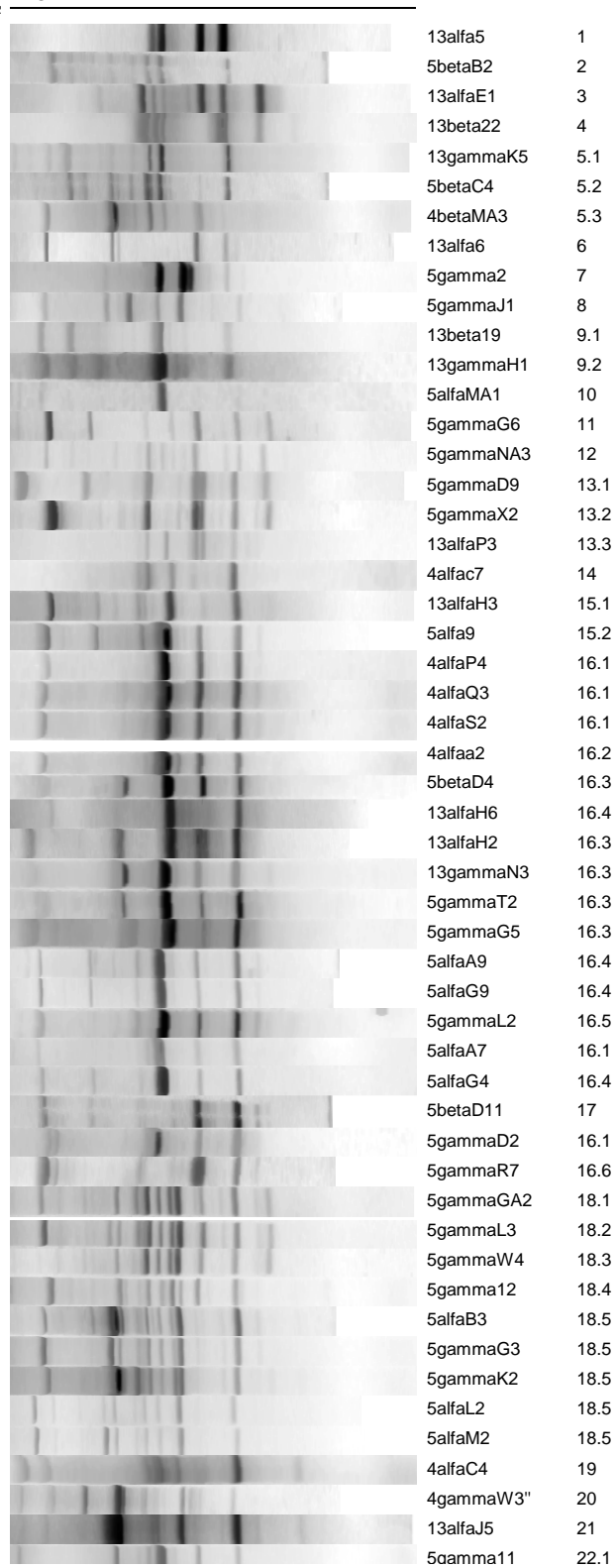
GelComparII similarity dendrogram

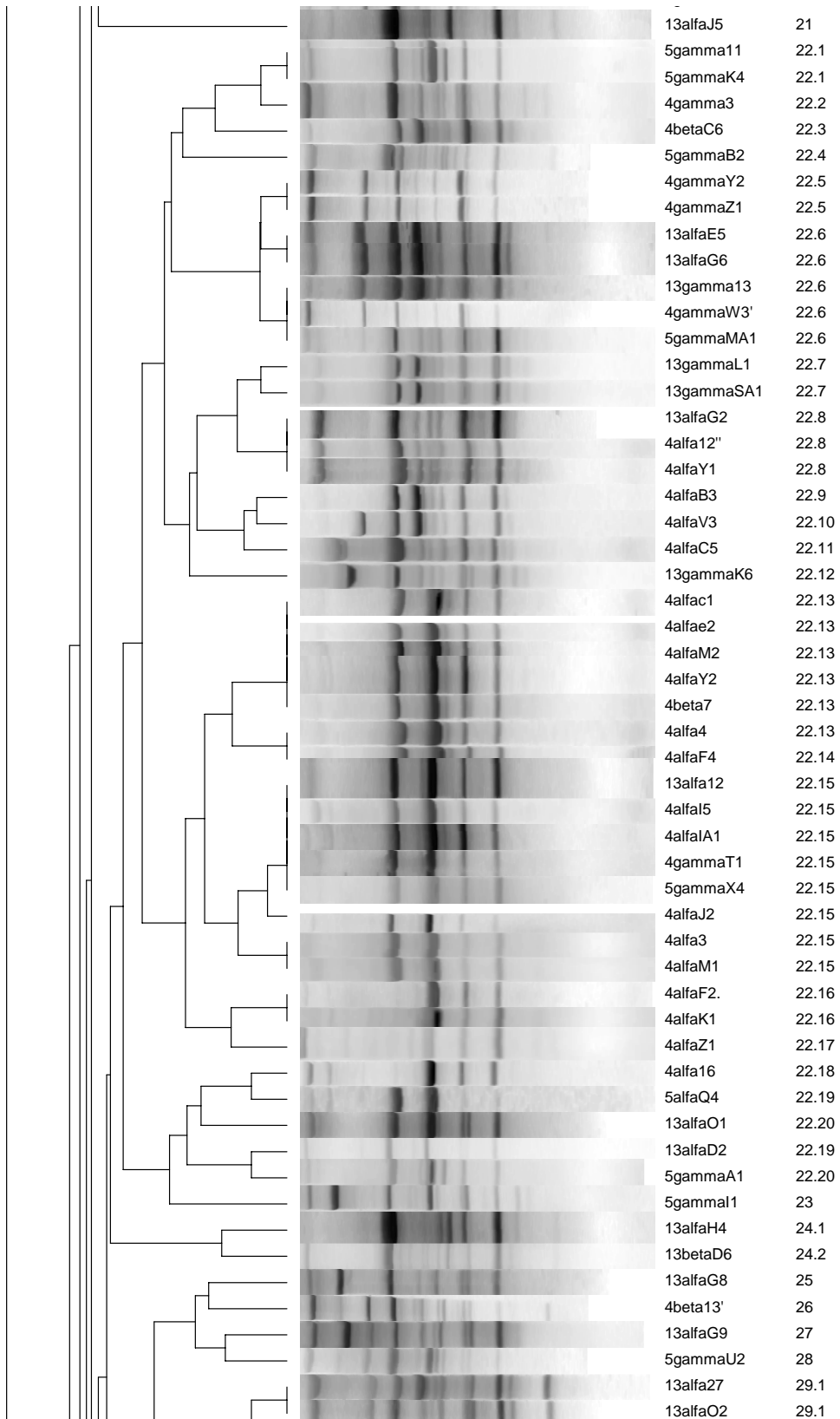
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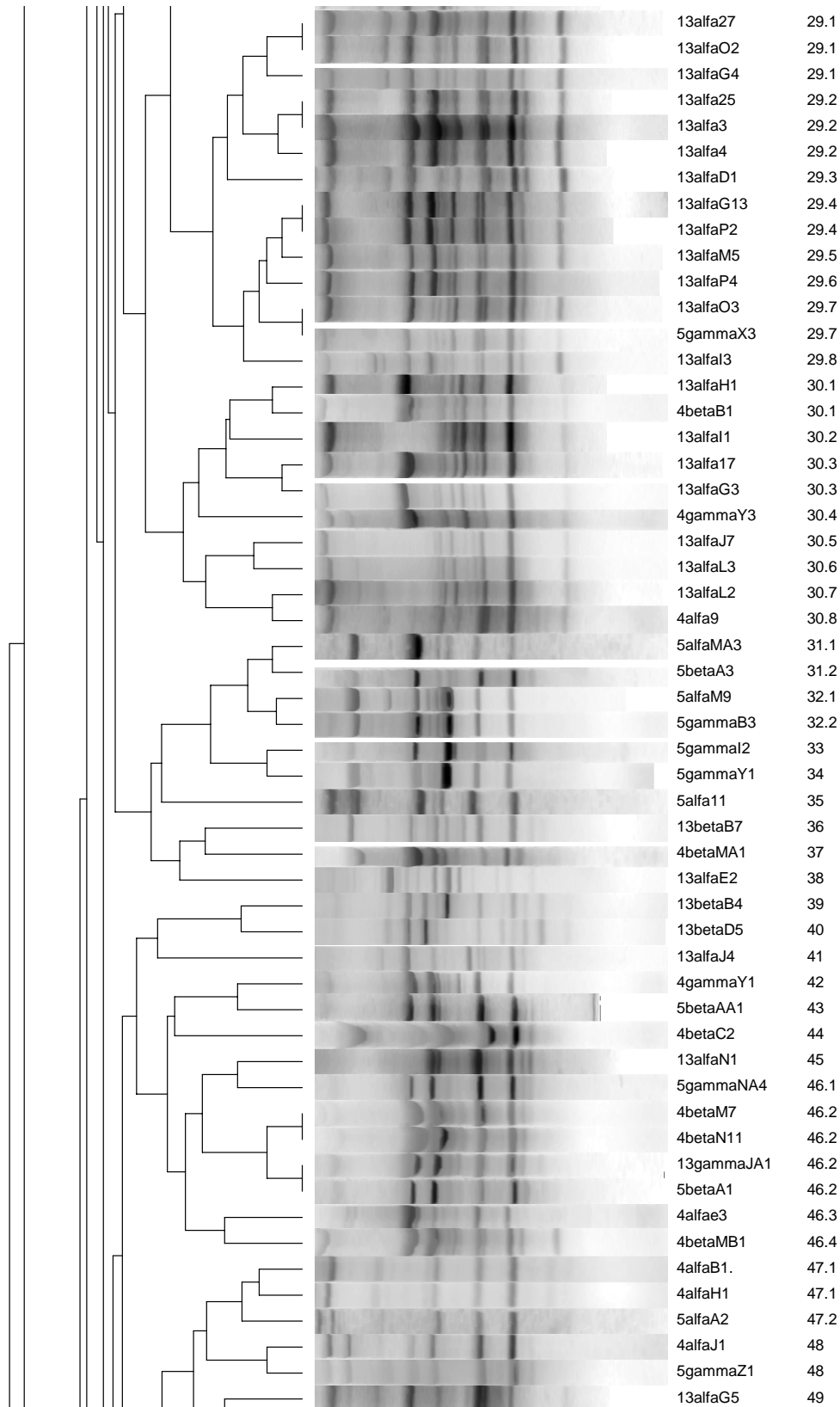
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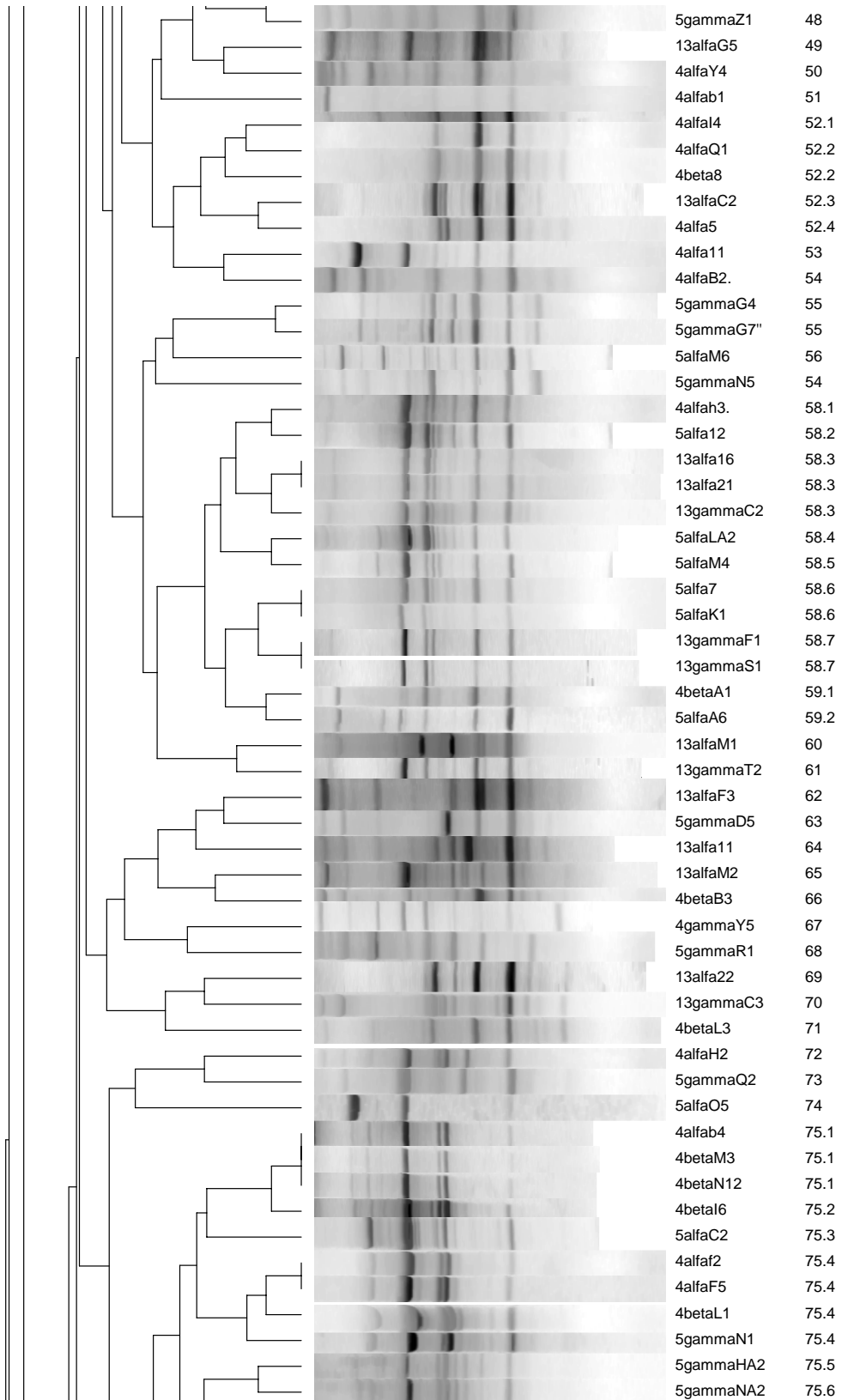


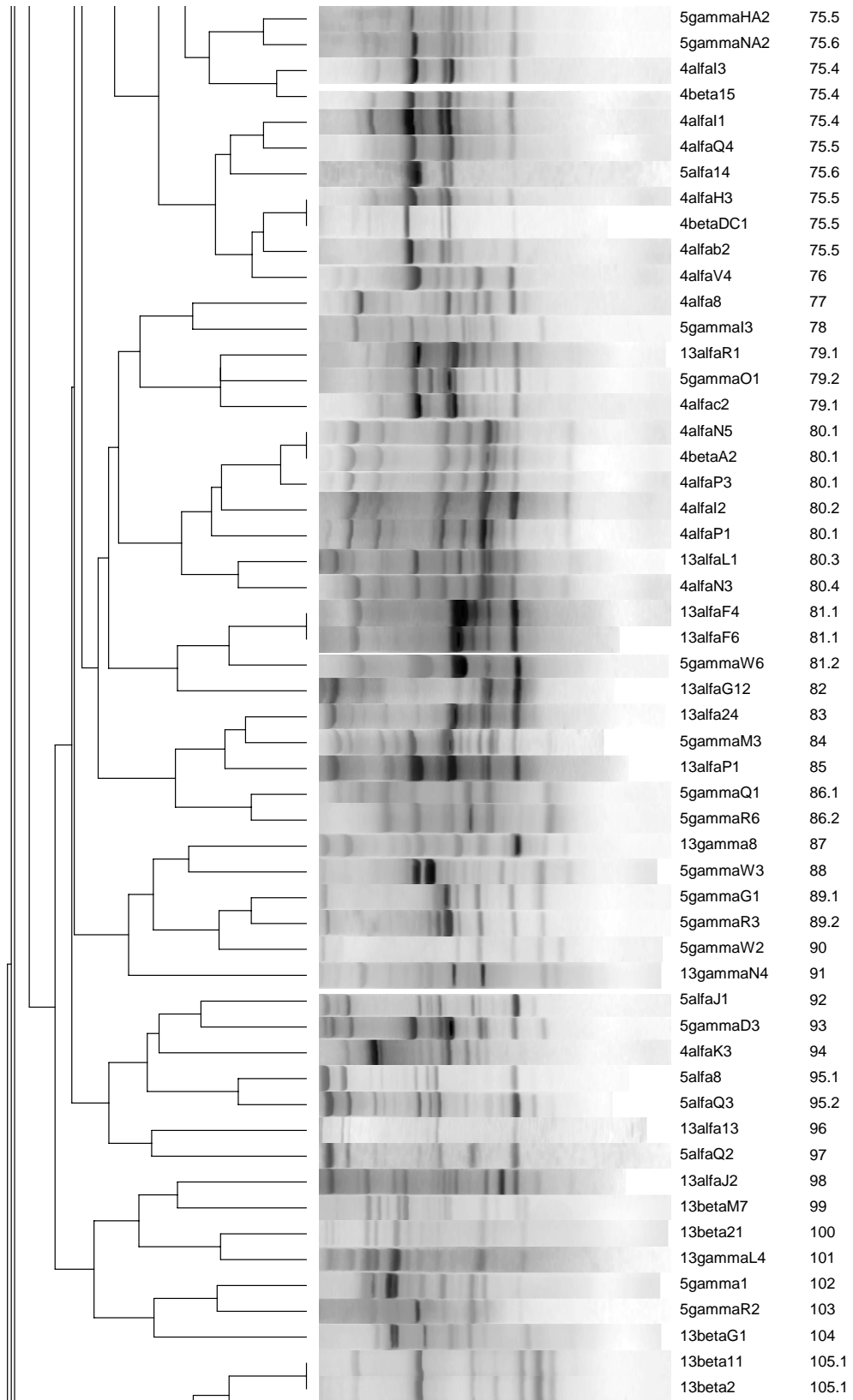
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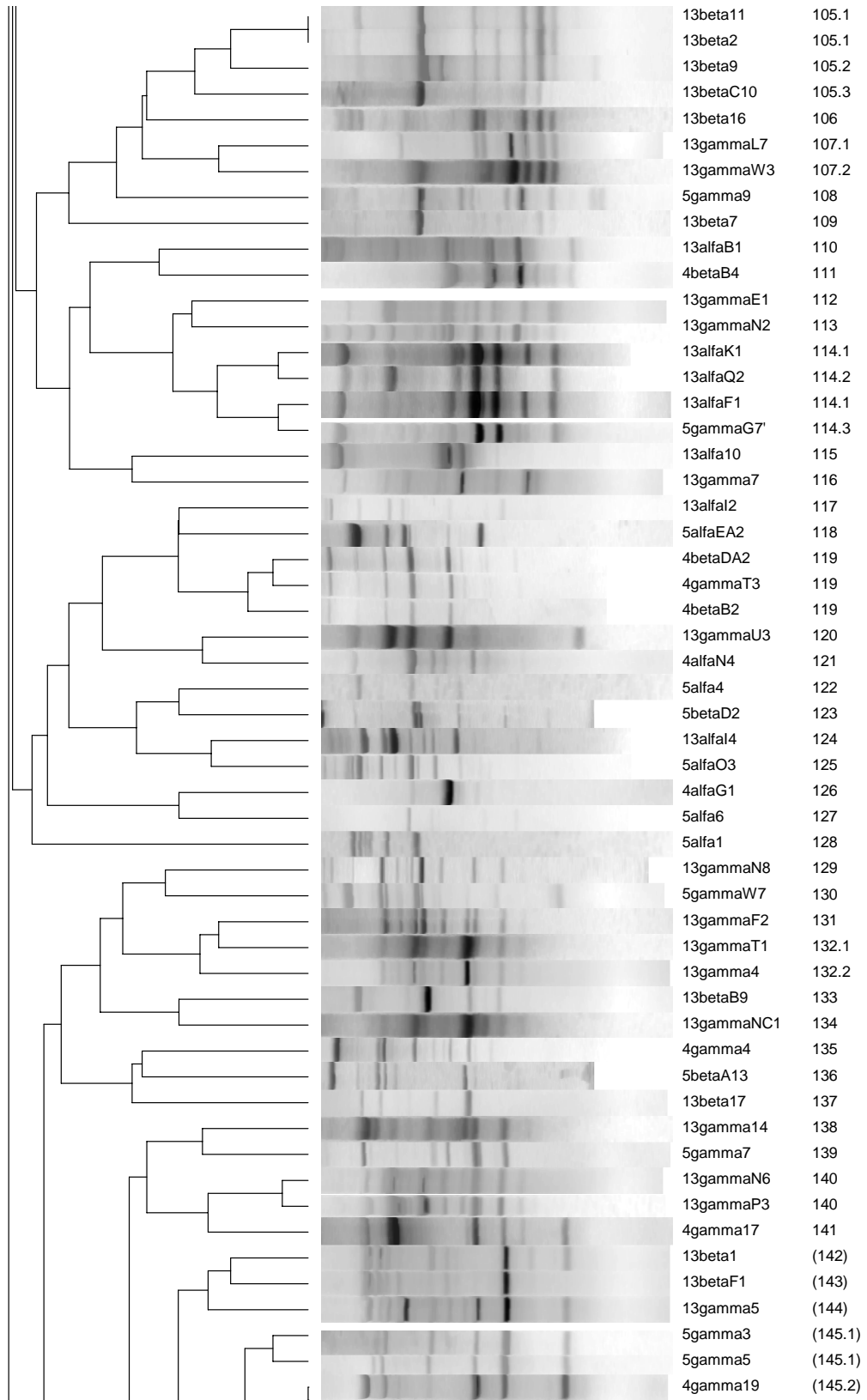


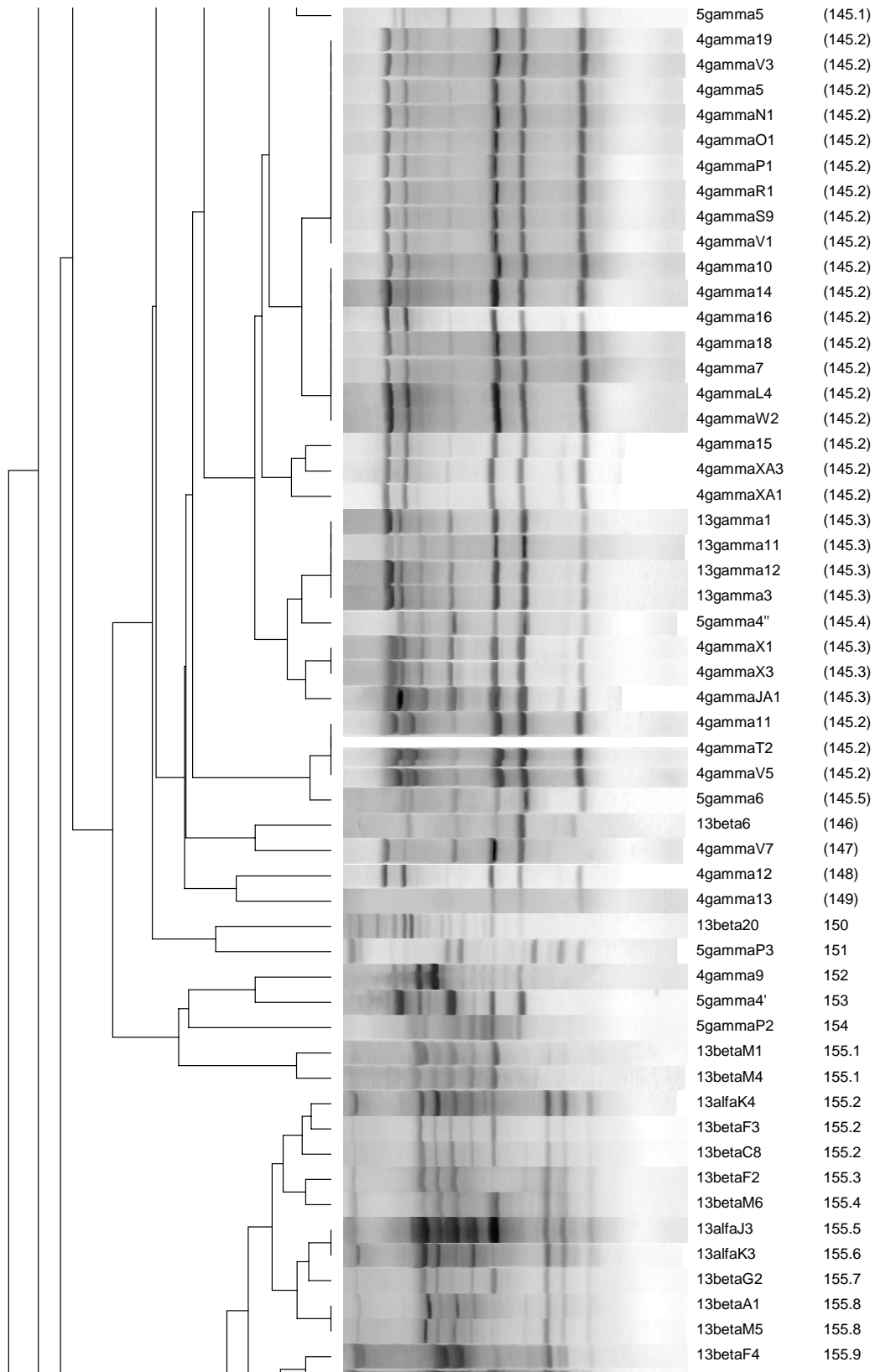


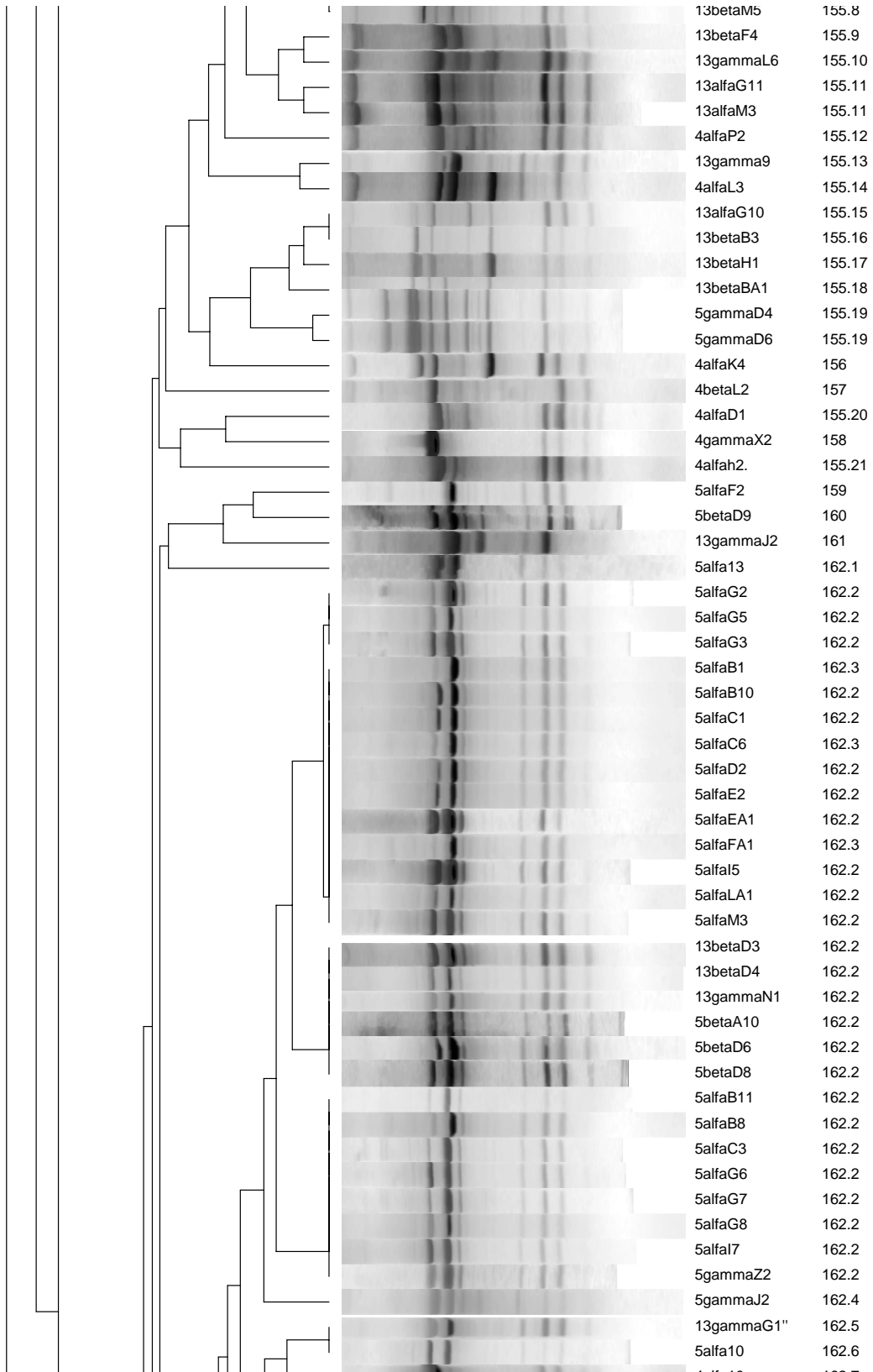


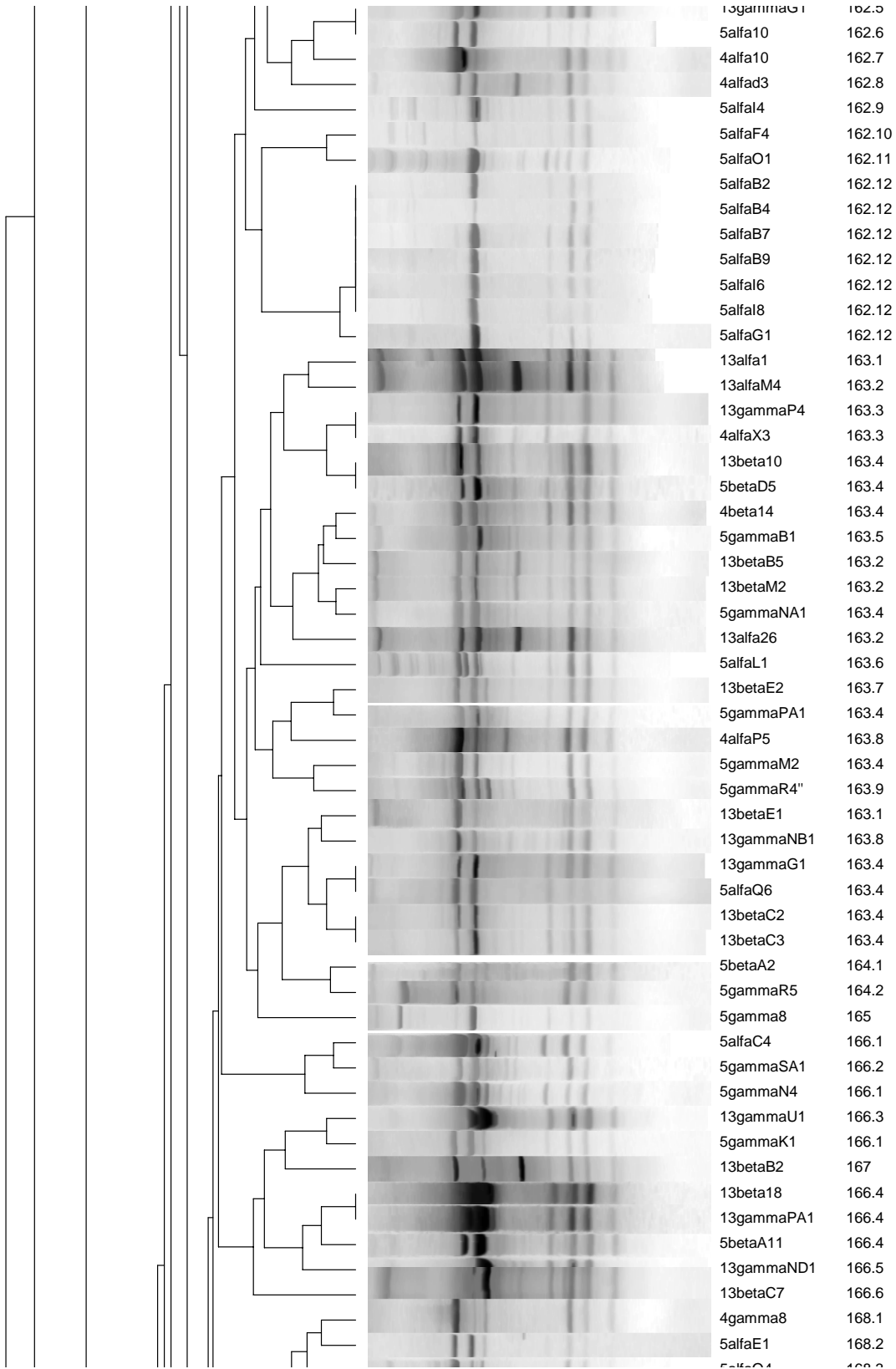


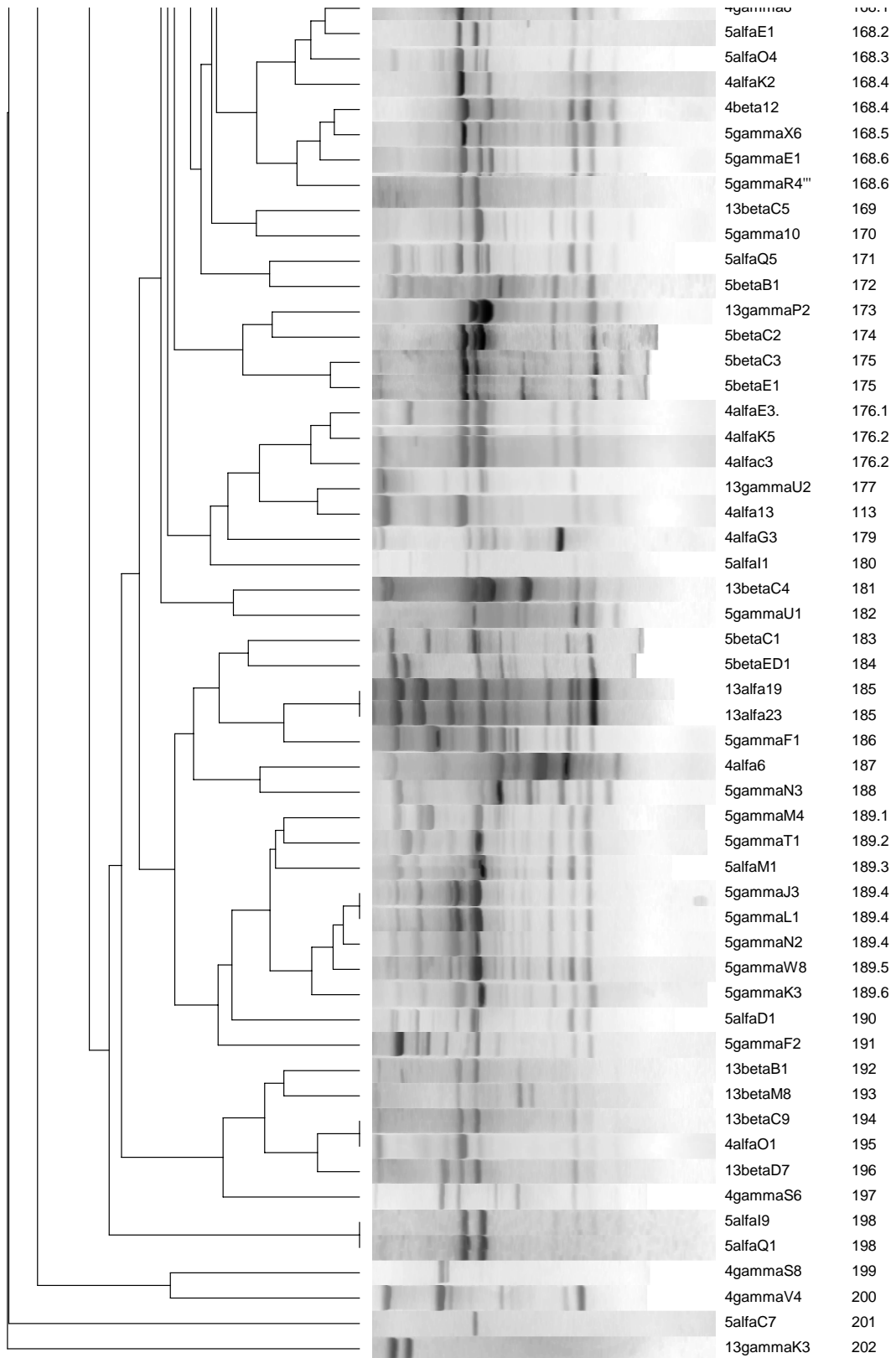












Appendix 2

Polone E. and Squartini A.

**La radice e i microrganismi della rizosfera: analisi dei
pattern di nodulazione di *Pisum sativum* in popolazioni
naturali di *Rhizobium leguminosarum* bv. *viciae***

ACCADEMIA DEI GEORGOFILI

ELISA POLONE – ANDREA SQUARTINI

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Giornata di studio: «*Le radici delle piante coltivate:
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INTRODUZIONE

L'azoto (N) è elemento di fondamentale importanza per tutti gli organismi viventi, essendo costituente di numerosi composti organici azotati essenziali per la vita, quali le proteine e gli acidi nucleici.

L'azoto, oltre che nelle rocce, si trova in abbondanza nell'aria che respiriamo (costituisce circa il 78% dell'atmosfera), tuttavia l'azoto gassoso, in forma di molecola biatomica (N_2), è assai poco reattivo perché i due atomi costituenti sono uniti da un triplo legame chimico ($N \equiv N$) molto forte. La scarsa reattività dell'azoto fa sì che esso sia poco diffuso in forma combinata non volatile nella crosta terrestre, ma dal momento che la maggior parte degli esseri viventi non è in grado di utilizzare direttamente azoto molecolare, esso deve prima essere convertito in composti inorganici, per esempio in forma di ammoniaca o di ione ammonio, di nitrati e di nitriti.

Per la riduzione dell' N_2 ad ammonio si deve compiere un salto energetico di 225 Kcal. Questa energia di attivazione è ottenibile a pressioni di 100 Mpa e 350° C, come nei costosi processi industriali di sintesi dell'ammonio, indirizzato alla fertilizzazione chimica azotata dei vegetali.

La stessa reazione, alcuni batteri, detti batteri azotofissatori, sono in grado di svolgerla a pressione atmosferica e temperatura ambiente. Tale fissazione può fornire fino a 700-1000 Kg di azoto fissato per anno per ettaro (Peoples et al., 1995). Si può pertanto comprendere l'importanza dei batteri azotofissatori per la vita di piante e animali.

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Gli azotofissatori sono esclusivamente procarioti; in base al loro rapporto con altri microrganismi, possiamo distinguerli in tre tipi:

- Diazotrofi liberi: si trovano nel suolo e nelle acque come organismi liberi, fissano quindi azoto indipendentemente dall'ambiente circostante e dalla presenza di altri microrganismi (ad esempio *Azotobacter*).
- Biocenosi diazotrofe: i batteri sono localizzati nella rizosfera di piante superiori e sono da queste influenzati attivamente (ad esempio *Azospirillum*).
- Simbiosi diazotrofe: i batteri instaurano rapporti di collaborazione endosimbiotica inducendo noduli in piante superiori (ad esempio *Rhizobium*).

Tra i tipi di fissazione biologica dell'azoto, quella simbiotica raggiunge la specializzazione più elevata e consente di ottenere le maggiori potenzialità di fissazione grazie al reciproco vantaggio per pianta e microrganismo.

Sebbene la maggior parte delle iniziali ricerche fosse rivolta a sistemi azotofissatori meno importanti dal punto di vista agrario, quali *Clostridium*, *Azotobacter* e *Klebsiella*, l'esistenza di un enzima (nitrogenasi) relativamente simile nei microrganismi capaci di fissare l'azoto atmosferico, ha consentito di acquisire nuove conoscenze anche sull'associazione *Rhizobium-Leguminosae*. L'enzima nitrogenasi catalizza la reazione di riduzione di N_2 a NH_3 , quest'ultima viene incorporata negli aminoacidi a livello del citoplasma nell'ospite.

Pertanto, la simbiosi *Rhizobium-Leguminosae* deve essere vista come un mezzo prezioso a disposizione dell'uomo per ridurre l'uso di concimi azotati nonché, di conseguenza, gli effetti nocivi di un loro eccesso sulla qualità ambientale.

La famiglia delle Leguminosae è quella che per eccellenza dimostra attitudine a formare simbiosi. Essa è divisa in tre sottofamiglie, la più antica, quella delle Cesalpinoideae (2000 specie) mostra solo il 23% di specie formanti noduli, laddove le più moderne Mimosoideae (3000 specie) e Papilionoideae (13000 specie) sono nodulate per il 90% e 97% rispettivamente (Bryan et al., 1996).

La famiglia delle Rhizobiaceae comprende i generi *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Allorhizobium*, a crescita veloce (con un tempo di generazione di 2-4 ore), *Bradyrhizobium* e *Azorhizobium*, a crescita lenta (con un tempo di generazione di 4-8 ore).

MATERIALI E METODI

Ceppi batterici e condizioni di crescita

Rhizobium leguminosarum bv. *viciae* è stato coltivato alla temperatura di 30° C nel mezzo BIII (0,23 g/l di K_2HPO_4 , 0,10 g/l di $MgSO_4 \cdot 7H_2O$, 1,10 g/l di Na-glutammato, 10 g/l di mannitolo, 1 ml/l di soluzione stock di vitamine 1000X [in mg/l: riboflavina 20, acido paminobenzoico 20, acido nicotinico 20, biotina 20, tiamina-HCl 20, piridossina HCl 20, pantotenato di calcio 20, inositolo 120; il tutto disciolto in tampone 0,05M Na_2HPO_4 pH 7, sterilizzato per filtrazione -0,22 μm - e aggiunto al mezzo autoclavato], 1 ml/l di soluzione stock di oligoelementi 1000X [$CaCl_2$ 5g/l, H_3BO_3 145 mg/l, $FeSO_4 \cdot 7H_2O$ 125 mg/l, $CoCl_2 \cdot 6H_2O$ 59 mg/l, $CuSO_4 \cdot 5H_2O$ 5 mg/l, $MnCl_2 \cdot 4H_2O$ 4,3 mg/l, $ZnSO_4 \cdot 7H_2O$ 108 mg/l, Na_2MoO_4 125 mg/l, nitrilo triacetato 1g/l – aggiustato a pH 5 prima della sua aggiunta per evitare la precipitazione –]. Il pH è stato portato a 6,9.

Il mezzo colturale solido è stato ottenuto aggiungendo al terreno liquido 16 g/l di agar. È stato aggiunto anche il colorante congo red (25 mg/l) che generalmente non colora *Rhizobium*.

Descrizione del sito di semina

Il campo che costituisce il sito di semina è situato nella stazione sperimentale della Facoltà di Agraria dell'Università di Padova. Esso comprende 16 parcelle di un metro di lato, delimitate da pareti in cemento che penetrano nel suolo per circa un metro, mantenendo sufficientemente isolato il terreno contenuto nella parcella.

Procedura di campionamento delle radici

Sono state prelevate 3 piante della varietà *Curico* dalla parcella n. 4, e 3 piante della stessa varietà dalla parcella n. 5.

Le piante sono state estratte dal terreno con l'uso di una vanga, alzando la zolla sottostante la radice, cercando di mantenere il più integro possibile l'apparato radicale. Si è poi proceduto a una prima grossolana eliminazione manuale della terra esterna all'apparato radicale. Successivamente le piante, con l'apparato radicale ancora compreso nella zolla di terreno, sono state immerse

in un recipiente con acqua. Alla pianta immersa venivano fatti compiere piccoli movimenti per consentire la rimozione della terra. Sono stati sufficienti 2 di questi lavaggi per pianta per ottenere piante sufficientemente pulite. In seguito le piante sono state etichettate con il numero della parcella seguito dalla lettera greca α , β o γ e trasportate in laboratorio adagiandone l'apparato radicale su una vasca contenente acqua sul fondo.

Sterilizzazione

L'apparato radicale di ciascuna pianta è stato immerso prima in etanolo al 95% (20 sec.) e successivamente in sodio ipoclorito al 5%: il tempo di immersione è stato variabile da 1 a 3 minuti. Sono stati poi eseguiti 6 lavaggi con acqua distillata sterile.

Al termine della sterilizzazione gli apparati radicali sono stati separati dalla parte aerea con un taglio all'altezza del colletto e sono stati trasferiti su piastre quadrate (23 x 23cm) sterili in plastica. Con bastoncini cotonati sterili si sono asciugati gli eccessi d'acqua. Si è poi proceduto alla separazione delle singole radici laterali con l'aiuto di pinze sterili in modo da distribuirle uniformemente sulla piastra. Si è dunque passati all'attribuzione del nome di ciascuna radice secondaria secondo il prospetto di nomenclatura.

Nomenclatura noduli

Sono state assegnate alle radici secondarie le lettere dell'alfabeto dalla A alla Z in grafia maiuscola partendo dall'apice e risalendo al colletto e scrivendole con marker indelebile sulla piastra in plastica a fianco di ogni radice. Per apparati radicali con radici secondarie in numero maggiore di 23 si sono adoperate in aggiunta le lettere dell'alfabeto in grafia minuscola. Nel caso di radici terziarie si è fatto seguire alla lettera della radice secondaria un'altra lettera dell'alfabeto dalla A alla Z; radici quaternarie sono state rappresentate da una terza lettera. I noduli sono stati numerati progressivamente dalle porzioni distali a quelle prossimali delle radici.

Acquisizione dell'immagine dell'apparato radicale

È stata acquisita l'immagine digitale dell'apparato radicale, dopo la sua nomenclatura con l'uso di uno scanner Epson Perfection 1240U.

Procedura di campionamento dei noduli

L'apparato radicale è stato inumidito con acqua sterile con l'uso di pipetta pasteur sterile per evitare il disseccamento dei noduli. Sotto cappa sterile ogni nodulo è stato asportato dalla radice con bisturi flambato, misurato su griglia millimetrata in piastra petri sterile e inserito con pinza sterile in tubo cryovial sterile da 2ml. All'interno del tubo il nodulo è stato schiacciato con il dorso della stessa pinza. Il tubo è stato chiuso e contrassegnato con il codice di nomenclatura. Lo stesso codice è stato riportato su un modulo aggiungendo informazioni su dimensioni e forma del nodulo e, se presenti, caratteri particolari. Si è aggiunto a ciascun tubo 1ml di mezzo YMB sterile e il tubo è poi stato agitato manualmente. Si è proceduto alla collocazione dei tubi in scatole di cartone, le quali sono state disposte verticalmente su agitatore a 30 °C per 7 giorni. Trascorso il periodo di incubazione, i tubi sono stati osservati contro luce a occhio nudo per valutare la torbidità della soluzione, assegnando un segno positivo (+) o negativo (-) nel caso in cui la soluzione si presentava rispettivamente più o meno torbida rispetto all'acqua. I tubi di segno - sono stati nuovamente collocati in incubatore a 30 °C per ulteriori 2 giorni prima di ricontrollarne la torbidità, mentre ai tubi di segno + è stato aggiunto 1ml di glicerolo 80% sterile; i tubi sono poi stati agitati manualmente per qualche secondo e si sono attesi 15 minuti prima di collocarli nell'ultrafrigo ad una temperatura di -80 °C.

*Analisi rep-PCR di isolati di *Rhizobium leguminosarum* bv. *viciae**

Una quantità (prelevata con un'ansa sterile) di cellule batteriche proveniente da una coltura cresciuta su piastra di BIII CR per circa 3 giorni è stata risospesa in 50 µl di una soluzione di lisi (0,25% sodio dodecil solfato [SDS], 0,05 M NaOH) e agitata per 60 sec. su vortex.

La sospensione batterica è stata scaldata per 15 minuti a 95 °C e successivamente centrifugata per 10 minuti in Eppendorf 5415D, in modo da ottenere la sedimentazione dei residui cellulari. 10 µl di surnatante sono stati trasferiti in una microprovetta contenente 90 µl di H₂O demonizzata sterile. Un µl di questa soluzione è stato aggiunto ai 24 µl della miscela di amplificazione (14,9 µl di H₂O bidistillata sterile, 2,5 di soluzione tampone per l'amplificazione 10X [AB Analitica, Padova], 4 µl di una miscela di deossinucleotidi trifosfati [dATP, dGTP, dTTP, dCTP] [AB Analitica, Padova] 1,25 mM ciascuno, 2,5 µl di una soluzione 20 µM di primer, 0,1 µl di una soluzione 0,5U/ µl di Taq polimerasi [AB Analitica, Padova]).

A ogni analisi è stata preparata una miscela di amplificazione (master mix) in cui ciascun componente è presente in quantità sufficiente per esaminare tutti i campioni e successivamente sono state preparate aliquote da 24 µl a ciascuna delle quali è stato aggiunto il DNA.

Il primer utilizzato è stato BOXA1R (CTACGGCAAGGCGACGCT-GACG) (Versalovic et al., 1994): contenuto in GC del 68,2% e T_m di 67,7 °C.

I frammenti di DNA amplificati sono stati separati mediante gel d'agarosio (1.5%) contenente 0.3 µg/ml di etidio bromuro, visualizzato mediante radiazione ultravioletta con lunghezza d'onda di 254 nm e fotografato con apparato digitale Kodak EDAS290, salvando le immagini in formato TIFF.

Analisi di immagine computer-assistita

Il livello di similarità dei tracciati elettroforetici, i conseguenti dendrogrammi e le matrici numeriche di distanza genetica tra i profili impiegate nei confronti per l'analisi ecogenetica, sono stati ottenuti analizzando le immagini digitali dei gel con il programma GelComparII (Applied Maths, Kortrijk, Belgio).

RISULTATI E DISCUSSIONE

Coltivazione Piante

Piante di pisello cv. Curico, sono state seminate in pieno campo in data 15 maggio 2002 ed estratte a 33 giorni dalla semina con l'intero apparato radicale dal quale sono stati isolati i noduli. Il terreno è una miscela di provenienza alloctona, ed è stato posto in sito all'inizio degli anni '90. A tale epoca si può quindi assumere che la composizione microbiologica fosse la stessa in tutte le parcelle. Le parcelle interessate dallo studio del presente progetto sono la n. 4 e la n. 5, le stesse sono state, negli anni dal 1994 al 1999 sottoposte a rilascio di ceppi selezionati di *Rhizobium leguminosarum* bv. *viciae* (ceppi 1110 e 1114, per la parcella 4, e 1114 per la parcella 5) e a coltivazione di pisello per quattro cicli (parcella 5) o per cinque cicli (parcella 4). La microflora naturale appartenente alla specie di rizobio simbiote del pisello, ha quindi ripetutamente ricevuto una selezione alla moltiplicazione in contesto simbiotico con la possibilità di una concomitante diversificazione genetica.

Isolamento batteri

Un totale di 581 noduli sono stati escissi, e da essi si è proceduto all'isolamento degli endosimbionti batterici, coltivando i rizobi su mezzo liquido YMB, dalla cui coltura sono stati ottenuti gli stocks per la crioconservazione con aggiunta di glicerolo. Le corrispondenti colture per l'analisi di caratterizzazione genetica sono state successivamente ottenute facendo moltiplicare colonie su piastre di mezzo minimo BIII con rosso Congo. Il numero di isolati batterici che hanno dato luogo a crescita sul mezzo YMB è inferiore al numero totale di noduli in quanto: a) alcuni noduli possono non contenere batteri in quanto si trovano in stadi troppo precoci della propria organogenesi, e non sono stati ancora invasati; b) alcuni noduli, e in particolare quelli di dimensioni molto ridotte o quelli senescenti in fase di sfaldamento istologico, possono essere più sensibili ai trattamenti di sterilizzazione superficiale (indispensabili per eliminare gli altri batteri esterni di rizosfera e ottenere colture pure di endosimbionti). Inoltre nonostante la avvenuta crescita su mezzo YMB in alcuni casi non è stata ottenibile la formazione di colonie su piastra del mezzo BIII, più selettivo (importante per confermare la purezza delle colture e l'aspetto della colonia). A tale proposito, anche alla luce dei recenti esempi di nodulazione di leguminose da parte di batteri diversi dai rizobi, si potrebbe ipotizzare che una minoranza dei noduli potrebbe essere stata invasa da microrganismi d'altro tipo incapaci di crescere sul mezzo minimo BIII basato sul mannitolo quale unica fonte di carbonio, e più selettivo per i rizobi.

Caratterizzazione degli isolati mediante analisi del polimorfismo di amplificazione

Dopo una fase di scelta dell'oligonucleotide ideale per il fingerprinting dei rizobi isolati, in cui sono stati confrontati i primers per l'amplificazione NPC (TGGCACGACGCTTGAAAATT) e BOXA1R (CTACGGCAAGGCGACGCTGACG) la scelta è caduta sul secondo, in virtù della regolarità e riproducibilità di risposta. Successivamente sono stati quindi prodotti i lisati cellulari di tutti gli isolati dei quali erano state ottenute colonie su mezzo BIII, e si è proceduto alle amplificazioni PCR, i cui profili sono stati visualizzati per elettroforesi la cui immagine al transilluminatore è stata acquisita in formato digitale TIFF per essere processata con software GelComparII. Il fingerprinting caratteristico di ciascun individuo ("prova

del DNA”) permette di riconoscere i casi di identità, inoltre il grado di disuguaglianza nella posizione delle bande e nel loro numero può essere quantificato e ricondotto a distanze numeriche che esprimono una corrispondente distanza genetica tra gli isolati. L'efficacia della amplificazione PCR col primer BOXA1R è stata sufficientemente elevata e un totale di 372 profili sono stati ottenuti.

Nomenclatura degli isolati batterici

L'esame sistematico del dendrogramma e la comparazione visiva delle bande, e delle zone di transizione da gruppo a gruppo di profili ha permesso di determinare, quanti e quali noduli ospitassero lo stesso tipo di isolato. A elettroforetogrammi uguali o divergenti per non oltre il 10% (corrispondente al livello di variabilità inter-replicati inerente al metodo di analisi) è stato attribuito lo stesso identificativo. Si è adottata una nomenclatura numerica progressiva a decorrere dalla sommità del dendrogramma. Oltre alla attribuzione assoluta di differenza, è stato introdotto un criterio sistematico per mantenere nel numero attribuito anche l'indicazione dell'appartenenza a un sottogruppo o famiglia i cui isolati condividano un elevato grado di somiglianza. Questo consiste in un prefisso costante seguito dai diversi suffissi progressivi, in numero uguale al numero di isolati appartenenti alla famiglia di profili designata dal prefisso. Ad esempio gli isolati da 22.1 a 22.36 consistono in un gruppo di 36 isolati non identici ma geneticamente imparentati tra loro in maniera soggettivamente apprezzabile dall'osservazione della conservazione delle loro bande nelle stesse posizioni, mentre isolati designati da una singola cifra, non appartengono a famiglie con relazioni altrettanto evidenti. A ogni conto, poiché il dendrogramma contiene di per sé un principio di ordinalità, la nostra modalità di attribuzione rispetta anche il fatto che due numeri aritmeticamente vicini corrispondano a due ceppi più simili tra loro di quelli relativi a due numeri fra loro più distanti.

Analisi del pattern di nodulazione per ciascun ceppo

Il confronto dei dati permette le considerazioni, sintetizzate nella presente tabella, che riassume il numero di noduli, il numero di profili riscontrati per pianta e la loro ripartizione proporzionale in profili unici e profili comuni. Da un totale di 372 isolati di altrettanti noduli dei quali è stato ottenuto

	Parcella 4	Parcella 5
noduli/parcella	155	217
profili/parcella	95 (1 ogni 1.63 noduli)	129 (1 ogni 1.68 noduli)
unici per parcella	77 (81,0%)	111 (86,0%)
comuni 4 e 5	18	(8,7%)

l'elettroforetogramma BOXA1R, sono stati identificati 205 tipi, ovvero 205 elettroforetogrammi, pari a 205 *ceppi*, definiti in quanto *unità tassonomiche operazionali fenotipicamente diverse al fingerprinting molecolare*. Questo dato costituisce la diversità biologica del campione considerato, concetto equivalente a quello della "ricchezza di specie", ma relativo in questo caso ai ceppi di una stessa specie, (*R. leguminosarum* bv. *viciae*). È noto da numerosa letteratura come i rizobi del suolo tendano in generale a essere rappresentati da alcuni ceppi competitivi che nodulano numerose volte e su più piante, tali casi sono ben visibili anche nel nostro studio e verranno adeguatamente discussi, ma è interessante come prima osservazione soffermarsi invece su quanto riguarda i ceppi non-dominanti. A tale proposito infatti, il dato più saliente che emerge da queste analisi scaturisce dalla considerazione di quanti, dei tipi riscontrati, siano stati reperiti *soltanto nei noduli dell'apparato radicale di un dato individuo di pisello*. Per la pianta 4alfa, ad esempio, 38 profili sui 53 presenti (pari al 71,7%) sono specifici. In generale un alto valore di casi individuo-specifici di simbionti si riscontra per tutte le piante osservate, con un massimo del 78% (pianta 5alfa) e un minimo del 59% (pianta 4beta). È possibile notare inoltre che i valori più alti sono propri delle piante dalle quali sono stati analizzati più noduli e si ritiene quindi rappresentino con maggior accuratezza la reale proporzione dei profili pianta-specifici, cioè si avvicinino al valore massimo del numero di ceppi che teoricamente potrebbero nodulare uno stesso individuo vegetale. Si osserva quindi che ogni esemplare di pianta pur condividendo esse genere, specie, biovar, cultivar, partita di semente, e, per quelle all'interno di una stessa parcella anche, la medesima zolla di terra, siano invase in maniera apparentemente specifica da popolazioni di simbionti molto distinte le une dalle altre, come se ogni pianta costituisse una sorta di isola in cui le interazioni genotipo/genotipo a livello individuale siano le principali regolatrici dell'interazione molecolare tra micro- e macro-simbionti. Ma a ciò va aggiunto che gran parte di questo effetto è legato al fatto che, di per sé, *la maggior parte dei noduli contengono un ceppo il cui profilo viene riscontrato una sola volta in assoluto*. Per rendere ragione di questo fattore è possibile elencare per ogni pianta il numero di profili unici (il profilo è reperito solo in quella data pianta

e solo in uno dei noduli da essa formati) e calcolarne la percentuale. Questo dato, utile a saggiare il grado di esaustività del progetto nell'estrarre progressivamente casi nuovi dal terreno mediante l'uso delle piante quali esche specie-specifiche, non è però adatto a osservare quanti ceppi nodulano una data pianta una volta sola, misura espressa invece dal numero dei profili singoli (il profilo è reperito in una o più piante ma solo in un nodulo per pianta). La loro percentuale sul numero di noduli ci informa di quanto questi nodulatori sporadici incidano sul totale di batteri ospitato da una pianta, mentre la percentuale sul numero di profili esprime la fetta di diversità ivi rappresentata da questi ceppi. Un ulteriore parametro utile è il rapporto tra numero profili e numero noduli, che può raggiungere il valore teorico massimo di 1 nel caso ogni nodulo ospitasse un profilo diverso. Il valore medio tra tutte le piante è 0,55, con notevoli fluttuazioni tra pianta e pianta.

I valori dei profili singoli, che si attestano consistentemente tra il 70 e l'80%, indicano come per la maggior parte dei rizobi di questa specie l'incontro con l'ospite sia un fenomeno probabilistico individuale. Questo indica che la prevalente situazione di profilo singolo o addirittura unico, di ceppi riscontrati associati a ogni individuo di vegetale, non sia tanto il risultato di interazioni di specificità genotipo batterio/genotipo leguminosa, quanto quello della casualità di incontro tra radici e membri di popolazioni microbiche estremamente diversificate. I dati indicano che in generale ogni nuova pianta seminata, che formi una media di circa cento noduli, reperisce con le ramificazioni del proprio apparato radicale circa sessanta nuovi ceppi non osservati in nessuna delle piante precedenti.

In tal senso, è possibile fare stime circa la reale entità della diversità biologica dei ceppi di *Rhizobium leguminosarum* bv. *viciae* presenti in questi terreni; considerando che le tre piante di pisello provenienti da una stessa parcella condividevano uno spazio di 25 cm² di superficie, e che la profondità raggiungibile dagli apparati radicali nel mese successivo alla semina, coincidente in questa prova con la finestra temporale di opportunità per la nodulazione, è di circa 25 cm, si può assumere che le radici delle piante di una parcella abbiano a disposizione un volume di suolo di 15625 centimetri cubici (25³). Questo volume, di oltre 15 litri di suolo, contiene circa altrettanti kg di peso secco di terreno. Conoscendo la quantità di cellule di *Rhizobium* di questa specie per grammo di peso secco del terreno di queste parcelle, che nel 1993, prima delle coltivazioni si piselli su queste parcelle era di $5,2 \times 10^3$ cellule per grammo di peso secco di suolo (V. Corich, tesi di Dottorato di Ricerca, Università di Padova, 1996), si può stimare che nel volume considerato

esistano almeno $7,8 \times 10^7$ cellule di rizobio della specie in questione. Ma, considerando che cinque stagioni di coltura di piante ospiti si sono susseguite tra l'epoca dell'analisi suddetta e la presente prova, si può assumere che i rizobi per grammo siano con molta probabilità aumentati, e poiché in molti suoli europei la specie in questione può toccare le 10^7 cellule per grammo di terreno, il numero dei rizobi nodulanti totali a disposizione delle piante di CV curico in uno spazio di 25 cm^2 potrebbe facilmente essere intorno alle 10^{11} cellule. È pertanto altamente plausibile che il numero di ceppi distinti all'interno di tale popolazione giustifichi la alta diversità osservata nei noduli. In termini pratici, esistendo un ovvio limite fisico di ingombro alla possibilità di coesistenza di piante di pisello su una stessa superficie, si può assumere che, in un terreno come il presente, che ha ricevuto almeno 5 stagioni di colture di piselli in passato, per ciascuna pianta sarà sempre garantita una quota di almeno il 60% di noduli occupati da ceppi unici.

Passando invece alle considerazioni relative ai ceppi non-unici, questi come detto possono essere considerati rappresentare quella compagine dotata di un grado più o meno elevato di competitività, o di attitudine alla dispersione all'interno dell'ambiente considerato, ivi inclusa la capacità di diffusione da una parcella all'altra. Questo comportamento, che culmina nella definizione di ceppi "dominanti", è verificabile riscontrando i casi di profili reperiti su più di un individuo di pianta. I casi definibili comuni, sono quelli reperiti trasversalmente su più piante e rappresentano i tipi rispondenti alle suddette caratteristiche, il cui grado di sviluppo è misurato dal numero di casi osservati e dalla larghezza di distribuzione raggiunta (numero di piante, in cui il profilo è riscontrato).

CONCLUSIONI

Analizzando le comunità di *Rhizobium leguminosarum* biovar *viciae* nodulanti piselli nelle parcelle di terreno oggetto dell'indagine sono state isolate ed individuate, a partire da 581 noduli, un totale di 111 unità tassonomiche fenotipicamente distinguibili in base al fingerprinting del DNA.

I ceppi definiti dalla definizione suddetta appaiono rappresentare una limitata porzione della diversità biologica totale dei ceppi presenti nel sito, come testimoniato dalla elevata percentuale, prossima all'80%, di isolati identificati in un solo nodulo in ciascuna delle piante analizzate.

Mentre la maggioranza degli isolati appartiene a tipi unici, i ceppi ricorrenti, dominanti o competitivi, comuni a più piante e parcelle appartengono a un gruppo ristretto generalmente rappresentato da famiglie di profili simili, la cui distribuzione specifica dimostra avvenuti processi di dispersione e differenziazione genetica intercorsi durante i quindici anni successivi alla divisione della miscela di terreno originario in parcelle circoscritte.

RIASSUNTO

Analisi dei pattern di nodulazione di Pisum sativum in popolazioni naturali di Rhizobium leguminosarum bv. viciae.

Nel presente lavoro si è effettuato l'isolamento in coltura pura dei microrganismi simbiotici da 581 noduli formati su sei piante di pisello precedentemente seminate in parcelle di terreno dell'Azienda Agraria della Facoltà. Successivamente si è proceduto alla caratterizzazione genotipica di ciascun isolato mediante un metodo di fingerprinting PCR basato sull'appaiamento arbitrario dell'oligonucleotide BOXA1R. I profili elettroforetici ottenuti sono stati confrontati con il programma GelComparII e trasformati in entrate di una matrice di similarità dalla quale è stato possibile ottenere il dendrogramma delle relazioni reciproche tra gli isolati in termini di distanza genetica. Questa analisi ha permesso di stabilire il grado di ceppo-specificità dei batteri associati ai diversi individui di pianta e gli effetti di separazione fisica delle stesse, quando appartenenti a parcelle di terreno distinte.

ABSTRACT

Analysis of the pea nodulation patterns on natural Rhizobium leguminosarum bv. viciae populations

The microbial inhabitants of 581 pea root nodules formed on 6 plants purposely sown in a soil within the University's experimental agricultural station were isolated and characterized upon DNA-based BOXA1R PCR-electrophoretic fingerprinting. Band profiles were analyzed by GelComparII image analysis software converting differences into a numerical matrix yielding their similarity dendrogram in terms of genetic fingerprint distances. The level of strain-specific association with plant specimens and soil parcels have been assessed.

BIBLIOGRAFIA

- BRYAN J. A., BERLYN G. P., GORDON J. C. (1996): *Toward a new concept of the evolution of symbiotic nitrogen fixation in the Leguminosae*, «Plant and Soil», 186, pp. 151-159.
- PEOPLES M. B., HERRINDGE D. F., LADHA J. K. (1995): *Biological nitrogen fixation: an efficient source of nitrogen for sustainable agricultural production*, «Plant and Soil», 174, pp. 3-28.
- VERSALOVIC J., SCHNEIDER M., DE BRUIJN F. J., LUPSKI J. R. (1994): *Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction*, «Methods in Molecular and Cellular Biology», 5, pp. 25-40.

Appendix 3

**Corich V., Giacomini A., Vendramin E., Vian P., Carlot M.,
Concheri G., Polone E., Casella S., Nuti M. P. and Squartini**

A.

**Long term evaluation of field-released genetically
modified rhizobia.**

Long term evaluation of field-released genetically modified rhizobia

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This is the report of the first open field release of genetically modified microorganisms (GMMs) in Italy. It covers ten years of monitoring, and follows in-field GMM dynamics from strain release to disappearance below detection limits, as well as assessment of impact on resident microorganisms. The bacteria released belong to the nitrogen fixing legume endosymbiont *Rhizobium leguminosarum* bv. *viciae*, and were engineered with non-agronomically-proficient traits, in order to assess their behavior and fate without GMM-specific positive feedback from the plant. A DNA cassette containing mercury resistance and β -galactosidase genes was introduced in either plasmid-borne or chromosomally integrated versions, in order to test the resulting strain stability. A synthetic promoter was used to drive the *lacZ* gene, conferring high catabolic activity to the GMM. Two different wild-type *Rhizobium* backgrounds were tested, comparing a non-indigenous vs. an indigenous, highly competitive strain. The latter had much greater persistence, since it was able to survive and establish at technically detectable levels for over four years after release. Selection factors, such as reiterated presence of the plant host, or lactose substrate supply, enhanced long-term survival to different extents. The lactose treatment showed that even a single trophic supplementation can surpass the benefits of symbiotic interaction for a period of several years. Concerning impact, the GMMs did not alter substantially the other soil community general microbiota. However, there were some significant differences in microbiota as a consequence of the *Rhizobium* inoculation. This effect was observed with either the WT or GMM, and was more evident in the release of the indigenous *Rhizobium*. Moreover, as the indigenous GMM had its parental, dominant wild-type in the same soil, it was possible to evaluate to what extent the GMM version could result in parent displacement (“self-impact”), and how much the two rhizobia would additively contribute to nodulation.

Keywords: rhizobia / GMM / impact / risk assessment / environmental release

INTRODUCTION

This work originated within the framework of the pre-normative risk assessment guidelines issued in Europe at the onset of the nineties (Nuti et al., 1994). We have since then devoted our effort to the construction and monitoring of model GMMs whose fate has been investigated in open field trials through a ten-year time span, from the first release to their technical extinction, as defined by the detection limits of available methodologies.

We have recently reviewed the collective knowledge gathered after 24 field releases in the Italian territory (Nuti et al., 2003). The present report is the detailed complete outcome of the earliest of these experiments, started in parallel with our previously published joint trials with genetically modified (GM) *Azospirillum brasilense* and

Pseudomonas fluorescens (Basaglia et al., 2003; Corich et al., 1995; Nuti et al., 1997; Resca et al., 2001).

Rhizobium leguminosarum bv. *viciae*, the nitrogen-fixing root nodule microsymbiont of pea and faba bean legume plants, was chosen as host strain for the recombinant genes. It constitutes an example for which a large body of ecological and genetic knowledge is available. In particular, being a widely applied agricultural inoculant, of interest in the areas of biotechnology and marketing, its behavior in soil in relation to massive release, has long been studied. The bacteria were engineered with the same genes to be used as markers for their subsequent tracking. No traits linked to the promotion of crop productivity were at this stage inserted, in order to analyze the sole issue of biosafety.

The genetic markers were selected to fulfill the requirements of: (1) neutrality with respect to the

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environmental selective conditions, in order to not confer advantages or deficiencies to the GMM under normal situations; (2) selectability at the recovery stage, in order to allow a clean and unambiguous monitoring of the released strains when re-isolated from soil; (3) absence of antibiotic resistance genes, to avoid spread of traits prone to jeopardize human clinical therapy. The chosen genes included mercury resistance (the *mer* operon from Tn1831) and a *lacZ* gene driven by a synthetic promoter. Such a promoter was designed in order to ensure a high level of *lac* gene expression. This trait was deemed necessary to differentiate the tagged GMM from background *lac*⁺ soil microbiota. It was constructed after aligning sequences of published prokaryotic promoters, and extracting the highest scoring consensus for each base position upstream the transcriptional start site. The resulting construct proved to be very efficient, and its gene expression level was stronger than the *lac* and *tac* promoters in *Rhizobium* and *E. coli* (Giacomini et al., 1994). The gene cassette including the *lac* and *mer* determinants was introduced into *Rhizobium leguminosarum* either in plasmid-borne form, or integrated into the chromosome. Two alternative regulation modes were devised by either interposing or omitting a *lac* operator between the synthetic promoter and the reporter gene. Before the field release stage, the resulting GMM strains were tested for over 500 days in microcosms in three different inoculant carrier substrates, assessing their survival, genetic stability and interspecies gene exchange (Corich et al., 1996 and 2000). We found that the stability of these genetic modifications was strongly dependent on the means of insertion into the bacterium genome. Chromosomal integration gave the most stable result, while the absence of operator in a plasmid-borne gene cassette was the one most prone to loss by segregation.

The present paper presents a survey carried out over a ten-year period (1994–2004), starting with the open field release of the GMMs in an agricultural setting in the presence of their legume host plants. Their persistence, as well as the impact on resident microbial groups were evaluated. An analysis of the ecological implications was also made by comparing the behavior of a GM *Rhizobium* isolated in foreign environment (allochthonous) with that of an indigenous strain of the same species, isolated from the release site (autochthonous) and tagged with the same genetic cassette.

RESULTS

Release, persistence and impact of an allochthonous (non-native) GMM

Prior to the release, an assessment of the indigenous microflora was carried out in the field of choice, yielding the

following results, expressed as CFU.g⁻¹ (dry weight) of soil: aerobic bacteria total count: $2.06 \times 10^7 \pm 5.00 \times 10^5$ (means of three field plot replicates \pm SD); fungi: $2.19 \times 10^4 \pm 8.84 \times 10^2$; streptomycetes: $2.69 \times 10^6 \pm 1.86 \times 10^6$; fluorescent pseudomonads: $5.63 \times 10^4 \pm 4.42 \times 10^4$; spore-forming bacteria: $2.17 \times 10^6 \pm 2.15 \times 10^5$; *Rhizobium* group: $8.88 \times 10^5 \pm 5.30 \times 10^4$. Besides scoring the soil rhizobial population by congo red-negative CFU counts on YMA, we performed pre-release MPN assessments for *R. leguminosarum* bv. *viciae*, *R. leguminosarum* bv. *trifolii*, *Sinorhizobium meliloti* and *Bradyrhizobium japonicum*, which gave, respectively, 5.2×10^3 , 2.8×10^2 , 2.8×10^4 , 3.0×10^1 cells per gram of soil dry weight. Subsequently, the three previously described GMM derivatives of *Rhizobium leguminosarum* bv. *viciae* 1003 were released. These are strains 1110, 1111, 1112, that carry Hg resistance and *lacZ* driven by a strong synthetic promoter, and that had been previously monitored in microcosm trials (Corich et al., 1996). Amounts of cells inoculated per seed were calculated as colony forming units per mL of inoculum suspension. The results are the following: strain 1003: $6.35 \times 10^6 \pm 9.19 \times 10^5$; strain 1110: $5.45 \times 10^6 \pm 1.20 \times 10^5$; strain 1111: $2.40 \times 10^6 \pm 1.98 \times 10^5$; strain 1112: $6.45 \times 10^6 \pm 3.54 \times 10^4$. Plating on media containing X-Gal allowed estimation of the possible loss of the introduced β -galactosidase marker at the moment of inoculation. Revertants, consisting in the fraction of cells having lost the trait, were observed only in strain 1111 constitutively-expressing *lacZ*, and amounted to $4.20 \times 10^5 \pm 4.24 \times 10^4$ of the above total cells. The use of such an unstable construct was envisaged as a negative control for its more stable versions (strains 1111 and 1112). The first sampling to monitor released bacteria was performed 10 days after sowing at the time of pea shoot emergence. Root development was already profuse and allowed rhizospheric soil isolation. The results showed a good initial colonization of the rhizosphere, and gave the following counts expressed as CFU.g⁻¹ of soil dry weight; strain 1003 (wt): $4.68 \times 10^4 \pm 2.26 \times 10^4$; strain 1110: $6.72 \times 10^5 \pm 5.76 \times 10^5$; strain 1111: $1.28 \times 10^6 \pm 8.95 \times 10^5$; strain 1112: $2.15 \times 10^5 \pm 5.76 \times 10^4$. Concerning the three GMM strains, their revertants having lost the *lacZ* marker were observed at a detectable level only for strain 1111, in which they were $2.83 \times 10^5 \pm 2.49 \times 10^5$, equal to about 22%.

In terms of genetic stability, as expected, strain 1111, which bore the plasmid with the constitutively (over)expressed *lacZ* construct showed fast plasmid loss, leading to revertants that account for a one-log decrease of the GMM. On the contrary, both strain 1110, which bore an identical plasmid differing only by the presence of the *lac* operator, and strain 1112 with the chromosomally-inserted version of the same genes, maintained their phenotype. The GMM presence in

Long term evaluation of GM rhizobia

Table 1. Bacterial strains and plasmids.

Strains	Relevant traits	Source or reference
<i>R. leguminosarum</i> bv. <i>viciae</i>		
1003	wild type (The Netherlands), rif ^r	Corich et al., 1996
1110	1003 pDG3, Hg ^r /lacZ ⁺⁺	Corich et al., 1996
1111	1003 pDG4, Hg ^r /lacZ ⁺⁺⁺ (constitutive)	Corich et al., 1996
1112	1003::Hg ^r /lacZ ⁺⁺	Corich et al., 1996
Agri10	wild type (Italy) rif ^r	this work
1114	Agri10 pDG3, Hg ^r /lacZ ⁺⁺	this work
Nb1	Wild type (England)	J. Beringer
<i>Escherichia coli</i>		
JM 109 pDG3	donor for conjugation of pDG3	Corich et al., 1996
HB101 pRK2013	helper for conjugation of pDG3	Figurski and Helinski, 1979
Plasmids		
pDG3	IncQ, Hg ^r , lacI ^q /synthetic promoter/lacO/lacZ (IPTG-inducible)	Giacomini et al., 1994
pDG4	IncQ, Hg ^r , lacI ^q /synthetic promoter/lacZ (constitutively expressed)	Giacomini et al., 1994

rif^r: Rifampicin-resistant; lacZ: β-galactosidase gene; Hg^r: mercury resistance; IncQ: incompatibility group Q; lacI^q: lactose operon repressor.

the rhizosphere was monitored again 50 days after sowing and, at this stage, all strains had already dropped below the detection limit allowed by plate counts (< 10¹ CFU.g⁻¹ soil dry weight). Both the wild-type and the three types of GMMs had undergone the same rapid decrease, which was presumably due to the low fitness of their background strain with respect to the new environment. In fact, an analysis of the root nodule content from the same plants, carried out at 50 days after sowing, on about 1200 nodules (300 per each of the four treatments), showed an occupancy averaging 0.67% and not exceeding 1.7%, which confirmed the low competitiveness of the strains. In detail, strain 1003 was found in two nodules, strain 1110 in none, strain 1111 in one nodule and strain 1112 in five nodules.

In addition, this effect observed in field conditions is not due to an intrinsically poor efficiency of all strains derived from 1003, since in axenic nodulation trials with autoclaved soil, each of them had proven able to nodulate peas profusely, indicating that this aspect of symbiotic performance was not affected by the genetic modification (data not shown). Lateral spread was negligible, as the analysis of another 650 nodules from the trap pea plants belt surrounding the plots did not yield strains carrying the phenotypic markers of the released ones. The fate of the released GMMs was further checked five months after release in a greenhouse study with soil gathered from the release site, by both selective plating and nodulation tests with faba bean and pea. No residual nodulating GMMs were rescued.

The disappearance of the GMMs was tested and confirmed one year after release, by re-sowing peas on some of the plots previously inoculated with strain 1112, which had given the highest values of persistence and nodulation. When the nodule content was analyzed, no GMMs could be reisolated.

Thirty months after the release, the possible residual presence of GMM DNA in soil was tested by PCR amplification using two primers designed to detect the *lac-mer* gene cassette, thus targeting specifically the released strain. The efficiency of the method had been successfully tested on soil freshly supplemented with serially diluted suspensions of the GMM of choice. The field soil in which strain 1112 was released gave negative results, confirming the descent below detection of the GMM and of its recombinant DNA. The detection limits of PCR approaches to track released GM rhizobia have been addressed and range between 20 and 300 culturable cells per gram, depending on the tagged gene (Cullen et al., 1998).

As scheduled, the possible impact of released GMMs on resident microbiota was assayed by culturable colony counts of selected groups 30 days after release, and is shown in Table 2. The period of 30 days was chosen as a compromise between a time not too far from release but sufficient to reveal induced changes. In order to extract possible significant differences between treatments, a one-way chi-square test for equal proportions was run. As explained, the variance (square of the standard deviation) in this data set was larger than the data

Table 2. Allochthonous GMM impact: resident culturable microbiota in pea rhizosphere soil assessed 30 days after the first GMM release.

	Bare soil control	Plant control	Plants inoculated with strain 1003	Plants inoculated with strain 1110	Plant inoculated with strain 1111	Plant inoculated with strain 1112
Total bacteria	2.19×10^7 $\pm 7.95 \times 10^5$	1.28×10^{8a} $\pm 1.21 \times 10^7$	1.86×10^{8a} $\pm 3.24 \times 10^7$	9.69×10^{7a} $\pm 2.87 \times 10^7$	8.21×10^{7a} $\pm 1.76 \times 10^6$	2.19×10^{8a} $\pm 1.39 \times 10^7$
Fungi	2.42×10^4 $\pm 7.95 \times 10^2$	1.67×10^5 $\pm 8.23 \times 10^4$	3.98×10^5 $\pm 5.63 \times 10^4$	1.56×10^5 $\pm 8.84 \times 10^4$	2.05×10^5 $\pm 6.16 \times 10^4$	2.06×10^5 $\pm 2.78 \times 10^4$
Fluorescent	2.70×10^4	1.71×10^6	2.64×10^6	1.02×10^6	1.41×10^6	4.53×10^6
Pseudomonads	$\pm 1.27 \times 10^4$	$\pm 6.53 \times 10^5$	$\pm 2.11 \times 10^5$	$\pm 3.31 \times 10^4$	$\pm 8.79 \times 10^3$	$\pm 3.32 \times 10^6$
Spore-forming	7.87×10^6	3.42×10^6	6.32×10^6	4.22×10^6	5.91×10^6	3.63×10^6
Bacteria	$\pm 3.18 \times 10^6$	$\pm 1.21 \times 10^6$	$\pm 1.48 \times 10^6$	$\pm 1.77 \times 10^6$	$\pm 4.40 \times 10^5$	$\pm 9.72 \times 10^5$
<i>Rhizobia</i>	1.97×10^5 $\pm 3.98 \times 10^4$	1.42×10^{7b} $\pm 3.68 \times 10^6$	3.44×10^{7b} $\pm 1.15 \times 10^7$	1.24×10^{7b} $\pm 3.64 \times 10^6$	1.11×10^{7b} $\pm 6.16 \times 10^5$	4.24×10^{7b} $\pm 3.82 \times 10^6$

Data are expressed as CFU.g⁻¹ (dry weight) of rhizosphere soil.

Values labelled with the same letter indicate data used for the analysis of a given variable (^a total bacteria, ^b rhizobia).

Significant differences (FREQ procedure) arise for the two variables "total bacteria" (P = 0.0495) and "rhizobia" (P = 0.0092) upon applying a one-way chi-square test for equal proportions run within all the plant plots data on the fourth root of the raw data.

averages; therefore the analysis called for the appropriate transformation. As the data spanned several orders of magnitude the fourth root was elected as the most suitable one. The unaveraged raw data were used, and the significance tested in two assemblies, the first including also the bare soil, in which case all groups except fungi and spore-forming bacteria gave significant differences. These can be explained by the simple plant effect. Thus in the second data assembly we omitted the soil control and recorded a barely significant difference for the total bacteria (P = 0.0495), and a highly significant difference only for the rhizobia group (P = 0.0092), which is in part consistent with their inoculation. As rhizobia are also a (minor) portion of the total bacterial count, the slight significance of the latter is also in line with such rhizobial fluctuation.

Release, persistence and impact of an autochthonous (native) GMM

Having witnessed the fast disappearance of rhizobia whose background strain (1003, originating from northern Europe) was not isolated from the same zone chosen for the release, we inserted the same genetic modifications into a *Rhizobium* indigenous to the site, and compared its behavior with that of the foreign GMMs. In order to enhance and bring above detection limits any possible impact of the GMM, we used a very competitive native strain, namely the one that had proven able to occupy most of the nodules on two different kinds of host plants grown in the same soil. The identification and isolation of this dominant candidate was done by com-

Table 3. Frequencies of unique DAF3-amplified RAPD fingerprints of *R. leguminosarum* isolated from nodules of pea or faba bean after the first GMM release.

RAPD Profiles	Pea (250 nodules)	Faba bean (32 nodules)
Profile #1	53.8%	44%
Profile #2	9.7%	13%
Profile #3	9.3%	19%
Profile #4	10.9%	6%
Profile #5	1.2%	9%
Profiles #6 to #33	< 1%	n.d.

n.d.: Not determined.

paring the RAPD electrophoretic profiles of 250 isolates from pea nodules, and of 32 isolates from faba bean. The primer of choice was the decamer DAF3 (Tichy and Simon, 1994). Thirty-three different profiles were detected among the symbionts of the pea host, while a total of nine profiles were enumerated from the smaller sample of faba bean nodules. The percentage at which the five most abundant cases occur in both hosts, is shown in Table 3.

Most profiles (#6 to #33), accounting for 15% of the pea nodule total occupancies, were found not more than once. At least five profiles were found in both host plants. Their respective frequency was in some cases highly variable. For example, such is the case of profile #5, observed in less than 1% of the pea cases, but accounting for 9% of the faba bean isolates. Nevertheless, the most abundant profile was the same for both plants. Profile #1, present in 54% and 44% of pea and faba bean nodules, respectively, was therefore selected as the dominant, symbiotically

Table 4. Autochthonous GMM release. Persistence of the wild-type and GMM strains, in plots sown with pea or faba bean, or amended with lactose in substitution for the host plant.

Strain	Sampled material	15 days after release	50 days after release
Agri10	Pea rhizosphere	$6.41 \times 10^6 \pm 3.48 \times 10^6$	$3.97 \times 10^6 \pm 4.29 \times 10^5$
	Faba bean rhizosphere	$5.35 \times 10^6 \pm 1.71 \times 10^6$	$3.63 \times 10^6 \pm 2.15 \times 10^5$
	Soil	$3.87 \times 10^4 \pm 1.30 \times 10^4$	$1.16 \times 10^4 \pm 1.29 \times 10^3$
	Soil + lactose	$2.92 \times 10^4 \pm 1.49 \times 10^3$	$1.07 \times 10^5 \pm 2.13 \times 10^4$
1114	Pea rhizosphere	$3.43 \times 10^6 \pm 6.80 \times 10^5$	$1.17 \times 10^6 \pm 1.57 \times 10^5$
	Faba bean rhizosphere	$2.09 \times 10^6 \pm 9.47 \times 10^5$	$3.70 \times 10^5 \pm 8.83 \times 10^4$
	Soil	$3.45 \times 10^3 \pm 9.37 \times 10^2$	$1.34 \times 10^4 \pm 3.15 \times 10^3$
	Soil + lactose	$2.45 \times 10^4 \pm 5.06 \times 10^3$	$1.56 \times 10^4 \pm 4.75 \times 10^3$

proficient, *R. leguminosarum* bv. *viciae* in the soils of the Legnaro experimental station. This background was considered to be the best example of high local adaptation, to be compared to the performance recorded with strains derived from strain 1003, whose low fitness could have been due to the different habitat of origin. The dominant *Rhizobium* strain carries four large plasmids ranging from 180 to 550 Md (data not shown). A spontaneous rifampicin-resistant derivative of the strain was obtained (Agri10), from which a genetically modified derivative was constructed by conjugal transfer of plasmid pDG3, carrying the mercury-lactose gene cassette, giving rise to strain 1114. This construct is thus equivalent to the previously released strain 1110, with the difference of an indigenous background. Strain Agri 10 was also pre-tested in pot nodulation tests on faba bean grown in the soil of the future release, and compared to another control strain, Nb1, kindly provided by John Beringer, which was a dominant nodule occupant for peas in its British isolation site. After 30 days, strain Agri 10, inoculated using 1×10^6 cells per seed, was found in 88% of *Vicia faba* nodules, while Nb1, in separate pots, invaded no more than 10%. This result further emphasizes that nodulation is quantitatively not determined by universal competitiveness, but rather by the degree of local adaptation to soil and host.

As the new release was scheduled for the fall season, requiring an overwintering pea cultivar, strains Agri 10 and 1114 were tested again in pot nodulation tests, using an inoculum of 8×10^6 , and 6×10^6 cells per seed respectively, applied to two different winter pea cultivars: Rampicante Telefono, and Mezzarama Senatore. The performance on each cultivar was different in terms of nodulation and the genetic stability of the GMM. Cultivar Rampicante Telefono had 20% of nodules occupied by strain Agri10 (wt), or 40% by strain 1114. The marker stability (percentage of isolates maintaining the introduced trait) of the latter strain after nodulation was 85%. Cultivar Mezzarama Senatore had 24% of nodules occupied by strain Agri10 (wt), or 53% by strain 1114. The

marker stability of the latter after nodulation was 100%. This cultivar was selected as host for the new field release, which took place on October 16th 1995. The different plasmid stability observed is presumably due to differential pressure that each host cultivar can exert during the fast bacterial replication occurring in the infection stage.

The operations followed the protocol described for the first campaign, with the modification of a higher titer of the inoculum suspension, providing $1.72 \times 10^7 \pm 3.06 \times 10^6$ and $1.94 \times 10^7 \pm 2.20 \times 10^6$ cells per seed, for strains Agri 10 and 1114 respectively. The higher amounts were used because of the less favorable seasonal parameters. In comparison with the first release, although the number of strains was reduced to two, the number of variables was increased by using two host plants, pea and faba bean, and by testing the effect of enhanced selective conditions for the GMM in certain selected plots. In this respect, considering that the gene cassette conferred a very high β -galactosidase activity (Corich et al., 1996; Giacomini et al., 1994), we amended individual field plots by sprinkling 4 liters of a 5% lactose solution per square meter. On these plots, the strains were released without host plants. A further control consisted of plots of bare soil not supplemented with lactose. The experimental scheme is summarized in Table 4, in which the results of two subsequent monitoring plantings, at 15 and 50 days after release, are shown. Both the parental and the GMM strain showed a persistence about 2 log higher than that seen for strain 1003 in the previous release. Their colonization of soil and rhizosphere appeared to be stable 50 days from sowing. The genetic stability of the GMM as judged by maintenance of marker phenotypes in 100 colonies from the different treatments, ranged from 96.5 to 100%, with no differences between the day 15 and day 50 sampling times. As regards the effects on indigenous microbiota, the data were gathered at the same time intervals, and are shown in Table 5. The Chi-square test verifying the hypothesis of equal proportions was run as in the case of the first release. Again the soil and plant assemblies were

Table 5. Autochthonous GMM impact: resident culturable microbiota in pea rhizosphere soil assessed 50 days after strain release.

	not inoculated	Agri10	1114
Faba bean			
Bacterial total count	$8.71 \times 10^7 \pm 1.97 \times 10^7$ a	$1.73 \times 10^8 \pm 8.58 \times 10^7$ a	$1.52 \times 10^8 \pm 3.51 \times 10^7$ a
fungi	$5.12 \times 10^4 \pm 6.80 \times 10^3$	$6.45 \times 10^4 \pm 1.67 \times 10^4$	$1.35 \times 10^5 \pm 2.40 \times 10^4$
<i>Streptomyces</i>	n.d.	n.d.	n.d.
Fluorescent pseudomonads	$5.55 \times 10^5 \pm 2.85 \times 10^5$ b	$2.00 \times 10^6 \pm 9.58 \times 10^5$ b	$1.27 \times 10^6 \pm 2.40 \times 10^5$ b
Spore-forming bacteria	$4.58 \times 10^6 \pm 1.15 \times 10^6$	$5.16 \times 10^6 \pm 9.94 \times 10^5$	$1.49 \times 10^7 \pm 8.25 \times 10^6$
<i>Rhizobia</i>	$4.03 \times 10^6 \pm 1.24 \times 10^6$ c	$1.10 \times 10^7 \pm 6.01 \times 10^6$ c	$9.36 \times 10^6 \pm 2.87 \times 10^6$ c
Pea			
Bacterial total count	$1.61 \times 10^8 \pm 2.58 \times 10^7$ d	$1.73 \times 10^8 \pm 5.45 \times 10^7$ d	$1.54 \times 10^8 \pm 3.65 \times 10^7$ d
fungi	$1.39 \times 10^5 \pm 4.68 \times 10^3$	$7.69 \times 10^4 \pm 4.62 \times 10^4$	$9.21 \times 10^4 \pm 1.60 \times 10^4$
<i>Streptomyces</i>	n.d.	n.d.	n.d.
Fluorescent pseudomonads	$1.13 \times 10^6 \pm 1.72 \times 10^5$ e	$5.77 \times 10^6 \pm 1.39 \times 10^6$ e	$3.06 \times 10^6 \pm 8.47 \times 10^5$ e
Spore-forming bacteria	$6.79 \times 10^6 \pm 1.17 \times 10^6$ f	$6.97 \times 10^6 \pm 1.11 \times 10^6$ f	$6.05 \times 10^6 \pm 3.25 \times 10^6$ f
<i>Rhizobia</i>	$2.32 \times 10^7 \pm 6.62 \times 10^6$ g	$4.27 \times 10^7 \pm 1.88 \times 10^7$ g	$2.04 \times 10^7 \pm 4.74 \times 10^6$ g
Soil			
Bacterial total count	$3.30 \times 10^7 \pm 1.55 \times 10^7$	$3.49 \times 10^7 \pm 7.03 \times 10^6$	$3.93 \times 10^7 \pm 1.66 \times 10^7$
fungi	$8.31 \times 10^4 \pm 1.48 \times 10^4$	$8.43 \times 10^4 \pm 4.60 \times 10^3$	$5.03 \times 10^4 \pm 1.41 \times 10^4$
<i>Streptomyces</i>	$3.97 \times 10^6 \pm 9.57 \times 10^5$	$3.41 \times 10^6 \pm 1.03 \times 10^6$	$2.70 \times 10^6 \pm 1.56 \times 10^6$
Fluorescent pseudomonads	$8.94 \times 10^3 \pm 1.08 \times 10^3$	$4.73 \times 10^3 \pm 1.35 \times 10^3$	$4.65 \times 10^3 \pm 1.09 \times 10^3$
Spore-forming bacteria	$1.84 \times 10^7 \pm 8.15 \times 10^6$	$1.75 \times 10^7 \pm 4.11 \times 10^6$	$2.28 \times 10^7 \pm 8.96 \times 10^6$
<i>Rhizobia</i>	$6.02 \times 10^5 \pm 8.77 \times 10^4$ h	$5.58 \times 10^5 \pm 6.55 \times 10^4$ h	$3.51 \times 10^5 \pm 4.80 \times 10^4$ h
Soil + lactose			
Bacterial total count	$3.23 \times 10^7 \pm 6.28 \times 10^6$	$5.17 \times 10^7 \pm 3.56 \times 10^7$	$2.81 \times 10^7 \pm 4.82 \times 10^6$
fungi	$6.81 \times 10^4 \pm 1.75 \times 10^4$	$5.17 \times 10^4 \pm 9.27 \times 10^3$	$4.46 \times 10^4 \pm 7.72 \times 10^3$
<i>Streptomyces</i>	$2.94 \times 10^6 \pm 3.32 \times 10^5$	$2.21 \times 10^6 \pm 3.82 \times 10^5$	$1.96 \times 10^6 \pm 6.86 \times 10^5$
Fluorescent pseudomonads	$4.57 \times 10^3 \pm 1.28 \times 10^3$	$3.27 \times 10^3 \pm 5.35 \times 10^2$	$1.29 \times 10^3 \pm 4.30 \times 10^2$
Spore-forming bacteria	$2.11 \times 10^7 \pm 3.95 \times 10^6$	$1.63 \times 10^7 \pm 6.26 \times 10^6$	$1.96 \times 10^7 \pm 6.32 \times 10^6$
<i>Rhizobia</i>	$2.11 \times 10^6 \pm 4.75 \times 10^5$ h	$1.94 \times 10^6 \pm 6.53 \times 10^5$ h	$1.96 \times 10^6 \pm 6.82 \times 10^5$ h

Values labelled with the same letter indicate data used for the analysis of a given variable. a: $P = 0.0243$, b: $P = 0.056$, c: $P = 0.0497$, d: $P = 0.0474$, e: $P = 0.0073$, f: $P = 0.0024$, g: $P = 0.0013$, h: $P = 0.0107$, report the P values obtained upon a one-way chi square test for equal proportions run within the three column plots of the same plant (*V. faba* a-c; *P. sativum* d-g) or within all the soil plots (h) on the fourth root of the raw data.
n.d.: Not determined.

tested both in combination and separately. When testing differences within the faba bean plots, significant values arose for total bacteria, and borderline values for rhizobia and pseudomonads. For peas, a higher level of significance involved total bacteria, rhizobia, pseudomonads, and spore-forming bacteria. The proportions of the latter appear reduced, giving the indication of a possible negative impact, which was however associated with both the GMM strain 1114 and its non-GMM control Agri10.

In order to refine the information relative to the effects on closely related organisms, besides the congo red YMA plate counts of overall rhizobia, we determined the most probable numbers of *Sinorhizobium meliloti* by alfalfa

nodulation tests with dilution series of the rhizosphere soil from pea and faba bean. The results (not shown) indicate the absence of effect. The low numbers, comparable to, or even lower than those previously obtained for these soils, also reveal that the two heterologous host plants (specific for the viciae biovar of rhizobia) did not seem to have a stimulatory rhizosphere effect on *S. meliloti*.

Self-impact: the effects in nature of an autochthonous GMM on its own wild-type

There is however one soil micro-organism on which the introduction of the released strains Agri 10 and 1114 was

inevitably bound to have an effect. That is their natural wild-type parental strain. Our setup made it possible to evaluate the outcome of an encounter between the inoculated strains and their indigenous, hitherto dominant, equivalent (the wild-type parent). The ecological question behind such analysis was whether a massively released, competitive *Rhizobium* would have displaced its resident counterpart, along with other natural strains, or whether, on the contrary, the plant would have nevertheless imposed the preservation of a share for diverse rhizobial strains in terms of nodule occupancy.

The GMM effect on its parent version could have been exerted mainly as competition for nodulation, which was the strain's most evident feature. In order to assess to what extent inoculating plants with an amount of 6×10^6 cells per seed of strain 1114 would result in substitutive out-competition or, instead, in an additive effect, 60 faba bean nodules were screened for strain identity by RAPD fingerprinting and marker resistance. In the uninoculated control the parent dominant strain occupied 40% of the nodules. The remaining 60% was split between several (about 15) different natural strains, each present at low frequencies ranging from 0.5 to 5%. In the inoculated plots, the GMM derivative took 50% of the nodules, its resident parent was still able to nodulate 15%, (*i.e.* one third of the remaining 50%), while the final 35% were occupied by the other natural profiles.

Long-term persistence of the GMM autochthonous strain 1114

The survival of the introduced GM strain 1114 was assessed by periodical monitoring, which was extended in particular in nine soil plots, in triplicate replicates, constituting the three above described treatments (inoculum on pea seed, or on bare soil, or on bare soil amended with lactose). Analyses were carried out through a period of nine years (1995–2004), *i.e.* until the last detectable GMM cell had disappeared from all plots. Figure 1 shows the GMM strain dynamics expressed as rifampicin and mercury resistant CFUs per gram of soil dry weight. The first three data sets (15, 50, and 150 days after inoculation) refer to the release season itself. In these, the values come either from rhizosphere soil (pea-sown plots) or from plain soil (plots with bare soil or bare soil plus lactose). In the subsequent period, in order to keep rhizobia within monitoring range, we planted peas again in the spring of 1996, 1997, and 1999. This operation was intended to provide to all plots periodical bursts of enhancement for the rhizobia. The plants were meant to act as general amplifiers for *R. leguminosarum* populations in all plots. The practice made it possible to sustain detectability of GMM strains through time, and to follow their dynamics in relation to the three initial differential

treatments. The data from day 210 onwards are therefore all from pea rhizosphere soil, including the plots in which the strain had initially been released on soil without plants. This plant-enhanced monitoring strategy also allowed assessment of the extent of nodulation by the persistent GMM.

When comparing plots where strain 1114 was released with peas with those where it was dispensed in soil without plants, it is clear that the initial plant effect was clearly supportive. The strain in bare soil showed lower values, and even when plants were added in the following seasons it could never be rescued back to numbers comparable to those found in the plot where pea was present right from the beginning. During the fourth year, the numbers in the bare soil plot fell below the detection limit. A very interesting result was observed in the three replicates of the plot where unsown soil had been amended with lactose (4 L.m^{-2} of a 5% lactose solution). This treatment was intended to test the possible selective advantages given by a strong β -galactosidase activity, which was part of our introduced marker gene cassette. In fact, *lacZ* in pDG3 is driven by a synthetic consensus promoter that confers a greater than hundred-fold increase over the basal activity level (Giacomini et al., 1994). A remarkably positive effect was observed, as the strain initially suffered only a moderate decrease compared to the dramatic one seen in the adjacent plain bare soil. Rhizobia of strain 1114 were thus able to overwinter at stable numbers in soil, *i.e.* above 10^4 per gram, which is just one log lower than the numbers obtained at the same date around aged pea roots in the plant plot (Fig. 1). The recovery promoted by plants in the following season boosted the rhizobia from the lactose-amended plots to numbers far higher than those that had survived in the presence of plants. Also, the sampling of the two following dates demonstrated that even three years later, the initial nutritional advantage allowed strain 1114 to survive in the long run much better than having been exposed to its symbiotic host.

The decline below detection levels of the introduced GMM 1114 in the release site appears to have occurred between spring 1999 and summer 2002, at which stage a new round of pea cultivation and the analysis of 581 new nodules from the three treatments' plots did not produce any isolate with the *lac-mer* phenotypic markers of strain 1114. Interestingly, RAPD PCR typing of all isolates (Polone et al., in preparation) showed that even the original parent strain of Agri10 and 1114 was no longer dominant in the field, having dropped to 1.33% of the nodule occupancies. Therefore the disappearance of the GMM is also to be viewed as part of a general substitutional succession of rhizobial populations, which became evident in the seventh year after the initial assessment. In order to test for possible back-fluctuations and

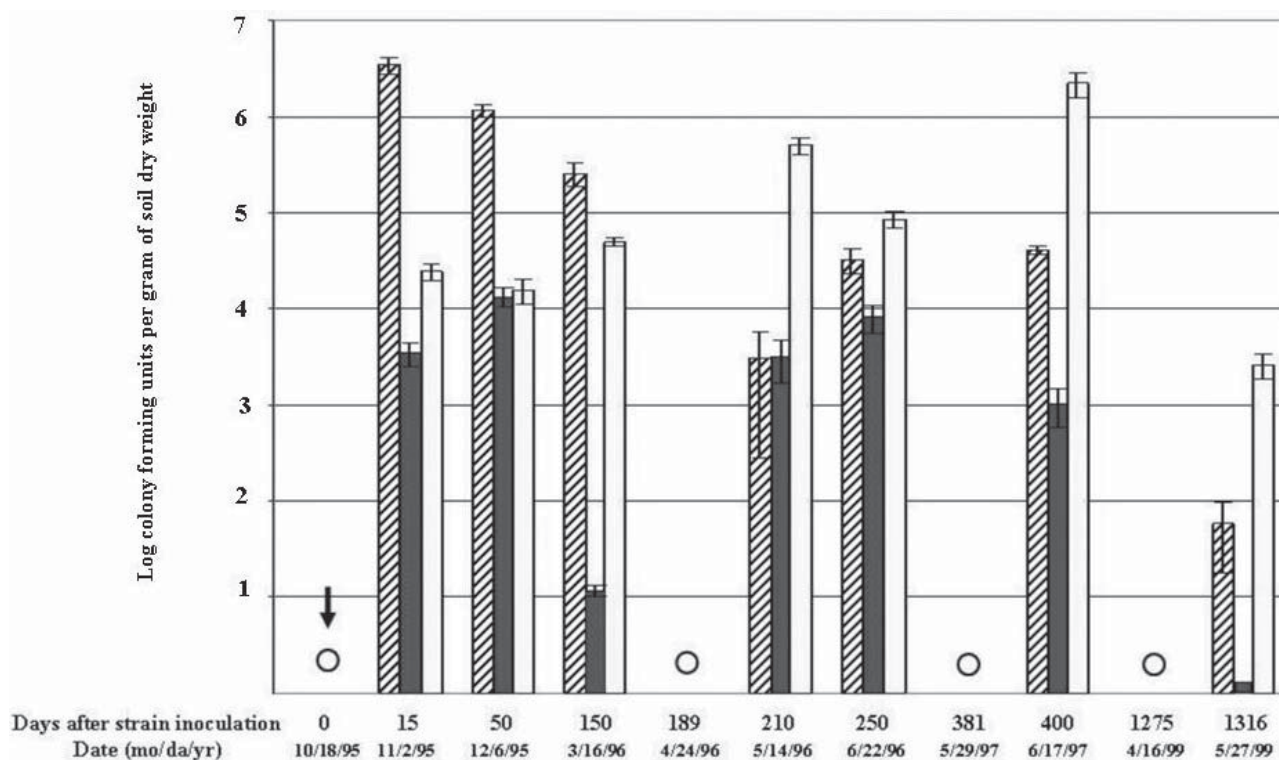


Figure 1. GMM dynamics through time. The Y-axis reports, in logarithmic scale, *Rhizobium leguminosarum* strain 1114 recoverable colony forming units that maintained rifampicin and Hg resistance markers. Data are expressed as the log of CFUs per gram of soil (dry weight) collected from the plots. Time data points are on the X-axis. The last monitored point, falling in the ninth year since release of strain 1114 (June 2004) was not plotted, since detectable GMM CFUs were no longer present in any of the plots. The arrow indicates the time of strain release. Open circles symbolize pea sowing. Histogram bars are means of values from three replicates. Hatched bars: initial release on pea seed. Solid bars: initial release on bare soil. Open bars: initial release on bare soil previously irrigated with a 5% lactose solution. The last value for bare soil (solid middle bar at day 1316) is not drawn to scale; it corresponds to the fall of the CFUs below the detection limit for this plot.

persistence in non-symbiotic conditions, peas were sown and the rhizosphere in the three treatments was again sampled in the tenth year since release, in late spring 2004. Strain 1114 was not detectable.

Nodulation was also monitored through the years (Tab. 6). Interestingly, the GMM, which had performed competitively on the plants where it was released (55% on pea and 52% on faba bean), persisted the following spring in a relatively competitive fashion. At that time it occupied 44% of the spring cultivar nodules in the plot where peas were previously present, 36% of nodules of plants sown in the soil-lactose plots, and 11.7% of nodules in the previously bare soil plots. However, while the introduced plasmid was almost fully retained (99%) during the first winter nodulation, when peas were sown again in all plots in the subsequent years, its stability showed a marked decrease, as all *rif^r* cases isolated from nodules of the peas and bare soil plots, were devoid of pDG3 (as verified by the absence of mark-

ers and by plasmid profiling). Only in the lactose plot could the plasmid still be found associated to 5% of the nodule-rescued *rif^r* colonies. The competitive contribution to nodulation by strain 1114 decreased in years in parallel with the drop of its rhizosphere persistence. Data from plants sown in the fourth year show that only in the plot initially amended with lactose were there still enough GMM cells to yield detectable pea nodulation, with a frequency of 0.5%. No nodules from plants of the other plots contained the GMM strain. Nevertheless, despite the low nodule score, all these cases had retained the *LacZ Hg^r* plasmid. This may correspond to a strategy aiming at keeping the genes for heterotrophic survival (*lacZ*), throughout a period when the opportunity for host plant symbiosis was encountered only every other year.

Besides nodules, plasmid stability was assessed also in the isolates from bulk soil and rhizosphere soil. Interestingly, the introduced plasmid appeared practically stable (90–100%), regardless the treatment and the time

Long term evaluation of GM rhizobia

Table 6. Autochthonous GMM release: nodulation dynamics and post-nodulation genetic stability over a seven year time course. The percent nodule occupancy of the parental and GMM strain during periodical legume sowings after strain release is indicated. The plasmid stability % (*in italics*) for the GMM strain is measured as marker presence in bacteria rescued from surface sterilized nodules.

Initial treatment of plots (October 1995)	Strain					
	Agri 10 (rif ^r parental)			1114 (rif ^r Hg ^r lacZ ⁺⁺ GMM)		
	Peas sown	Bare soil	Bare soil + lactose	Peas sown	Bare soil	Bare soil + lactose
Winter pea crop pre-flowering (December 1995)	67%	(no plants)	(no plants)	55% (<i>99%</i>)	(no plants)	(no plants)
New pea crop pre-flowering (May 1996)	32%	23%	31%	44% (<i>0%</i>)	11.7% (<i>0%</i>)	36% (<i>5%</i>)
New pea crop pre-flowering (June 1997)	0%	0%	0%	0%	0%	3% (<i>100%</i>)
New pea crop pre-flowering (May 1999)	n.d.	n.d.	n.d.	0%	0%	0.5% (<i>100%</i>)
New pea crop pre-flowering (June 2002)	n.d.	n.d.	n.d.	0%	0%	0%

n.d.: Not determined.

from release, up to the last instance where strain 1114 was encountered (May 1999). This in-soil genetic stability is remarkably different from the one observed for the corresponding nodulating isolates described above, in which, already after the first spring round of symbiosis, only 0–5% of the nodule-associated 1114 had retained plasmid pDG3 (Tab. 6).

DISCUSSION

The first consideration regards the fate of the strain of foreign origin. Notwithstanding its proficiency in its land of isolation or in axenic soil, when released in our station its performance was poor, both in terms of symbiosis and persistence. The new environment and the competition with resident rhizobia appeared to severely affect its outcome. Moreover, the three GMM versions displayed differential stability of the markers, depending on the position of these within the genome and on their gene regulation. Such behavior confirms in soil the genetic stability we observed with the same strains within inoculant packages in the prior long-term (500 days) microcosm trial (Corich et al., 1996). Examples of variable persistence of GM rhizobia are found in the literature (Hirsch, 2004) and in some instances such behavior is site-dependent. An hitherto persistent strain of *R. leguminosarum* bv. *viciae*, carrying a transposon disappeared two weeks after release from an eutric cambisol in Dijon (Hirsch, 1996). A genetically modified strain of *S. meliloti* carrying an exogenous glucuronidase and increased expression of its proline dehydrogenase dropped

by five orders of magnitude in five months in a spanish soil (Van Dillewijn et al., 2001). The low persistence of a released *Rhizobium* is not necessarily linked to its being genetically impaired. For example, a recA-minus derivative of *S. meliloti* showed a stability similar to that of its wild-type, as it declined from 10⁶ to 10⁴ cfu.g⁻¹ of soil in two years time (Schwieger and Tebbe, 2000). Climatic conditions such as heavy rain after release negatively affect persistence (Hirsch, 1996), and so do cold winters, as shown in the boreal zone for GM *R. galegae* (Pitkajarvi et al., 2003). Besides survival, in terms of nodulation, released GM rhizobia, like all symbiotic inoculants, face the competition of indigenous members of the same species, as shown quantitatively for GM *S. meliloti* (Miethling and Tebbe, 2004).

Concerning impact, the following considerations can be made. While no major effect was expected by the poorly persisting allochthonous strain, on the contrary, the dominant indigenous one was in a better position to create potential disturbance. However, notwithstanding the ecological success that was maintained by the GMM derivative, neither its massive inoculation nor the presence of recombinant traits resulted in major perturbations on numbers of culturable soil inhabitants within the groups tested. The significance of some of the differences recorded is however informative of possible impacting interactions caused by rhizobial inoculants themselves (either GM or not) that should be elucidated better by studies at the qualitative level.

On the issue of impact, it must also be stressed that the genetic traits introduced were not of an inherently

harmful nature. Moreover, the limited power of single point quantitative CFU counts is in itself a strategy prone to overlook many subtle changes. There are no examples in the literature of negative impacts due to GM rhizobia yet, while in some cases a beneficial effect on mycorrhizal formation has been recorded with GM *S. meliloti* improved for its nodulation competitiveness (Tobar et al., 1996).

The absence of a strong “self-impact” on the GMM’s parental kin constitutes one of the interesting results stemming from these trials. An already dominant strain, even when further inoculated in high numbers, did not displace in a proportional manner either its parent or the other minority profiles from the final nodule score. Indeed the percentage of nodules cumulatively taken by the dominant parent plus its GMM version, reached 65%. Within this figure the contribution of the original parent strain alone is 15%. The remaining 35% of the nodules contained a series of strains with different profiles (about 13) each present with frequencies from 3 to 6%. Therefore there was not a straight additive effect but rather a partial competitive substitution of the soil dominant strain by its massively inoculated counterpart. It is very interesting to note that such inoculum was able to substitute both its parent and the other rhizobia to the same extent, leading to a 25% decrease for each (from 40% to 15% for the wt parent and from 60% to 35% for the other profiles). From MPN data we also know that the efficient indigenous strain was part of a natural soil *Rhizobium* population of around 5.6×10^5 cells per gram of soil dry weight. Considering its success even when starting from these numbers, one could have expected that adding a large inoculum of its GMM derivative (6×10^6 cells per seed), would result in an overwhelming competitive effect. However, the analysis shows that even when saturated with a very infective strain the plants still ended up with a fair proportion of the nodules induced and invaded by a variety of resident, less competitive, *R. leguminosarum* bv. *viciae* strains. Moreover, a good share of nodules was still accessed by the soilborne dominant parent. This result provides insight into the general mechanisms ruling rhizobial competition for nodulation. It could be postulated that the plant may play an active role in controlling the strain diversity of the bacteria eventually admitted in its nodules, irrespective of their numbers in soil or rhizosphere. The phenomenon is in line with previous reports dealing with alfalfa rhizobia (Paffetti et al. 1998).

In the long term persistence analysis of strain 1114, we observe that the initial lactose supplementation was positively correlated with its survival in the plots. The effect appears superior also to that of an initial presence of the host. We observe that in terms of recoverable colony forming units up to four years after the

release, trophic selection proved at least ten-fold more effective than plant host presence, and nearly a hundred-fold superior when compared to totally neutral environmental conditions consisting in the bare soil neither sown with legumes nor supplemented with lactose. This effect is unlikely to be caused by persisting resource, as lactose is supposedly degraded shortly after supplementation, but rather by the lasting effect of its nutritional support to the lac-proficient populations during the first winter after release. The superior effect of lactose supplementation is possibly explained by the fact that lactose enrichment was diffused through the whole soil volume, while the pea rhizosphere effect was limited to the network explored by root paths. In surveys on genetically modified *S. meliloti*, although not endowed with extra catabolic genes, strain persistence was found positively correlated with soil organic matter content (Da and Deng, 2002). More specifically, the issue of ecological selectivity in relation to released GMMs has been thoroughly addressed by van Veen et al. (1997). The persistence of rhizobia bearing genetic modifications has been related to many factors mainly regarding soil type, presence of host legume and competing co-specific strains (Hagen et al., 1997). In relation to lactose, microcosm soil trials performed in pea rhizospheres with *Pseudomonas fluorescens* carrying *lacZY*, during a 21 days incubation, showed that the substrate could enhance the GMM recovery by 0.3 of a log unit (Naseby and Lynch, 1998).

Coming to the issue of genetic stability, we observed (Tab. 6) that the recombinant plasmid was lost rapidly upon nodulation, while being retained stably by the GMM persisting in the soil. However, this is compatible with our previous results on *in-* and *ex-planta* genetic stability; in our prior investigations (Corich et al., 2001a) we reported that introduced plasmids suffered high frequency losses during spring pea nodulation by *R. leguminosarum* bv. *viciae*, and were in contrast maintained by cells remaining in the rhizosphere. In another report (Corich et al., 2001b), focusing on large natural *Rhizobium* plasmids, we studied the extent of plasmid loss and deletion in relation to nodulation. Large natural plasmids were much more stable than small, introduced plasmid vectors. The phenomenon appeared to be linked to nodulation, since the same vectors were nearly 100% stable during *in vitro* growth for over 200 generations of the rhizobia. In the present paper we also noticed (Tab. 6) that in the winter pea (sown October 1995), nodulation did not affect plasmid stability. This suggests that possibly cultivar- or spring temperature-related effects may be playing a role in destabilizing introduced replicons during host invasion.

In conclusion, interesting lessons on *Rhizobium* environmental dynamics can be drawn from these data. A trophic gene-for-substrate advantage, even with a single

instance of availability, can result in a long-term positive selection even stronger than that of plant symbiosis, and this can benefit future nodulation. Such knowledge is of prime interest for the design of improved agricultural inoculants and for strategies of competition *versus* indigenous strains.

A plasmid-curing effect, exerted particularly upon introduced replicons, during spring nodulation is confirmed.

Summarizing the effects on the assessment of environmental impact, we observe that, as far as our methods could reveal, neither the ecologically less fit GMM strain that was released first, nor the more adapted competitive autochthonous version, affected the microbiota in the site of introduction in ways that could be related to the genetic modification. Considering the nature of the released bacterial species, and the neutral incidence of the introduced genes in the chosen habitat, the absence of GMM-related impact is in line with the expectations. However, the small but statistically detectable alterations in the counts of some soil groups, observed particularly after the release of the locally dominant strain, indicate that a certain degree of impact on the soil communities can be exerted by the rhizobial inoculants themselves, irrespective of whether they are genetically modified or not.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Strains are listed in Table 1. *E. coli* was grown at 37 °C on Luria Broth LB (1% NaCl, 1% Bacto tryptone, 0.5% yeast extract). Rhizobia were routinely grown in Tryptone-Yeast, TY (Berlinger, 1974) or Yeast Mannitol, YM (Vincent, 1970). When appropriate, media were supplemented with 30 µg.mL⁻¹ (*E. coli*) or 60 µg.mL⁻¹ (*Rhizobium*) of rifampicin, and 3–5 µg.mL⁻¹ (*E. coli*) or 10 µg.mL⁻¹ (*Rhizobium*) of HgCl₂, 20 µg.mL⁻¹ isopropyl thiogalactoside (IPTG), and 20 µg.mL⁻¹ bromo-chloro-indolyl galactopiranoside (X-gal).

For the detection of indigenous soil culturable microbiota, different groups were selected with the following media and conditions. Aerobic bacterial population: Plate Count Agar (Difco, Detroit, Michigan) supplemented with 50 µg.mL⁻¹ cycloheximide; plates were incubated for 3 days at 28 °C. Spore forming bacteria: after heating the soil suspensions at 80 °C for 10 minutes, these were plated on Nutrient Agar (Difco, Detroit, Michigan) supplemented with 5 g.L⁻¹ glucose and 50 µg.mL⁻¹ cycloheximide; plates were incubated for 3 days at 28 °C. Microfungi: Potato Dextrose Agar pH 3.5 (Difco, Detroit, Michigan); plates were incubated

for 3 days at 28 °C. Streptomycetes: Complete Streptomyces Medium (CSM) (7 g.L⁻¹ glycerol, 0.7 g.L⁻¹ L-asparagine, 0.7 g.L⁻¹ K₂HPO₄, pH 7); plates were incubated for 14 days at 28 °C. Fluorescent pseudomonads: modified King's B medium (20 g.L⁻¹ proteose peptone, 10 g.L⁻¹ glycerol, 1.5 g.L⁻¹ KH₂PO₄, 1.5 g.L⁻¹ MgSO₄.7H₂O, pH 7.2), supplemented with 100 µg.mL⁻¹ cycloheximide, 50 µg.mL⁻¹ carbenicillin, 13 µg.mL⁻¹ chloramphenicol; plates were incubated for 2 days at 28 °C. Rhizobia: YM medium supplemented with 50 µg.mL⁻¹ cycloheximide and 25 µg.mL⁻¹ congo red; plates were incubated for 2 days at 28 °C. Media were solidified with the addition of 16 g.L⁻¹ agar.

Nodulation tests in axenic soil

Ten kg of soil from the release site were sieved and blended. The soil was subsequently sterilized under a vapor current by leaving it for 1 hour in an unpressurized autoclave at 100 °C. The operation was repeated after two days, to eliminate the progeny of germinated spores. Soil was transferred into 18 cm diameter clay pots. Three surface-sterilized pea seeds were sown per pot and inoculated with a suspension of 10⁸ bacterial cells per seed. Plants were incubated in a growth cabinet under the following program: 14 h light/23 °C, 10 h dark/18 °C, with a 70% relative humidity. Roots were inspected for nodules after 30 days.

Field studies: Most Probable Number (MPN) evaluation of rhizobia

The estimation of indigenous background counts of rhizobia in the field was carried out by serially diluting soil and performing nodulation tests up to the extinction limits as described by Bergersen (1980) for *R. leguminosarum* bv. *viciae*, *R. leguminosarum* bv. *trifolii*, *Sinorhizobium meliloti* and *Bradyrhizobium japonicum*, using respectively *Pisum sativum* cv. Curico, *Trifolium repens* cv. Dutch White, *Medicago sativa* cv. Roma, and *Glycine max* cv. Amelia as host legumes. Tests were carried out in 50 mL test tubes (or 200 mL Erlenmeyer flasks for peas) with agarized Fahraeus medium (Bergersen, 1980).

Field releases

The release took place on may 11th 1994 in Legnaro, 10 Km south-east of Padua within the experimental field facilities of the Faculty of Agriculture. The soil was of clay type (41% clay, 20% loam, 39% sand) with 3.65% organic matter, and pH 7.9 (Corich, 1995). The

plots are located in open terrain, and the soil had been laid in place in 1989 by mixing sand, peat and clay components of allochthonous origin. No other crops had been cultivated in these units prior to the present experiment. The soil was weeded and tilled in preparation for the trial. Pea or faba bean plants were sown in a grid of 16 field plots in three replicates, receiving either one of the three GMMs, or the wild-type. Controls included three replicates of uninoculated peas, and one plot of uninoculated unsown soil. Each plot had an area of 1 m², whose perimeter was framed by a 1-m deep, 2-cm thick concrete wall. Eighty pea seeds per plot were hand sown in ten rows at a depth of 4 cm in pre-drilled holes. Seeds in the same row were spaced 10 cm apart, rows were spaced 8 cm from each other, and border rows were at 10 cm from the plot's edge. Three replicate plots per treatment were set up. The bacterial inoculum consisted of 1 mL of cell suspension in saline solution containing amounts on the order of 10⁶ colony-forming units (CFUs), and was pipetted in the soil hole before placing each seed. Each block was kept covered with a wire mesh until plant emergence to protect seeds from rodents. The site was fenced, trespassing was warned against by appropriate signs, and the area was covered with a loose net to prevent birds from feeding and carrying over seeds and bacteria. In order to further ensure soil confinement of the GMMs and to test their lateral spreading potential, the site was planted with a surrounding double belt of pea host plants meant to act as trap crop to intercept and reveal possible horizontal escape of GMMs. Selected plots were watered with 4 L.m⁻² of a 5% lactose solution, before bacterial release.

Sampling GMMs from rhizosphere and from soil

Rhizosphere-associated bacteria were isolated as follows: plants were carefully extracted and excess soil was roughly shaken off roots, further non-rhizospheric soil was eased off with a spatula, leaving only the most proximal layers of soil closely adhering to the surface. Roots were subsequently immersed in 50 mL of saline solution in Erlenmeyer flasks, which were shaken at 150 revolutions.min⁻¹ for 2 h. A final fast agitation was given by hand for 1 min. Serial dilutions, the first of which was vortexed for one minute, were plated. Soil dry weight was measured after drying soil from the suspension in tin trays at 80 °C for 72 h in a dry oven.

In order to isolate bacteria from bare soil, 1 kg was randomly collected by sequential random picks at a maximum depth of 15 cm. 20 g of sieved, mixed soil were transferred to a beaker, and saline solution was added up to a volume of 200 mL, and mixed for 1 h with a magnetic stirrer. Serial dilutions in physiological solution

were carried out, and plated on media containing the appropriate selection. Soil was dried and weighed as above. When necessary for the detection of cells present in low amounts, less diluted suspensions were plated.

Bacterial isolation from nodules

Nodules were excised and surface-sterilized by a treatment including 5 sec in 70% ethanol and 60 sec in 1 mg.mL⁻¹ HgCl₂. Ten washes with sterile H₂O were performed prior to transferring each nodule to a microtiter well containing 80 µL of sterile saline solution. Each nodule was then squashed with forceps and dilutions of the resuspended content were plated on YM or TY plates.

Randomly Amplified Polymorphic DNA (RAPD) analysis

A loopful from a 3-day old bacterial colony was resuspended in 50 µL lysis buffer (0.25% sodium dodecyl sulphate, 0.05 M NaOH) in an eppendorf tube and vortexed for 1 min. The sample was heated at 95 °C for 15 min and centrifuged for 10 min to pellet debris. Ten µL from the supernatant were diluted into 90 µL H₂O. One µL of such dilution was added to 24 µL of PCR mix (14.9 µL H₂O, 2.5 µL 10 X amplification buffer (AB Analytica, Padova, Italy), 4 µL of a nucleotide triphosphate mix (dATP, dCTP, dGTP, dTTP, each at 1.25 mM), 2.5 µL of a 20 µM primer solution, and 0.1 units of *Taq* polymerase (AB Analytica, Padua, Italy)). The amplification program involved: 94 °C for 2 min 30 sec; 42 °C for 1 min; 65 °C for 2 min (1st cycle); 94 °C for 30 sec, 42 °C for 1 min; 65 °C for 2 min (2nd to 45th cycle); followed by 72 °C for 5 min. Amplified fragments were analyzed by electrophoresis in 1.2% agarose gel.

Soil DNA extraction and GMM-specific PCR amplification

Three plants per plot were randomly selected, avoiding the border rows. Rhizosphere soil was isolated as described above for the sampling of GMMs, and the soil suspension was pelleted at 22 100× *g*, weighed and resuspended in H₂O to a concentration of 0.5 g.mL⁻¹. When bulk soil was tested, no prior treatment was done before resuspension. The extraction was performed on volumes containing 1 g of soil, with the method of Tas et al. (1995). Briefly, 1 g of soil was suspended in 2.5 mL of lysis buffer (0.12 M Na₂HPO₄ [pH 8.0], 1% [wt/vol] sodium dodecyl sulfate [SDS], 0.1 mg of proteinase K per mL) and incubated at 37 °C for 1 h with occasional shaking; 450 µL of 5 M NaCl were then

added to the samples and mixed thoroughly. Next, 375 μL of CTAB-NaCl solution (10% cetyltrimethylammonium bromide in 0.7 M NaCl) were added to the mixture, which was carefully mixed again and incubated at 65 °C for 20 min. The samples were then extracted with an equal volume of chloroform. A 0.5-mL volume of Phase Lock Gel was added (5 Prime 3 Prime, Inc., Boulder, CO). The aqueous phase was transferred to a fresh test tube, and DNA was precipitated with 0.7 volume of isopropanol. The pellet was dried under vacuum, and dissolved in 200 μL of sterile distilled water.

PCR amplification using two specific 20-mer primers, the first matching the synthetic promoter domain, the second within the *lacZ* gene, (GAAGCGATAATGCGGTC-GAC, and GGTATAATATGCGCCATTAAGC) was carried out with a program based on the one described above for the RAPD technique, with the modification of the temperatures of annealing and elongation, set at 57 °C and 72 °C, respectively. Amplified products were analyzed by electrophoresis in 1.2% agarose gel.

Plasmid profiles

The plasmid pattern of rhizobia was visualized according to Eckhardt (1978) with the modifications introduced by Espuny et al. (1987) consisting in a horizontal run with a longitudinal strip of SDS-containing gel behind the wells.

Construction of strains Agri10 and 1114

The strain chosen as background, which was the most frequent RAPD profile among pea-nodule occupants, was grown in TY broth, and cells were plated on TY agar supplemented with rifampicin in order to select spontaneous resistant mutants. This selection yielded strain Agri10. Strain 1114 was obtained by inserting plasmid pDG3, featuring a mercury and *lacZ* cassette under the control of a synthetic promoter, on a broad host range incQ vector (Giacomini et al., 1994) into the rifampicin resistant strain Agri 10. The plasmid was transferred from *E. coli* to *R. leguminosarum* by triparental conjugation as previously described (Corich et al., 1996). Briefly, overnight 10 mL liquid cultures of the donor, the recipient and the helper HB101 pRK2013 were centrifuged, resuspended in 50 μL of saline solution and mixed on TY plates. After an overnight incubation at 30 °C, the patch was resuspended, serially diluted in saline solution and plated on selective plates containing 10 $\mu\text{g}\cdot\text{mL}^{-1}$ mercury and 60 $\mu\text{g}\cdot\text{mL}^{-1}$ rifampicin.

Statistical analyses

Counts of resident microbiota after GMM release were analyzed for impact effects by one-way chi-square test

for equal proportions by using the SAS Stat Software version 6 (SAS Institute Inc. Cary, NC). As the variance of data (square of the standard deviation) was larger than their averages, an appropriate transformation was applied prior to the analysis, and upon testing different ones, the fourth root extraction gave the finest discriminatory resolution. The fourth root is also a recommended choice for the transformation of data sets spanning orders of magnitude, as was the case with the present ones. The un-averaged values from each different replicate were transformed and fed into the SAS FREQ procedure.

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REFERENCES

- Basaglia M, Casella S, Nuti MP, Peruch U, Poggiolini S, Vian P, Vanderleyden J, De Troch P, Vameralli T, Mosca G (2003) Field release of genetically marked *Azospirillum brasilense* in association with *Sorghum bicolor* L. *Plant Soil* **256**: 281–290
- Bergersen FJ (1980) Methods for evaluating biological nitrogen fixation. John Wiley & Sons, Chichester, New York, Brisbane, Toronto
- Beringer JE (1974) R-factor transfer in *Rhizobium leguminosarum*. *J. Gen. Microbiol.* **84**: 188–198
- Corich V (1995) Immissione nell'ambiente di *Rhizobium leguminosarum* bv. *viciae* geneticamente modificati. Ph.D. thesis. University of Padova
- Corich V, Giacomini A, Concheri G, Ritzerfeld B, Vendramin E, Struffi P, Basaglia M, Squartini A, Casella S, Nuti MP, Peruch U, Poggiolini S, De Troch P, Vanderleyden J, Fedi S, Fenton A, Moenne-Loccoz Y, Dowling DN, O'Gara F (1995) Environmental impact of genetically modified *Azospirillum brasilense*, *Pseudomonas fluorescens* and *Rhizobium leguminosarum* released as soil/seed inoculants. In Jones D, ed, Biosafety results of field tests with genetically modified plants and microorganisms, Monterey Ca., USA, November 19–23 1995

- Corich V, Bosco F, Giacomini A, Basaglia M, Squartini A, Nuti MP** (1996) Fate of genetically modified *Rhizobium leguminosarum* biovar *viciae* during long-term storage of commercial inoculants. *J. Appl. Bacteriol.* **81**: 319–328
- Corich V, Giacomini A, Basaglia M, Vendramin E, Vian P, Carlot M, Squartini A, Casella S, Nuti MP** (2000) The field release and monitoring of rhizobial strains marked with *lacZ* and mercury resistance genes. In Jansson J, Van Elsas JD, Bailey M, eds, Tracking genetically engineered microorganisms, Landes Biosciences, Georgetown, TX, USA
- Corich V, Giacomini A, Basaglia M, Vendramin E, Vian P, Carlot M, Squartini A, Casella S, Nuti MP** (2001a) Aspects of marker/reporter stability and selectivity in soil microbiology *Microb. Ecol.* **41**: 333–340
- Corich V, Giacomini A, Carlot M, Simon R, Tichy H-V, Squartini A, Nuti MP** (2001b) Comparative strain-typing of *Rhizobium leguminosarum* bv. *viciae* natural populations. *Can. J. Microbiol.* **47**: 580–584
- Cullen DW, Nicholson PS, Mendum TA, Hirsch PR** (1998) Monitoring genetically-modified rhizobia in field soils using the polymerase chain reaction. *J. Appl. Microbiol.* **84**: 1025–1034
- Da HN, Deng SP** (2002) Surviving and persistence of genetically modified *Sinorhizobium meliloti* strain 104 A14 in soil. *Appl. Soil Ecol.* **22**: 1–14
- Eckhardt T** (1978) A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. *Plasmid* **1**: 584–588
- Espuny MR, Ollero FJ, Bellogin RA, Ruiz-Sainz JE, Perez Silva J** (1987) Transfer of the *Rhizobium leguminosarum* biovar *trifolii* symbiotic plasmid pRtr5a to a strain of *Rhizobium* sp. that nodulates on *Hedysarum coronarium*. *J. Appl. Bacteriol.* **63**: 13–20.
- Figurski DH, Helinski DR** (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc. Nat. Acad. Sci. USA* **76**: 1648–1652
- Giacomini A, Ollero FJ, Squartini A, Nuti MP** (1994) Construction of multipurpose gene cartridges based on a novel synthetic promoter for high-level gene expression in Gram-negative bacteria. *Gene* **144**: 17–24
- Hagen M, Pühler A, Selbitschka W** (1997) The persistence of bioluminescent *Rhizobium meliloti* strains L1 (RecA-) and L33 (RecA+) in non-sterile microcosms depends on the soil type, on the co-cultivation of the host legume alfalfa and on the presence of an indigenous *R. meliloti* population. *Plant Soil* **188**: 257–266
- Hirsch PR** (1996) Population dynamics of indigenous and genetically modified rhizobia in the field. *New Phytol.* **133**: 159–171
- Hirsch PR** (2004) Release of transgenic bacterial inoculants - rhizobia as a case study. *Plant Soil* **266**: 1–10
- Miethling R, Tebbe C** (2004) Resilience of a soil-established, genetically modified *Sinorhizobium meliloti* inoculant to soil management practices. *Appl. Soil Ecol.* **25**: 161–167
- Naseby DC, Lynch JM** (1998) Impact of wild type and genetically modified *Pseudomonas fluorescens* on soil enzyme activities and microbial population structure in the rhizosphere of pea. *Mol. Ecol.* **7**: 617–625
- Nuti MP, Squartini A, Giacomini A** (1994) European Community regulation for the use and release of genetically modified organisms (GMOs) in the environment. In O'Gara F, Dowling D, Boesten B, eds, Molecular Ecology of Rhizosphere Microorganisms, VCH Publ., Weinheim, Germany
- Nuti MP, Basaglia M, Bonfante P, Casella S, Corich V, Dal Maistro L, Giacomini A, Martini I, Peruch U, Poggiolini S, Squartini A, Vian P** (1997) Field release of genetically modified biofertilizers and phytostimulators. In Matsui S, Miyazaki S, Kasamo K, eds, Proceedings of the 4th International Symposium on the Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms, published by the Japan International Research Center for Agricultural Sciences (JIRCAS), pp 101–111
- Nuti MP, Russo A, Toffanin A, Casella S, Corich V, Squartini A, Giacomini A, Peruch U, Basaglia M** (2003) What did we learn from 24 field releases of GMMs in Italy? In Lelley T, Balasz E, Tepfer M, eds, Ecological Impact of GMO Dissemination in Agro-Ecosystems, Facultas Verlags, Wien, p 45–54
- Paffetti D, Daguin F, Fancelli S, Gnocchi S, Lippi F, Scotti C, Bazzicalupo M** (1998) Influence of plant genotype on the selection of nodulating *Sinorhizobium meliloti* by *Medicago sativa*. *Antonie van Leeuwenhoek* **73**: 3–8
- Pitkajarvi J, Rasanen LA, Langenskiod J, Wallenius K, Niemi M, Lindstrom K** (2003) Persistence, population dynamics and competitiveness for nodulation of marker gene-tagged *Rhizobium galegae* strains in field lysimeters in the boreal climatic zone. *FEMS Microbiol. Ecol.* **46**: 91–104
- Resca R, Basaglia M, Poggiolini S, Vian P, Bardin S, Walsh UF, Enriquez Barreiros CM, O'Gara F, Nuti MP, Casella S, Peruch U** (2001) An integrated approach for the evaluation of biological control of the complex *Polymixa betae*/Beet Necrotic Yellow Vein Virus, by means of seed inoculants. *Plant Soil* **232**: 215–226
- Schwieger F, Tebbe CC** (2000) Effect of field inoculation with *Sinorhizobium meliloti* L33 on the composition of bacterial communities in rhizospheres of a target plant (*Medicago sativa*) and a non-target plant (*Chenopodium album*)—linking of 16S rRNA gene-based single-strand conformation polymorphism community profiles to the diversity of cultivated bacteria. *Appl. Environ. Microbiol.* **66**: 3556–3565
- Tas E, Saano A, Leinonen P, Lindström K** (1995) Identification of *Rhizobium* in peat-based inoculants by DNA hybridization and PCR-application in inoculant quality control. *Appl. Environ. Microbiol.* **61**: 1822–1827
- Tichy H, Simon R** (1994) Genetic typing of microorganisms: Current concepts and future prospects. In O'Gara F, Dowling D, Boesten B, eds, Molecular Ecology of Rhizosphere Microorganisms, VCH Publ., Weinheim, Germany
- Tobar RM, Azcón-Aguilar C, Sanjuan J, Barea JM** (1996) Impact of a genetically modified *Rhizobium* strain with

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- improved nodulation competitiveness on the early stages of arbuscular mycorrhiza formation. *Appl. Soil Ecol.* **4**: 15–21
- van Dillewijn P, Soto MJ, Villadas PJ, Toro N** (2001) Construction and environmental release of a *Sinorhizobium meliloti* strain more competitive for alfalfa nodulation. *Appl. Environ. Microbiol.* **67**: 3860–3865
- van Veen JA, van Overbeek LS, van Elsas JD** (1997) Fate and activity of microorganisms introduced into soil. *Microbiol. Mol. Biol. Rev.* **61**: 121–135
- Vincent JM** (1970) A manual for the practical study of the root-nodule bacteria. IBP Handbook no. 15, Blackwell Scientific Publications, Oxford

Appendix 4

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PCR primers based on different portions of insertion elements can assist genetic relatedness studies, strain fingerprinting and species identification in rhizobia.

PCR primers based on different portions of insertion elements can assist genetic relatedness studies, strain fingerprinting and species identification in rhizobia

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Abstract

Using the sequence of an insertion element originally found in *Rhizobium sullae*, the nitrogen-fixing bacterial symbiont of the legume *Hedysarum coronarium*, we devised three primer pairs (*inbound*, *outbound* and *internal* primers) for the following applications: (a) tracing genetic relatedness within rhizobia using a method independent of ribosomal inheritance, based on the presence and conservation of IS elements; (b) achieve sensitive and reproducible bacterial fingerprinting; (c) enable a fast and unambiguous detection of rhizobia at the species level. In terms of taxonomy, while in line with part of the 16S rRNA gene- and glutamine synthetase I-based clustering, the tools appeared nonetheless more coherent with the actual geographical ranges of origin of rhizobial species, strengthening the European–Mediterranean connections and discerning them from the asian and american taxa. The fingerprinting performance of the outward-pointing primers, designed upon the inverted repeats, was shown to be at least as sensitive as BOX PCR, and to be functional on a universal basis with all 13 bacterial species tested. The primers designed on the internal part of the transposase gene instead proved highly species-specific for *R. sullae*, enabling selective distinction from its most related species, and testing positive on every *R. sullae* strain examined, fulfilling the need of PCR-mediated species identification. A general use of other IS elements for a combined approach to rhizobial taxonomy and ecology is proposed.

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Keywords: *Rhizobium*; IS element; Strain fingerprinting; Rhizobial genetic diversity

1. Introduction

The characterization of prokaryotes has achieved some of its most insightful tools thanks to the progressive development of molecular biology. In this respect, a major gear shift is undoubtedly represented by the rise of PCR that, among its countless applications, has led to the generation of gene- or genome-targeted strategies,

which have greatly sharpened our power of studying, comparing and sorting bacteria. Within the field of DNA-based methods, originally featuring genomic restriction digests, specific probe hybridizations and plasmid profiling (later joined by pulsed-field gel electrophoresis), the polymerase chain reaction (PCR) has added a wide array of alternatives. Some of these allow strain-level typing, such as AP-PCR [1], RAPD [2], DAF [3], AFLP [4], REP, ERIC and BOX PCR techniques [5–11]. Polymerase-mediated approaches have also given an impulse to species-level taxonomy focused on the ribosomal operon, as exemplified by ARDRA

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[12] and by the PCR-mediated adaptation to microbial ecology of previously devised techniques such as DGGE [13,14] and SSCP [15,16]. Moreover, the latter series of tools can be employed also at the community level on uncultured or unculturable pools of taxa, both for analytical comparisons and with preparative taxonomical cloning/sequencing aims. This available platform of methods allows an increasing flexibility of approaches to be chosen according to the different scopes sought. In this respect, the diverse nitrogen-fixing endosymbionts of legumes, collectively referred to as rhizobia, have been proven susceptible to studies carried out with several of the above methods [17–22]. The present study stems from observations on an insertion sequence, originally found in a *Rhizobium sullae* background, whose diffusion and conservation however turned out to be exploitable for studies either within or beyond the rhizobial branches.

A microbial ecologist dealing with species diversity can address one or more of three major aspects: (1) distinguish bacteria by a strain-level differentiating method; (2) establish to which extent the different species are related, using a suitable phylogenetic measure; (3) unambiguously track one particular taxon within a complex community. In the present paper, we have explored the possibility of simultaneously addressing all these aims by working on the same genetic segment with three different PCR-based approaches. The target is an insertion element, *ISRh1*, found hosted in the nitrogen-fixing root nodule microsymbiont of the legume *Hedysarum coronarium* [23]. We have recently described these bacteria as the novel species *R. sullae* [24]. In this report, we describe how the partial conservation of this element in other species of rhizobia can be exploited as a 16S-independent relatedness-telling device, whose results can be compared with those of canonical vertical phylogeny indicators. The study of an insertion element as an aid to support phylogeny has been considered in light of the following evidences; notwithstanding the fact that its intrinsic mobility hinders the assessment of a solely-vertical evolutionary line, this turns into an advantage when seeking the traces of time-independent horizontal gene transfer events. The element moves, but this happens via direct contact and is linked to the transfer of genetic material, bringing about an acquired relatedness. It should be added that the *nod* genes themselves, often on mobile regions of the genome, are also used for phylogeny. Indeed, as the element is found preferentially clustered close to the *nod* genes within the symbiotic plasmid [23], the method appears particularly suited to reveal evolutionarily-recent horizontal gene transfers, as opposed to ribosomal sequence divergence-based trees. In this respect, it can also be considered that, unlike *nod*-genes, the IS element is not subject to gene product structural constraints exerted by the different host plants, making it more suitable for investigating

instances of sequence divergence as a function of the time after lateral gene transfer in rhizobia.

In addition, we demonstrate how outward-pointing primers based on the same element can work in a RAPD fashion and serve the need of universal fingerprint for virtually all species. Finally, we show how a fully species-specific amplicon, detecting only *R. sullae*, can be raised from the variable inner core of the transposase of the same IS element.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacterial strains used in this work are listed in Table 1.

Rhizobia were grown on yeast-mannitol agar (YMA), for 24 h at 30 °C. *Escherichia coli* was grown on LB at 37 °C. Other species tested were handled as total DNA, supplied by the Universities of Padova and Sassari.

2.2. Oligonucleotide primers

Sequences of the primers used for PCR are shown in Table 2. *Internal primers* were defined using software Primer3 (Whitehead Institute for Biomedical Research, Cambridge, MA). The primer design scheme for the three different applications is depicted in Fig. 1.

2.3. DNA extraction

Cells were lysed by resuspending a loopful of a plate-grown isolated colony in 50 µl of lysis buffer (0.25% SDS, 0.05 M NaOH) into a 1.5-ml polypropylene tube, stirring 60 s on a vortex and heating at 95° for 15 min. The lysate was centrifuged 15 min and 10 µl of supernatant was taken and added to 90 µl of sterile water. Lysates were stored at 4 °C prior to PCR.

2.4. PCR amplification

For the amplification of the full *ISRh1*, 1 µl of lysate containing the total DNA of bacterial isolates was run in a PCR 9700 Gen-amp Cyclor apparatus (Applied Biosystems), using 1 µM of each of the *inbound* primers (Table 2). The PCR program was: initial denaturation at 95 °C for 3 min; 35 cycles at 95 °C for 45 s, annealing at 59 °C (or at one of a series of different temperatures tested, ranging from 52 to 70 °C) for 30 s, elongation at 72 °C for 90 s, and a final extension at 72 °C for 10 min. Strain fingerprinting was performed using 1 µM of each of the *outbound* primers *ISRh1out22F* and *ISRh1out22R* (Table 2). The PCR program was as described for *inbound* primers, but with annealing at

Table 1

Bacterial strains. The geographical origin of the isolation of *R. sultae* strains is indicated

Bacterial strains	Source
<i>R. sultae</i> IS123 ^T (Southern Spain)	University of Padova
<i>R. sultae</i> RH44 (Southern Spain)	University of Padova
<i>R. sultae</i> HCNT1 (Volterra, Italy)	University of Padova
<i>R. sultae</i> RHF (Pisa, Italy)	University of Padova
<i>R. sultae</i> RH100 (Balearic Islands)	University of Padova
<i>R. sultae</i> HCPT1 (Volterra, Italy)	University of Padova
<i>R. sultae</i> VIL 3-2 (Villamagna, Italy)	University of Padova
<i>R. sultae</i> VIL 2 (Villamagna, Italy)	University of Padova
<i>R. sultae</i> HCNA (Volterra, Italy)	University of Padova
<i>R. sultae</i> 30-21 (Volterra, Italy)	University of Padova
<i>R. sultae</i> HCS2 (Volterra, Italy)	University of Padova
<i>R. sultae</i> HA7 (Volterra, Italy)	University of Padova
<i>R. sultae</i> 835 (Southern Spain)	University of Padova
<i>R. sultae</i> A6 (Constantine, Algeria)	University of Padova
<i>R. sultae</i> CSPM1 (Ottava, Sardinia, Italy)	ISPAAM Sassari
<i>R. sultae</i> 1Ab (Escolca, Sardinia, Italy)	ISPAAM Sassari
<i>R. sultae</i> 2Aa (Escolca, Sardinia, Italy)	ISPAAM Sassari
<i>R. sultae</i> 2Ba (Escolca, Sardinia, Italy)	ISPAAM Sassari
<i>R. sultae</i> 2Bb (Escolca, Sardinia, Italy)	ISPAAM Sassari
<i>R. sultae</i> 2Bc (Escolca, Sardinia, Italy)	ISPAAM Sassari
<i>R. sultae</i> 2Bd (Escolca, Sardinia, Italy)	ISPAAM Sassari
<i>R. sultae</i> 2Be (Escolca, Sardinia, Italy)	ISPAAM Sassari
<i>R. sultae</i> 2Bg (Escolca, Sardinia, Italy)	ISPAAM Sassari
<i>R. sultae</i> 2 Bh (Escolca, Sardinia, Italy)	ISPAAM Sassari
<i>R. sultae</i> 3Cd (Escolca, Sardinia, Italy)	ISPAAM Sassari
<i>R. sultae</i> 3Cf (Escolca, Sardinia, Italy)	ISPAAM Sassari
<i>R. leguminosarum</i> bv. <i>viciae</i> ATCC10004 ^T	USDA, Beltsville MD
<i>R. leguminosarum</i> bv. <i>viciae</i> 4γ11	University of Padova
<i>R. leguminosarum</i> bv. <i>viciae</i> 4γP1	University of Padova
<i>R. leguminosarum</i> bv. <i>viciae</i> 4γV5	University of Padova
<i>R. leguminosarum</i> bv. <i>viciae</i> 4γV1	University of Padova
<i>R. leguminosarum</i> bv. <i>viciae</i> 4γX2	University of Padova
<i>R. leguminosarum</i> bv. <i>viciae</i> 4αC2	University of Padova
<i>R. leguminosarum</i> bv. <i>viciae</i> 4αN5	University of Padova
<i>R. leguminosarum</i> bv. <i>viciae</i> 4αE2	University of Padova
<i>R. leguminosarum</i> bv. <i>viciae</i> 4αP3	University of Padova
<i>R. leguminosarum</i> bv. <i>viciae</i> 4αS2	University of Padova
<i>R. leguminosarum</i> bv. <i>viciae</i> 4αQ3	University of Padova
<i>R. leguminosarum</i> bv. <i>viciae</i> 4βI3''	University of Padova
<i>R. leguminosarum</i> bv. <i>viciae</i> 4αb2	University of Padova
<i>R. leguminosarum</i> bv. <i>viciae</i> 4αI5	University of Padova
<i>R. leguminosarum</i> bv. <i>viciae</i> 4βC6	University of Padova
<i>R. leguminosarum</i> bv. <i>viciae</i> 4βA1	University of Padova
<i>R. leguminosarum</i> bv. <i>viciae</i> 4β15	University of Padova
<i>R. leguminosarum</i> bv. <i>viciae</i> 4α13	University of Padova
<i>R. gallicum</i> bv. <i>gallicum</i> R602 ^T	USDA, Beltsville MD
<i>R. giardinii</i> bv. <i>giardinii</i> H152 ^T	USDA, Beltsville MD
<i>R. mongolense</i> 1844 ^T	USDA, Beltsville MD
<i>R. hainanense</i> 166 ^T	USDA, Beltsville MD
<i>R. etli</i> CFN 42 ^T	USDA, Beltsville MD
<i>R. galegae</i> HAMBI 540 ^T	USDA, Beltsville MD
<i>R. tropici</i> CIAT 899 ^T	USDA, Beltsville MD
<i>M. loti</i> NZP 2213 ^T	USDA, Beltsville MD
<i>M. loti</i> 2626	USDA, Beltsville MD
<i>S. meliloti</i> USDA 1002	USDA, Beltsville MD
<i>S. meliloti</i> ATCC 9930 ^T	USDA, Beltsville MD
<i>R. leguminosarum</i> bv. <i>trifolii</i> ATCC 14480 ^T	USDA, Beltsville MD
<i>R. leguminosarum</i> bv. <i>phaseoli</i> RCR 3644 ^T	USDA, Beltsville MD
<i>A. tumefaciens</i> P1	University of Padova
<i>Methylobacterium</i> sp. Cl06	University of Padova
<i>S. plymuthica</i> Br15	University of Padova
<i>B. thuringiensis</i> 61-10	University of Padova

Table 1 (continued)

Bacterial strains	Source
<i>Sphingomonas</i> sp. CL3	University of Padova
<i>P. graminis</i> Clb01	University of Padova
<i>E. coli</i> JM103	University of Sassari
<i>Neisseria</i> sp. SS1	University of Sassari
<i>S. aureus</i> SMI	University of Sassari

54 °C. For species identification, 1 μM of each of the *internal primers* was used. The PCR program was: initial denaturation at 95 °C for 3 min; 35 cycles at 95 °C for 45 s, 64 °C for 30 s, 72 °C for 90 s; and a final extension at 72 °C for 10 min. BOXA1R primers were used as described [8].

The PCR reaction mixtures contained 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each dATP, dCTP, dGTP and dTTP, 1 μM of each primer and 2.5 U Taq DNA Polymerase, recombinant (Invitrogen, Life Technologies).

2.5. Gel electrophoresis and documentation

The PCR products were loaded on a 1.5% agarose gel, run electrophoretically for 2 h at 100 V. The ethidium bromide-stained gel was visualized over an UV transilluminator and photographed by a Kodak DC290 digital camera.

2.6. DNA sequencing

PCR amplicon bands of interest were purified from agarose gels by using a Qiagen Quia quick PCR Purification Kit (Qiagen, GmbH, Hilden Germany) as described by the manufacturer, used for di-deoxy-cycle DNA sequencing with fluorescent terminators (Big Dye, Perkin–Elmer/Applied Biosystems, Foster City CA) and run in an Applied Biosystems ABI Prism 3730XL automated DNA sequencer. Chromatograms were analyzed by Chromas 2.23 software (Technelysium Pty Ltd., Tewantin Australia).

2.7. Software analyses

TIFF files of electrophoretograms to be compared by image analysis were processed by GelComparII (Applied Maths, Kortrijk Belgium).

Sequences of amplicons from gels were used either for BLAST analysis against the GenBank database (<http://www.ncbi.nlm.nih.gov/>) or at the *Rhizobium leguminosarum* bv. *viciae* 300 genome project website (http://www.sanger.ac.uk/Projects/R_leguminosarum/). Pair-wise alignment of selected sequences was run using the on-line EMBI-EBI ClustalW tool at the European Bioinformatics Institute Website (<http://www.ebi.ac.uk/>), applying the default values (gap open penalty = 15.0, gap extension penalty = 6.66).

Table 2
Oligonucleotides used as PCR primers

Primer	Definition	5'-3' sequence	Source or reference
ISRhIout22R	Outbound	CGCTGACAACGTGACATCACCG	This work
ISRhIout22F	Outbound	ATCAAACAACGTGACATCGCCG	This work
ISRhIin21F	Inbound	CGGCGATGTCACGTTGTTGA	This work
ISRhIin21R	Inbound	CGGTGATGTCACGTTGTCAGC	This work
ISRhIintL	Internal	GTCAGGCCTTGCTTCTTCAG	This work
ISRhIintR	Internal	CAGGCAGTTGTGCAGAAAGA	This work
BOXA1R	BOX PCR primer	CTACGGCAAGGCGACGCTGACG	[28]

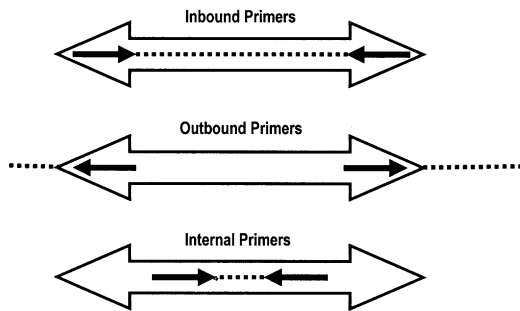


Fig. 1. Primer design rationale. The double-pointed block represents the insertion element *ISRhI*, black arrows symbolize primer orientation, dotted lines indicate PCR products: (top) *inbound primers*, yielding a whole copy of the IS element; (middle) *outbound primers*, generating variable-length fingerprinting; (bottom) *internal primers*, encompassing a species-specific amplicon.

3. Results and discussion

3.1. Assessing genetic relatedness of rhizobia (inbound primers)

Starting from our original DNA hybridization-based observation [23] that the 811 bp insertion element (termed *ISRhI*) is present, often in multiple copy, in all the symbiotically functional strains of *R. sullivanii* tested so far, but absent from the type strains of several other rhizobia, we designed a first pair of *inbound* primers, matching the left and right inverted repeats of *ISRhI* and pointing inwards, targeted to amplify the full *ISRhI* and to detect its possible presence in the different bacteria tested. As these are based on two nearly identical inverted repeats (one mismatch over 17 bases), their sequence is mostly colinear. Four extra bases, from within the non-inverted repeat parts of the element, have been included in each primer 3' end in order to enhance both stability and specificity. As PCR amplifications can be run under varying stringency conditions by testing different annealing temperatures, not only the presence of perfectly conserved copies of *ISRhI*, but also that of related and/or degenerated versions of it can be observed as bands of different intensities. Moreover, band sizes under or over 811 bp can indicate deletions

or rearrangements taken place within the element. Under these premises, amplifications were carried out to reveal rhizobia most closely related to each other according to this accessory, plasmid-riding, mobile element.

Among various bacteria tested, at an annealing temperature of 59 °C, *R. sullivanii*, *Rhizobium gallicum*, *Rhizobium galegae* and *R. leguminosarum* bv. *viciae* give a strong positive signal. For the latter, we recorded strains which do possess the element, including the type strain and strain 300, and others which do not, such as strain 248. Moreover, some of the positive cases of *R. leguminosarum* bv. *viciae* can, besides the 811 bp band, also display a shorter one, of about 240 bp long. Among the rhizobia not possessing *ISRhI*-like detectable elements are *Rhizobium giardinii*, *R. leguminosarum* bv. *phaseoli*, *Rhizobium mongolense* and *Rhizobium hainanense*. Weak PCR responses to *ISRhI* are present in others, such as *R. leguminosarum* bv. *trifolii* and *Rhizobium etli*. The only non-rhizobium in the test set giving a signal with bands of different size is *Staphylococcus aureus*. This is not unexpected, as the *ISRhI* element, since its discovery, was known to share a 55% protein level homology with IS431MEC and other transposons of *S. aureus* [23].

In order to entirely analyze the degree of conservation of *ISRhI* through rhizobia we proceeded with two different approaches. In the first place, we searched local alignment colinearity to *ISRhI* via BLAST on DNA databases. The availability of complete or partially sequenced genome projects (*Sinorhizobium meliloti*, *Bradyrhizobium japonicum*, *Mesorhizobium loti*, *R. leguminosarum* bv. *viciae* and *R. etli*) enabled to identify the complete sequence of the *ISRhI*-like element in all these strains, with the exception of *S. meliloti*, which fails to share such element. In the search, besides the straight query of *ISRhI*, we also devised a BLAST-Walking approach, i.e. taking the sequence of the identified hit as a new query for a further round of BLAST. This proved critical in order to reveal the presence of the element across species not displaying a high homology with the starting one. Specifically, the *ISRhI*-like sequence in *R. etli*, not conserved enough to be revealed by that of *R. sullivanii* in a direct BLAST, was detected using the *ISRhI* result of *B. japonicum* as a query.

In parallel, for the rhizobium species being tested positive by inbound primers-PCR, but for which no sequenced genomes are yet available, we extracted and sequenced individual amplicon bands from the gel. This enabled us to obtain and deposit the *ISRhI* sequences of the type strains of *R. galegae* (AY940433), *R. gallicum* (AY940432) and *R.l. bv. trifolii* (AY940434). The sequencing approach also allowed to check the sequence of occasionally observed bands whose size was different from the typical 811 bp size. In this respect, the above mentioned shorter band observed in *R. leguminosarum* bv. *viciae* was found to be a copy of the element having experienced an internal deletion. This was confirmed by investigating the complete sequenced genome of strain 300 in silico, in which, analogous to the situation de-

scribed for *R. sullae* IS123 [23], we found multiple plasmid pRL7-concentrated copies of the *ISRhI*-like element, some of which have deletions of variable extent. Sequencing also enabled us to inspect faint bands of a size comparable to that of *ISRhI*, appearing at low stringency PCR conditions. This allowed to rule out the relatedness to *ISRhI* of a second larger band observed in *R. leguminosarum* bv. *trifolii*, and of a faint band seen in *S. meliloti*.

The seven sequences of verified *ISRhI*-like elements obtained from the different rhizobia were subsequently aligned by the pairwise comparison ClustalW tool. The identity values are shown in Table 3, and the ClustalW-derived neighbour-joining tree is shown in the upper portion of Fig. 2, under which we report a 16S

Table 3
Percent identities upon ClustalW pairwise alignments for the *ISRhI*-like sequences from different rhizobia

<i>R. sullae</i>	100%								
<i>R. leguminosarum</i> bv. <i>viciae</i>	89.9%	100%							
<i>R. gallicum</i>	82.3%	83.5%	100%						
<i>R. galegae</i>	83.2%	84.2%	83.4%	100%					
<i>M. loti</i>	70.3%	70.9%	69.3%	71.1%	100%				
<i>B. japonicum</i>	58.8%	60.8%	59.8%	59.8%	60.6%	100%			
<i>R.l. trifolii</i>	57.1%	56.1%	56.5%	59.6%	53.0%	50.6%	100%		
<i>R. etli</i>	49.1%	49.7%	50.1%	49.3%	49.1%	58.7%	50.6%	100%	
	<i>R. sullae</i>	<i>R.l. viciae</i>	<i>R. gallicum</i>	<i>R. galegae</i>	<i>M. loti</i>	<i>B. japonicum</i>	<i>R.l. trifolii</i>	<i>R. etli</i>	

The strains used as well as their sequence references are listed in the legend of Fig. 2.

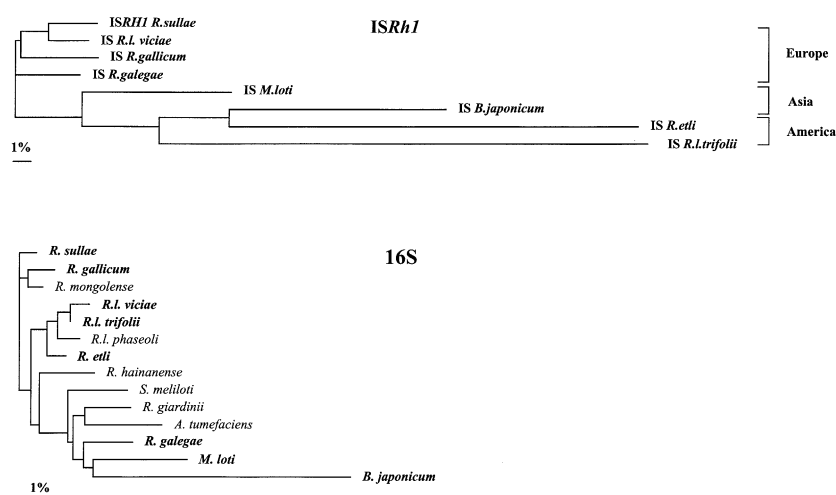


Fig. 2. Use of the *inbound primers* as phylogenetic tools for rhizobia and comparison with their 16S sequence tree. ClustalW sequence identity neighbour joining phylograms for the *ISRhI* (top) and 16S rRNA (bottom) aligned gene sequences. The outputs were produced using the on-line EMBI-EBI ClustalW tool at the European Bioinformatics Institute Website (<http://www.ebi.ac.uk>), applying the default values (gap open penalty = 15.0, gap extension penalty = 6.66). Phenetic clusters relevant to the geographical origin of the strains used are indicated on the right of the *ISRhI* tree. Sources of *ISRhI*-like sequences: AF023675 (*ISRhI*, *R. sullae*, strain IS123^T); <http://www.sanger.ac.uk/Projects/Rleguminosarum/> position 110128–110136 of plasmid pRL7; (*R. leguminosarum* bv. *viciae* strain 300); AY940432 (*R. gallicum* bv. *gallicum* R602^T); AY940433 (*R. galegae* HAMBI 540^T); BA000013, position 54155–54965 of plasmid pMLa (*M. loti* MAFF303099); BA000040, position 1841205–1842015 (*B. japonicum* USDA 110); U80928 position 66688–65877 of plasmid p42d (*R. etli* CFN42); AY940434 (*R. leguminosarum* bv. *trifolii* ATCC 14480^T). Source of 16S rRNA gene sequences: Y10170 (*R. sullae*); U89829 (*R.l. bv. viciae*); AY509900 (*R.l. bv. trifolii*); U29388 (*R.l. bv. phaseoli*); U28916 (*R. etli*); AF008130; (*R. gallicum*); U89817 (*R. mongolense*); U71078 (*R. hainanense*); X67222 (*S. meliloti*); X67226 (*R. galegae*); U86344 (*R. giardinii*); M11223 (*A. tumefaciens*); X67229 (*M. loti*); X66024 (*B. japonicum*). Taxa not in boldface in the 16S tree indicate those in which no *IS RhI*-like sequences are found, as enabled by inbound primers-PCR or by blast analysis vs. sequenced genomes.

sequence tree obtained using the same alignment parameters, and including species with strains testing positive (in boldface) or negative for *ISRh1*. Interestingly, the *ISRh1* tree closely clusters species in which the strains used for the alignment were isolated in Europe (*R. sulae* IS123, *R. leguminosarum* bv. *viciae* 300, *R. galegae* HAMBI 540^T, *R. gallicum* R602^T, originating from Spain, England, Finland and France, respectively), while those of asian origin (*M. loti* and *B. japonicum*) are progressively further apart, as well as the two instances from central (*R. etli* CFN42) or north America (*R. leguminosarum* bv. *trifolii* ATCC 14480^T). The actual geographical origin of the clover-nodulating type strain of the former *R. trifolii* has been a matter of debate, however, having been deposited in the 1960s by Dr. Ura Mae Means from the Beltsville headquarter of USDA, this is considered the most likely attribution.

The positive result of *R. galegae*, hosted by a western-range legume, although not complying with ribosomal taxonomy, is, however, but sitting in a relatively distant 16S branch, well supported by other phylogeny studies such as those of glutamine synthetase II, whose trees have revealed an otherwise unseen closeness of *R. galegae* to the *R. leguminosarum* branch [25]. Moreover, a recent study on plant-nodulating bacteria in Tunisia reported the isolation of *R. galegae* from mediterranean legumes [26]. Since the same range and habitat are shared by *H. coronarium*, the host plant of *R. sulae* in which the IS element was discovered, the evidence further supports the present geography-related inferences. In order to see the most conserved homologies within rhizobial members, we performed amplifications at increasing temperatures; shifting from 60 to 65 °C demonstrates that only the small band of *R. leguminosarum* bv. *viciae* and a fainter corresponding one in *R. sulae* are maintained as amplified signal, while 70 °C is the limit at and above which primer stability is exceeded (data not shown).

In essence, using a tool independent from usual phylogenetic markers, data indicate which rhizobia have inherited or maintained a conserved mobile element. These results can be compared, as shown, with those obtained by applying different taxonomical operators such as 16S and 23S rRNA gene sequences, glutamine synthetase I and II and *nif* and *nod*. It is known that rhizobium phylogenies, determined using different methods, are usually not in mutual agreement. As an example, the clustering obtained by aligning GSI is incongruent with that based on GSII [25]. The same applies for cladistics based on either of the two ribosomal subunits. All considered, the complexity of rhizobium diversity can benefit from the use of additional, independent taxonomical aids such as IS element homology, which may add pieces in the reconciling of different perspectives.

3.2. IS amplification fingerprinting of rhizobia and non-rhizobia (outbound primers)

As we had found several copies of *ISRh1*, scattered within a few kilobases of the *nod* gene region of *R. sulae* IS123, and observed rich hybridization patterns against genome digests of most *R. sulae* strains [23], we predicted that different band fingerprints of amplification could be obtained using primers pointing outwards from the IS ends, thus amplifying DNA portions lying between any two tandem copies of *ISRh1* occurring within polymerase processivity range. Therefore, a second pair of oligonucleotides (*outbound primers*) was designed, still based upon the inverted repeats sequences but complementary to the inbound pair. In this case, five extra bases were placed at the 5' end. Fig. 3 shows, in the left panels, the outbound fingerprints resulting from an array of different *R. sulae* strains isolated from *H. coronarium* nodules growing in different locations throughout the plant range. As a

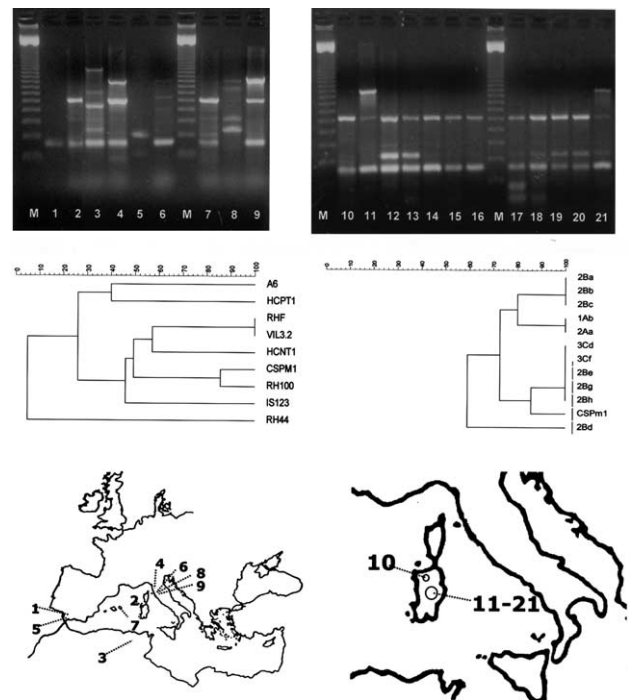


Fig. 3. Use of the *outbound primers* for the fingerprinting of *R. sulae*: profile complexity as a function of isolation range width. Left panels: Strains isolated from the whole Mediterranean area; right panels: strains isolated within the island Sardinia. Top panels: gel electrophoretic separation of PCR amplicons, raised by the *outbound primers* from genomic DNA of *R. sulae* strains; middle panels: GelComparII-derived dendrograms (Dice algorithm, tolerance 1%) expressing percent pattern similarity. Bottom panels: maps of the mediterranean basin (range of the host legume plant *H. coronarium*), fit to show the borders of each isolation area. Lane content: (1) strain IS123^T; (2) strain cspm1; (3) strain A6; (4) strain RHF; (5) strain RH44; (6) strain HCNT1; (7) strain RH100, (8) strain HCPT1; (9) strain VIL3.2; (10) strain cspm1; (11) strain 1Ab; (12) strain 3Cd; (13) strain 3Cf; (14) strain 2Ba; (15) strain 2Bb; (16) strain 2Bc; (17) strain 2Bd; (18) strain 2Be; (19) strain 2Bg; (20) strain 2Bh; (21) strain 2Aa and (M) Sigma 123 bp molecular weight ladder (doubling 123 bp increments).

comparison, the corresponding profiles obtained from a set of strains isolated from a defined small area within the island Sardinia are shown in the right panels. The higher degree of diversity across the geographically distant isolates is already evident by eye comparison and quantified by GelComparII software analysis, which shows the difference in terms of dendrogram branch horizontal extension as a direct function of genomic heterogeneity. The average distance extrapolated from the Dice matrix for the strains isolated within the Island Sardinia is 20.27, while that for the strains isolated from the whole mediterranean basin is 58.77.

When other bacterial species in the *outbound primers* analysis were included, we noticed that not only a fingerprint could be obtained from any rhizobium, but that even far and unrelated taxa gave clearly reproducible patterns, examples of which are shown in Fig. 4. Fingerprints were consistently observed with every species tested, including *Serratia plymuthica*, *Bacillus thuringiensis*, *Sphingomonas* sp., *Pseudomonas graminis*, *Agrobacterium tumefaciens*, *Methylobacterium* sp. *E. coli*, *Neisseria* sp. and *S. aureus*. Consequently, under the 59 °C annealing condition, which is a relatively low stringency for their sequence and length, it appears that these primers act in a fashion comparable to that of the general REP, ERIC or BOX prokaryotic fingerprinting elements. Indeed, this is no longer surprising, since the clarifying studies published by Gillings and Holley [27,28] showed that the mechanism by which ERIC elements usually work is not due to the matching of genuine ERIC sequences, but rather by partial homology annealing to sites shorter than their own size. Demonstrating the universal fingerprinting capability of different oligonucleotide sequences against various targets, including also eukaryotic species, the authors conclude that fingerprints given by primers of such length (>20 bases) are a highly reproducible variant of the randomly amplified polymorphic DNA methods (RAPD), which exploits sequences half the length. Other authors later have confirmed Gillings and Holley's results, also using rhizobia



Fig. 4. Use of the *outbound primers* for universal bacterial fingerprinting. PCR electrophoretic profiles of both rhizobial and phylogenetically distant bacterial species are shown. Lane content: (1) *R. sultae* VIL2; (2) *R. sultae* 835; (3) *R. sultae* RH19; (4) *R. sultae* HCNA; (5) *R. sultae* 30–21; (6) *R. sultae* HCS2; (7) *R. sultae* HA7; (8) *R. gallicum* R602^T; (9) *R. giardinii* H152^T; (10) *R. mongolense* 1844^T; (11) *R. hainanense* 166^T; (12) *S. plymuthica* Br15; (13) *B. thuringiensis* H01; (14) *Sphingomonas* sp. CL1; (15) *P. graminis* Clb01; (16) *Methylobacterium* sp. CL7; (17) *A. tumefaciens* P1 and (M) Sigma 123 bp molecular weight ladder (doubling 123 bp increments).

[17]. In the former study, Niemann and coworkers showed that, under regular PCR conditions, the ERIC2 primer (AAG TAA GTG ACT GGG GTG AGC G) could amplify sequences whose actual target was instead: ACT TCG TCG CCC GGG TTG AGC G, or also GCG AAG AAA CCA GTG GTG AGC G, implying that a mismatch up to 10 bases on the 5' end on a total of 22 would not affect the onset of the chain reaction in the absence of its primary target.

In order to verify the fingerprinting quality of the *ISRh1outbound primers* in terms of reproducibility and sensitivity, we compared their performance with that of the established BOX PCR [7], using either the BOXA1R primer or the outbound pair on a set of well-characterized strains of *R. leguminosarum* bv. *viciae*. Eighteen strains were tested, arranged in nine pairs, giving either identical or slightly different though related BOX-A1R fingerprints. Fig. 5 shows the comparisons. Overall,

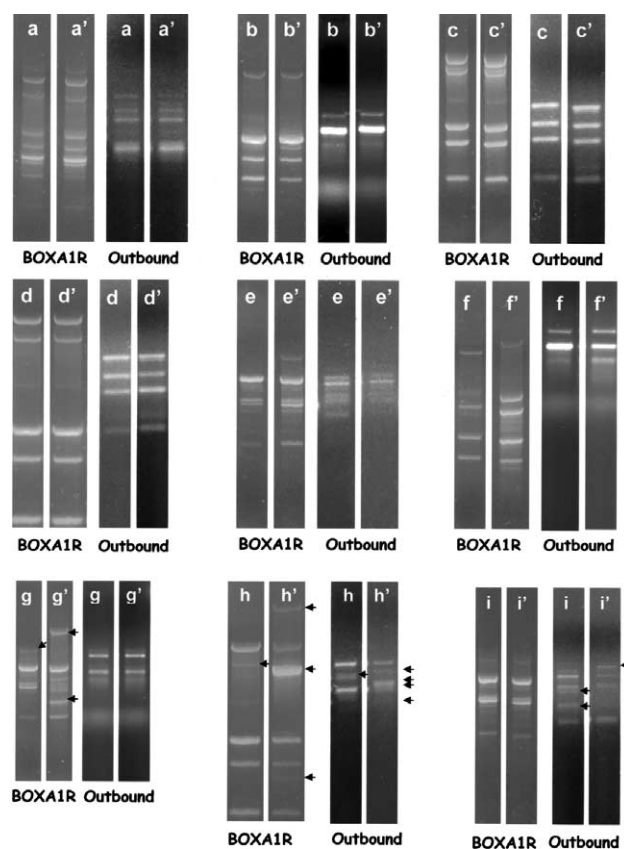


Fig. 5. Pairwise comparison of the *outbound primers* fingerprinting performance with that of BOX PCR. Eighteen strains of *R. leguminosarum* bv. *viciae*, belonging to nine pairs in which the two members of each pair share either an identical (a, b, c, d, i), a highly similar (e, f) or a slightly similar (g, h) BOX PCR profile (primer BOXA1R), are shown next to their corresponding outbound-PCR profiles. For strains belonging to pairs in which a marked difference is revealed by PCR the arrows indicate bands that differentiate the two cases: (a) strain 4 α P3; (a') strain 4 α N5; (b) strain 4 α Q3; (b') strain 4 α S2; (c) strain 4 γ 11; (c') strain 4 γ V5; (d) strain 4 γ P1; (d') strain 4 γ V1; (e) strain 4 α b2; (e') strain 4 β 15; (f) strain 4 β A1; (f') strain 4 β C6; (g) strain 4 α E2; (g') strain 4 α I5; (h) strain 4 γ X2; (h') strain 4 β I3?; (i) strain 4 α C2; (i') 4 α I3.

the *outbound primers* tested gave results equal to the BOX-A1R. In seven out of nine cases, the identity or the difference of the profiles were obtained by both tools; in one case, the slight difference detected by BOXA1R was not evident by the *outbound primers* (g), while in one other case, the outbound pair was able to trace a difference not visible by BOXA1R (i).

3.3. Specific detection of *R. sullae* (internal primers)

Upon blast sequence analysis of the whole *ISRh1* against databases and software-assisted primer design, we defined a third pair of primers, located within the core of the transposase open reading frame and encompassing a potential 310 bp amplicon. With respect to specifically raising the intended band as a positive signal for *R. sullae*, the suitability of the primers was tested by running PCR at different temperatures. Sixty-four degree Celsius turned out to be the critical temperature, as shown in Fig. 6, discriminating as positively amplifying the sole *R. sullae* and every tested strain of it. When

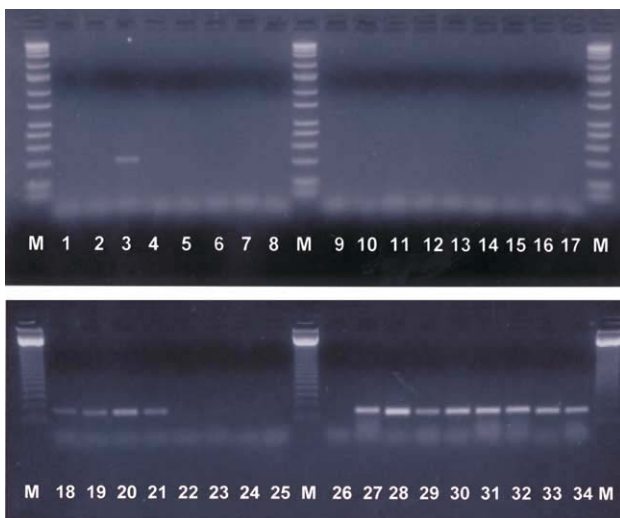


Fig. 6. Use of the *internal primers* for species detection/identification. *R. sullae*-specific PCR amplification of the 310 amplicon within the transposase of *ISRh1* (annealing at 64 °C). Lane content: (1) *R. leguminosarum* bv. *viciae* ATCC10004^T; (2) *R. leguminosarum* bv. *viciae* ss1; (3) *R. sullae* IS123^T; (4) *R. galegae* HAMB1 540^T; (5) *S. meliloti* ATCC 9930^T; (6) *R. leguminosarum* bv. *trifolii* ATCC 14480^T; (7) *R. leguminosarum* bv. *phaseoli* RCR 3644^T; (8) *R. tropici* CIAT 899^T; (9) *R. etli* CFN 42^T; (10) *E. coli* JM103; (11) *M. loti* 2626^T; (12) *M. loti* NZP 2213; (13) *R. leguminosarum* bv. *phaseoli* RCR 3644 T; (14) *R. leguminosarum* bv. *trifolii* ATCC 14480 T; (15) *B. thuringiensis* 61-10; (16) *Neisseria* sp. SS1; (17) *S. aureus* SM1; (18) *R. sullae* A6; (19) *R. sullae* RHF; (20) *R. sullae* RH44; (21) *R. sullae* HCNT1; (22) *R. leguminosarum* bv. *viciae* αM2; (23) *R. gallicum* R602^T; (24) *R. giardinii* H152^T; (25) *R. mongolense* 1844^T; (26) *R. hainanense* 166^T; (27) *R. sullae* RH19; (28) *R. sullae* RH100; (29) *R. sullae* HCPT1; (30) *R. sullae* VIL3.2; (31) *R. sullae* VIL.2; (32) *R. sullae* 835; (33) *R. sullae* IS123^T; (34) *R. sullae* HCNA; (M) top panel: Sigma DirectLoad Wide Range DNA Marker (50–10,000 bp); (M) bottom panel: Sigma 123 bp molecular weight ladder (doubling 123 bp increments).

testing lower temperatures (not shown), *R. leguminosarum* proved to be its closest relative regarding the homology for this region, since at non-stringent temperatures (down to 2° below 64 °C) the intensity of its band was as strong as that of *R. sullae*. However, the clean selectivity of the *internal primers* at the stringent temperature is evidenced by the lower panel, where every strain of *R. sullae* tested appears positive, while the five other species of rhizobia (including also *R. leguminosarum* and *R. gallicum*) are negative. Available genome sequence data for *R. leguminosarum* 300 allowed detailed analysis of the basis of such selective discrimination. In that strain, the regions corresponding to the forward and reverse *internal primers* have the following 5'-3' sequences, respectively: GTCAGGCCCTGTTTCTTCAG and TTTTCTGCGCAACTGCCTG, where the bases in italic and boldface differ from those present in the *R. sullae* *ISRh1*, indicating two mismatches per primer. A further comparison, also involving non-rhizobia (data not shown), confirms that the *ISRh1* *internal primers* are suitable tools to specifically discriminate rhizobia nodulating *H. coronarium* from other bacteria. Since, due to its resistance to several stressful conditions, including alkalinity, drought and salt, the plant is often introduced as a valuable forage crop for soil reclamation purposes, the fate of such agricultural endeavor often relies on the prompt establishment of the symbiotic interaction with the appropriate bacteria. Future work will involve testing whether these primers can be used to rapidly detect the presence of *R. sullae* in soil where the cultivation of the host plant is planned and where no prior information on the microsymbiont presence be available.

In conclusion, we have shown that a three-in-one PCR-based exploitation can be devised by deducing three different primer pairs from the same DNA insertion sequence, and that these can serve the different purposes of assisting genetic relatedness analysis, strain-level bacterial characterization and unambiguous species identification.

As rhizobial genomes harbor many different (and often rather specific) insertion sequences, we foresee the application of methods based upon the present example as helpful in taxonomical as well as agronomical studies on nitrogen-fixing symbioses.

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References

- [1] Welsh, J. and McClelland, M. (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* 18, 7213–7218.
- [2] Williams, J.G., Kubelik, A.R., Livak, K.J., Rafalsky, J.A. and Tingey, S.V. (1990) DNA polymorphisms amplified by arbitrary primers are useful genetic markers. *Nucleic Acids Res.* 18, 6531–6535.
- [3] Caetano-Anollés, G., Bassam, B.J. and Gresshoff, P.M. (1991) DNA amplification fingerprinting using very short arbitrary oligonucleotides. *Biotechnology* 9, 553–557.
- [4] Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Muiper, M. and Zabeau, M. (1995) AFLP: a new concept for DNA fingerprinting. *Nucleic Acids Res.* 21, 4407–4414.
- [5] Lupski, J.R. and Weinstock, G.M. (1992) Short, interspersed repetitive DNA sequences in prokaryotic genomes. *J. Bacteriol.* 174, 4525–4529.
- [6] Versalovic, J., Koeuth, T. and Lupski, J.R. (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res.* 19, 6823–6831.
- [7] Versalovic, J., Schneider, M., de Bruijn, F.J. and Lupski, J.R. (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol. Cell Biol.* 5, 25–40.
- [8] Hulton, C.S.J., Higgins, C.F. and Sharp, P.M. (1991) ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli*, *Salmonella typhimurium* and other enterobacteria. *Mol. Microbiol.* 5, 825–834.
- [9] Martin, B., Humbert, O., Camara, M., Guenzi, E., Walker, J., Mitchell, T., Andrew, P., Prudhomme, M., Alloing, G., Hakenbeck, R., Morrison, D.A., Boulnois, G.J. and Claverys, J.-P. (1992) A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. *Nucleic Acids Res.* 20, 3479–3483.
- [10] Rademaker, J.L.W. and de Bruijn, F.J. (1997) Characterization and classification of microbes by rep-PCR genomic fingerprinting and computer-assisted pattern analysis In: DNA markers: protocols, applications and overviews (Caetano-Anollés, G. and Gresshoff, P.M., Eds.), pp. 151–171. John Wiley and Sons, Inc., New York, NY.
- [11] Stern, M.J., Ames, G.F.L., Smith, N.H., Robinson, E.C. and Higgins, C.F. (1984) Repetitive extragenic palindromic sequences: a major component of the bacterial genome. *Cell* 37, 1015–1026.
- [12] Vaneechoutte, M., De Beenhouwer, H., Claeys, G., Verschraegen, G., De Rouck, A., Paepe, N., Elaichouni, A. and Portaels, F. (1993) Identification of *Mycobacterium* species by using amplified ribosomal DNA restriction analysis. *J. Clin. Microbiol.* 31, 2061–2065.
- [13] Burmeister, M., diSibio, G., Cox, D.R. and Myers, R.M. (1991) Identification of polymorphisms by genomic denaturing gradient gel electrophoresis: application to the proximal region of human chromosome 21. *Nucleic Acids Res.* 19, 1475–1481.
- [14] Muyzer, G., Dewaal, E.C. and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16S ribosomal-RNA. *Appl. Environ. Microbiol.* 59, 695–700.
- [15] Sheffield, V.C., Beck, J.S., Kwitek, A.E., Sandstrom, D.W. and Stone, E.M. (1993) The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions. *Genomics* 16, 325–332.
- [16] Widjoatmodjo, M.N., Fluit, A.C. and Verhoef, J. (1995) Molecular identification of bacteria by fluorescence-based PCR-single-strand conformation polymorphism analysis of the 16S rRNA gene. *J. Clin. Microbiol.* 33, 2601–2606.
- [17] Niemann, S., Dammann-Kalinowski, T., Nagel, A., Pühler, A. and Selbitschka, W. (1999) Genetic basis of enterobacterial repetitive intergenic consensus (ERIC)-PCR fingerprint pattern in *Sinorhizobium meliloti* and identification of *S. meliloti* employing PCR primers derived from an ERIC-PCR fragment. *Arch. Microbiol.* 172, 22–30.
- [18] Laguerre, G., Mavingui, P., Allard, M.R., Charnay, M.P., Louvrier, P., Mazurier, S.I., Rigottier-Gois, L. and Amarger, N. (1996) Typing of rhizobia by PCR fingerprinting and PCR restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: application to *Rhizobium leguminosarum* and its different biovars. *Appl. Environ. Microbiol.* 62, 2029–2036.
- [19] Perret, X. and Broughton, W.J. (1998) Rapid identification of *Rhizobium* strains by targeted PCR fingerprinting. *Plant Soil* 204, 21–34.
- [20] Richardson, A.E., Viccars, L.A., Watson, J.M. and Gibson, A.H. (1995) Differentiation of *Rhizobium* strains using the polymerase chain reaction with random and directed primers. *Soil Biol. Biochem.* 27, 515–524.
- [21] Sivakumaran, S., Lockhart, P.J. and Jarvis, B.D.W. (1997) Identification of soil bacteria expressing a symbiotic plasmid from *Rhizobium leguminosarum* bv. *trifolii*. *Can. J. Microbiol.* 43, 164–177.
- [22] Thies, J.E., Holmes, E.M. and Vachot, A. (2001) Application of molecular techniques to studies in *Rhizobium* ecology: a review. *Austral J. Exper. Agric.* 41, 299–319.
- [23] Meneghetti, F., Alberghini, S., Tola, E., Giacomini, A., Ollero, F.J., Squartini, A. and Nuti, M.P. (1996) Presence of unique repeated insertion sequences in nodulation genes of *Rhizobium 'hedysari'*. *Plant Soil* 186, 113–120.
- [24] Squartini, A., Struffi, P., Döring, H., Selenska-Pobell, S., Tola, E., Giacomini, A., Vendramin, E., Velázquez, E., Mateos, P.F., Martínez-Molina, E., Dazzo, F.B., Casella, S. and Nuti, M.P. (2002) *Rhizobium sullae* sp. nov. (formerly *Rhizobium 'hedysari'*): the root-nodule microsymbiont of *Hedysarum coronarium* L.. *Int. J. Syst. Evol. Microbiol.* 52, 1267–1276.
- [25] Turner, S.L. and Young, J.P.W. (2000) The glutamine synthetases of rhizobia: phylogenetic and evolutionary implications. *Mol. Biol. Evol.* 17, 309–319.
- [26] Zakhia, F., Jeder, H., Domergue, O., Willems, A., Cleyet-Marel, J.C., Gillis, M., Dreyfus, B. and De Lajudie, P. (2004) Characterisation of wild legume nodulating bacteria (LNB) in the infarid zone of Tunisia. *Syst. Appl. Microbiol.* 27, 380–395.
- [27] Gillings, M. and Holley, M. (1997) Repetitive element PCR fingerprinting (rep-PCR) using enterobacterial repetitive intergenic consensus (ERIC) primers is not necessarily directed at ERIC elements. *Lett. Appl. Microbiol.* 25, 17–21.
- [28] Gillings, M. and Holley, M. (1997) Amplification of anonymous DNA fragments using pairs of long primers generates reproducible DNA fingerprints that are sensitive to genetic variation. *Electrophoresis* 18, 1512–1518.

Appendix 5

Muresu R., Polone E., Sulas L., Baldan B., Tondello A., Delogu G., Cappuccinelli P., Alberghini S., Benhizia Y., Benhizia H., Benguedouar A., Mori B., Calamassi R., Dazzo F. B. and Squartini A.

Coexistence of predominantly nonculturable rhizobia with diverse, endophytic bacterial taxa within nodules of wild legumes

Coexistence of predominantly nonculturable rhizobia with diverse, endophytic bacterial taxa within nodules of wild legumes

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Abstract

A previous analysis showed that *Gammaproteobacteria* could be the sole recoverable bacteria from surface-sterilized nodules of three wild species of *Hedysarum*. In this study we extended the analysis to eight Mediterranean native, uninoculated legumes never previously investigated regarding their root-nodule microsymbionts. The structural organization of the nodules was studied by light and electron microscopy, and their bacterial occupants were assessed by combined cultural and molecular approaches. On examination of 100 field-collected nodules, culturable isolates of rhizobia were hardly ever found, whereas over 24 other bacterial taxa were isolated from nodules. None of these nonrhizobial isolates could nodulate the original host when reinoculated in gnotobiotic culture. Despite the inability to culture rhizobial endosymbionts from within the nodules using standard culture media, a direct 16S rRNA gene PCR analysis revealed that most of these nodules contained rhizobia as the predominant population. The presence of nodular endophytes colocalized with rhizobia was verified by immunofluorescence microscopy of nodule sections using an *Enterobacter*-specific antibody. Hypotheses to explain the nonculturability of rhizobia are presented, and pertinent literature on legume endophytes is discussed.

Introduction

The nitrogen-fixing symbiosis between plants of the family *Leguminosae* and prokaryotic partners is typically characterized by the formation of root or stem nodules that are induced and subsequently invaded by the specific microsymbionts. These include the well-known alphaproteobacterial group of *Rhizobiaceae* containing the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium* (*Ensifer*), *Mesorhizobium*, *Azorhizobium*, and *Allorhizobium*, along with other taxa such as *Methylobacterium* (Sy *et al.*, 2001) and *Devosia* (Rivas *et al.*, 2002), and members of the *Betaproteobacteria* such as *Burkholderia* (Moulin *et al.*, 2001) and *Ralstonia* (Chen *et al.*, 2001). The support of microscopy to examine nodule symbioses has gained new importance in

light of these findings, and various studies have coupled the visual approach with the molecular characterization of symbionts (Chen *et al.*, 2005; Elliott *et al.*, 2007). The traditional strategy used to investigate nodule-associated microbial symbionts involves their isolation and cultivation from internal tissues of surface-sterilized nodules (Vincent, 1970). The description of symbiotic partnerships for the various legumes has therefore traditionally relied, as a starting point, on the culturability of the bacterial occupant within the nodule when streaked on yeast–mannitol-based agar plates. Despite the physiological transformation of vegetative bacteria into nondividing bacteroids, it is normally observed that rhizobia are regularly cultured from surface-disinfected crushed nodules. This implies either that some vegetative rods (still confined or recently released from

infection threads) have not undergone the bacteroid conversion (Paa *et al.*, 1980; Timmers *et al.*, 2000), or that some bacteroids can be resuscitated back to culturable state, or both of these possibilities.

The vast majority of studies that have detected the taxa so far assigned have, however, dealt with cultivated species of legumes or with man-exploited natural plants. In contrast, little attention has been paid to the root-nodule symbionts of truly wild legumes, that is, those whose ecology is not directly affected by human action. As the *Leguminosae* family includes over 18 000 species (<http://www.ildis.org/Leguminosae/>), only a minor portion has been examined (mostly representing crops of agricultural interest), and knowledge of the biological diversity of interactions between legumes and microorganisms is still very limited. We previously addressed this issue by describing the unusual diversity of *Gammaproteobacteria* as the sole culturable nodule occupants within three wild *Hedysarum* species collected in various locations in Algeria (Benhizia *et al.*, 2004a, b). In those studies, rhizobia could not be cultured from any of the 52 nodules examined, leading to the hypothesis that some *Gammaproteobacteria* may represent an alternative endosymbiotic partner to rhizobia for these nodulated plants.

In addition to the theories and research on *Rhizobium*–legume interactions, a parallel and rarely converging field of knowledge is that of microbial endophytes. Evidence that the healthy plant interior can normally contain bacteria or fungi not necessarily related to a pathogenic context was first put forward by Perotti (1926), subsequently revisited by Old & Nicolson (1978), and is now well documented by many studies that have been reviewed over the years (Hallmann *et al.*, 1997; Sattelmacher, 2001). The majority of reports deal with culturable endophytes. Stems and roots of most plant species tested harbour a range of 10^3 – 10^6 live internal bacteria per gram of fresh weight, whose roles are related to various interactive phenotypes. Legumes are in this respect no exception: Gagné *et al.* (1987) found bacteria in the xylem of alfalfa roots; and Sturz *et al.* (1998) found 22 species within red clover. Sturz *et al.* (1997) showed that red clover harbours rhizobia of different species (not limited to the endosymbiont, *Rhizobium leguminosarum* bv. *trifolii*) not just in nodules but systemic throughout the plant. Elvira-Recuenco & Van Vuurde (2000) found that *Pantoea agglomerans* and *Pseudomonas fluorescens* were the most common endophytes in various pea cultivars. Dong *et al.* (2003) experimentally created the conditions in which inoculated enterobacteria achieved internal invasion of *Medicago sativa* and *M. truncatula*, and Kuklinsky-Sobral *et al.* (2004, 2005) demonstrated the plant growth promoting rhizobacteria (PGPR) properties of soybean endophytes.

Nodules themselves can be colonized internally by several bacterial genera unrelated to rhizobial symbiotic nitrogen

fixation. Philipson & Blair (1957) found diverse species, including Gram-positive bacteria, in roots and nodules of healthy red and subterranean clover plants. Sturz *et al.* (1997) showed that rhizobia recovery from red clover nodule tissue could yield up to 4.3×10^9 CFU g⁻¹ fresh weight, but that, at the same time, 3.0×10^5 CFU g⁻¹ of nonrhizobial endophytes, belonging to 12 different species, could be cultured from the same nodules. *Agrobacterium* sp. has been reported in nodules of tropical legumes (De Lajudie *et al.*, 1999). In bean nodules, Mhamdi *et al.* (2005) found, along with *Rhizobium*, *Agrobacterium*-like bacteria, and proved that these could invade new nodules upon coinoculation with rhizobia and affect their nodulation performance (Mrabet *et al.*, 2006). *Actinobacteria* such as *Streptomyces lydicus* have been reported to colonize pea nodules (Tokala *et al.*, 2002). Furthermore, Bai *et al.* (2003) showed that *Bacillus subtilis* and *Bacillus thuringiensis* can naturally coinhabit soybean nodules along with *Bradyrhizobium japonicum*, and that these Gram-positive bacteria can enhance plant productivity in coinoculation experiments. A more recent report (Zakhia *et al.*, 2006) described the association of 14 bacterial genera with wild legume nodules in Tunisia.

In order to gain a better understanding of the incidence and diversity of natural legume–endophyte associations, we examined the microbial occupants inside nodules and other plant tissues of 11 wild legume species collected in Sardinia (Italy) and Algeria, using both the standard colony isolation method and a direct PCR amplification of prokaryotic DNA from nodules and other tissues. In parallel, microscopy-based approaches were undertaken to document the microbial colonization within these legume tissues.

Materials and methods

Plant collection and nodule examination

An extensive search was conducted to locate plants of interest in suitable biotopes within Sardinia during the springs of 2004 and 2005. The legumes sampled, the nearest urban settlement, the geographical coordinates of the sampling site, and the number of nodules collected and analysed were as follows: *Hedysarum spinosissimum* (Giòscari, 40°42'N, 8°33'E; and Castelsardo 40°54'N, 8°41'E, 15 nodules), *H. glomeratum* (Pimentel, 39°29'N, 9°04'E, and Segariù, 39°34'N, 8°57'E, 13 nodules), *Hippocrepis unisiliquosa* (Castelsardo, 25 nodules), *Scorpiurus muricatus* (Castelsardo, 25 nodules), *Tetragonolobus purpureus* (Nurèci, 39°50'N, 9°01'E, 34 nodules), *Ornithopus compressus* (Bolòtana, 40°19'N, 8°57'E, 24 nodules), *Ornithopus pinnatus* (Bolòtana, 21 nodules), and *Psoralea bituminosa* (Castelsardo, five nodules). The collection and characterization of culturable bacteria (exclusively *Gammaproteobacteria*) from nodules of three Algerian species (*Hedysarum spinosissimum* ssp.

capitatum, *Hedysarum pallidum* and *Hedysarum carnosum*) have been previously described (Benhizia *et al.*, 2004a, b). In the present work, these three African species (plus the recently found Algerian-endemic species *Hedysarum naudinianum*; near Sétif, 36°12'N, 5°24'E, five nodules) have been further characterized by adding the direct-PCR bacterial identification and nodule microscopy approaches. According to the Italian botanical taxonomy (Pignatti, 1982), *H. glomeratum* (collected by us in Sardinia) is considered a synonym of *H. spinosissimum* ssp. *capitatum* (collected in Algeria). However, in view of several ecotypic differences and of the distance within the large range, we keep the distinction between the two species when describing the results.

Bacterial strain isolation and culture conditions

Root segments bearing nodules from the plants were washed free of soil under running water, then encaged in a fine-mesh steel holder and surface-sterilized by immersion in 95% ethanol for 20 s followed by 5% sodium hypochlorite for 3 min, and finally washed seven times with sterile distilled water. As an alternative surface sterilization procedure, in place of the NaClO step, after the ethanol treatment, nodules were immersed in 0.1% HgCl₂ for 2 min, and the number of H₂O washes was extended to 10. All treatments were performed under microbiologically controlled conditions. Tests to validate surface-sterilization of plant tissues were performed by touching the material several times on the surface of plate count agar (PCA, Difco) plates prior to isolation of the interior microbiota.

Surface-sterilized root nodules were transferred into empty sterile plastic dishes and cut in half with a flamed scalpel. One portion was processed for microscopy and the other was squashed in 50–150 µL of sterile physiological saline solution, the volume varying in proportion to the nodule size. About one-fifth of the resulting suspension volume was withdrawn for lysis and direct 16S rRNA gene PCR, with the aim of verifying the identity of the prevailing bacterial species by a culture-independent approach. The remaining portion from the squashed nodule suspension was streaked on yeast-mannitol agar (YMA) plates containing Congo-red (Vincent, 1970), on PCA, and on defined BIII-agar (Dazzo, 1982). As alternative rhizobial media with pH-buffering capability, YMA containing 4 g L⁻¹ calcium carbonate (Jordan, 1984) and medium I (Howieson *et al.*, 1988, also known as 1/2 Lupin agar) were also tested. The latter was modified using 0.17 g L⁻¹ K₂HPO₄ and 0.13 g L⁻¹ KH₂PO₄. Plates were incubated at room temperature for up to 2 weeks. All colonies obtained were purified and processed for DNA extraction.

The same protocols were used for the isolation of endophytic bacteria from preweighed and surface-sterilized portions of roots and stems.

Plant cultivation and nodulation tests

Mature pods were collected from wild plants in their natural habitat and kept dry at room temperature until use. Seeds were removed manually from pods, surface-sterilized by immersion in 70% ethanol for 30 s followed by stirring in 0.1% HgCl₂ for 7 min, and rinsed in seven changes of sterile deionized water. Seeds were preimbibed for 3 h in the final wash. Dormancy was broken by mechanical scarification with autoclaved material as follows: seeds were transferred over a ribbed rubber sole fitted in a polypropylene box and gently streaked for 5 s with bodywork-grade medium-grain sandpaper. Alternatively, a vernalization treatment was applied by storing seeds at –20 °C for 3 days. Germination and concomitant verification of surface sterility were obtained by spreading seeds on YMA plates wetted with 10 drops of sterile water, and incubating inverted for 3 days in the dark. Germinated seedlings were transferred aseptically to sterilized plastic Leonard jars containing a water-washed, oven-dried, quartziferous sand–vermiculite 1/3 mixture, fed from the bottom with nitrogen-free Fähræus solution. The rooting mixture was rehumidified with 1/10 vol of sterile nitrogen-free Fähræus solution and autoclaved in plastic biohazard-type bags prior to transfer to the top portion of the Leonard jar assembly. Bacteria were inoculated by dispensing a 1-mL suspension of an overnight-grown liquid culture in yeast-mannitol broth (YMB) medium (*c.* 10⁸ cells mL⁻¹). Seeds were covered with a layer of autoclaved gravel, and the entire Leonard jar assemblies were transferred to a growth cabinet (Angelantoni, Sas. Massa Martana, Italy) programmed for a 16-h daylight photoperiod at 23 °C, night temperature of 18 °C, and 60% constant relative humidity. Plants were inspected as early as after 40 days for nodule formation and grown for up to 3 months. For nodulation by natural symbionts, uninoculated seedlings were transplanted to jars containing field-collected soil instead of the sand–vermiculite mix.

Acetylene reduction activity (ARA) measurement

Nitrogenase activity was estimated by the acetylene reduction assay according to Somasegaran & Holben (1985) using a TRACE GC 2000 gas-chromatographer (Thermo Finnigan) equipped with a flame ionization detector.

Nodule microscopy

Single-stain light microscopy and electron microscopy

Whole nodules and nodule halves obtained as described above were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 6.9) for 24 h at 4 °C and postfixed for 2 h at 4 °C

in 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated in a graded ethanol series, and then embedded in araldite resin. Thin sections (1 μm), obtained with a Reichert–Jung ultramicrotome, were stained with 1% toluidine blue for light microscopy. Ultra-thin sections (0.05 μm) were collected on copper grids, stained with uranyl acetate followed by lead citrate at room temperature, and then examined with a Hitachi 300 transmission electron microscope operating at 75 kV.

Double-stain light microscopy

Nodules were washed in running tap water and fixed overnight in 1.5% glutaraldehyde in 200 mM phosphate buffer, pH 7.2. Samples were degassed for 5 min under vacuum and dehydrated in an ethanol series from 30% through 95%, then embedded in LR white resin. One- to 1.5- μm sections were obtained using glass knives on a Reichert Om U3 ultramicrotome (C. Reichert Optische Werke, Vienna, Austria), and dried on a microscope slide by placing each section on a drop of 30% acetone in distilled water and transferring to a 60 °C hot plate dryer. Dry sections were stained by flooding with Astra-Blue (1% dissolved in 1% acetic acid) for 2 min, rinsed with distilled water, and double-stained with Basic Fuchsin (0.07% in H_2O) for 2 min at the same temperature.

Sections were also stained on the hot plate with Aniline Blue Black (0.5% dissolved in 3.5% acetic acid) for 5 min, rinsed, and then stained with Basic Fuchsin (0.07% in H_2O) at room temperature for 2 min (Kraus *et al.*, 1998). Slides were placed on top of a moistened paper tissue and covered with a Petri dish lid to prevent drying during staining. All of the above stains were purchased from Merck/BDH (Darmstadt, Germany). After staining, samples were rinsed in water, mounted with Eukitt medium (Sigma Chem. Corp, Saint Louis, MO), and examined by bright-field microscopy using a Leitz Dialux 22 microscope.

Detection of *Enterobacter agglomerans* within nodules by immunofluorescence microscopy

Nodules were rinsed in water, dried on paper tissue, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.2, and embedded overnight in paraffin (Paraplast Plus, Sigma). Sections (*c.* 7 μm thick) were collected on microscope slides coated with poly-L-lysine, dried, treated with xylol (four washes), 1:1 xylol: absolute ethanol (EtOH), absolute EtOH, 95% EtOH, 90% EtOH, 70% EtOH, 50% EtOH; H_2O ; phosphate-buffered saline (PBS, Dazzo, 1982). Treated sections were soaked in 100 mM glycine (*p*-hydroxyphenylaminoacetic acid) for 1 h to cover the residual fixer response, treated with 3% bovine serum albumin (BSA) in PBS for 1 h at 37 °C to block nonspecific staining, incubated for 1 h at 37 °C

with the primary antibody (mouse IgG2a monoclonal antibody to *E. agglomerans* NCTC9381, Acris Antibodies GmbH, Hiddenhausen, Germany) diluted 1:20 in 1% BSA in PBS, washed in PBS four times, incubated for 1 h at 37 °C with the secondary antibody in 1% BSA in PBS (goat anti-mouse polyvalent immunoglobulins, fluorescein isothiocyanate-conjugated, Sigma Chemical Co.), washed in PBS four times, treated for 15 min in 0.1% Toluidine Blue to quench basal autofluorescence, washed extensively in PBS, and mounted in Mowiol with 1 $\mu\text{g mL}^{-1}$ 4',6-diamidino-2-phenyl-indole. Samples were observed under an Olympus BX51 epifluorescence microscope equipped with a $\times 100/1.35$ oil iris PlanApo objective. At least 1000 cells were scored for signals by each of two independent examiners. Only signals presenting rod-like shapes were considered. Selected images were acquired using a Magnafire camera (Optronics, Goleta, CA).

Fluorescent stain microscopy

Nodules were fixed overnight using 4% paraformaldehyde in phosphate buffer, pH 7.4, and embedded in paraffin as described above. Four-micrometer-thick sections were mounted on slides coated with 3-aminopropyl-triethoxysilane (Sigma). The sections were deparaffinized by two extractions in xylene, followed by two extractions with absolute ethanol and then a single wash with 95% EtOH, 90% EtOH, 80% EtOH, 70% EtOH, 50% EtOH, and finally PBS. Endophytic bacteria were then stained with propidium iodide solution (1 $\mu\text{g mL}^{-1}$) containing antifade (23 mg mL^{-1} DABCO, 80% glycerol, 2 mM Tris-HCL pH 8). Samples were examined under an Olympus BX51 epifluorescence microscope as described for immunofluorescence.

DNA extraction

Cells were lysed by resuspending a loopful of plate-grown isolated colonies in 50 μL of lysis buffer [0.25% sodium dodecyl sulphate (SDS), 0.05M NaOH] in a 1.5-mL polypropylene tube, followed by stirring for 60 s on a vortex and heating at 95 °C for 15 min. The lysate was centrifuged for 15 min, and 10 μL of the supernatant was mixed with 90 μL of sterile water. Lysates were stored at 4 °C prior to PCR. For direct PCR analysis, nodules were squashed in 50 μL of sterile water, and 10 μL of the suspension was transferred to 50 μL of lysis buffer and treated according to the protocol described above for DNA isolation.

PCR amplification of the 16S rRNA gene and amplified ribosomal DNA restriction analysis (ARDRA)

One microliter of the lysate containing the total DNA of each bacterial isolate was treated in a PCR BioRad I-Cycler using the two 16S rRNA gene-targeted universal

bacterial primers 63F (5′CAGGCCTAACACATGCAAGTC) (Marchesi *et al.*, 1998) and 1389R (5′ACGGGCGGTGTGTACAAG) (Osborn *et al.*, 2000) at 1 µM each in a 25-µL reaction volume, using the following program: initial denaturation at 95 °C for 2 min; 35 cycles at 95 °C for 30 s, 55 °C for 1 min, 72 °C for 4 min; and a final extension at 72 °C for 10 min. The PCR reaction mixture contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each of dATP, dCTP, dGTP and dTTP, 1 µM of each primer, and 2.5 U Taq DNA polymerase, recombinant (Invitrogen Life Technologies). Amplicons were digested overnight at 37 °C upon mixing 5 µL from the 25-µL reaction volume with 1 µL of CfoI enzyme (Pharmacia, Uppsala, Sweden) and 2 µL of 10 × reaction buffer. Digested DNA was loaded on a 1.5% agarose gel, run electrophoretically for 3 h at 110 V. The ethidium bromide-stained gel was visualized over an UV transilluminator and photographed with a Kodak DC290 digital camera. Upon ARDRA analysis the isolates were sorted and selected for sequencing.

Direct PCR from nodule extract

Ten microliters of the suspension resulting from squashing the nodule in 50–100 µL of sterile physiological solution was mixed with 50 µL of lysis buffer, and the same protocol as described above for DNA isolation was carried out.

DNA sequencing

One microliter of the amplicon resulting from the above-described PCR amplification was mixed with 1 µL containing 6.4 picomoles of the above-described forward primer 63F in a 0.2-mL polypropylene tube and then dried by incubating the open tube for 15 min at 65 °C in an I-Cycler thermal cycler. The template and primer mix was directly used for di-deoxy-cycle DNA sequencing with fluorescent terminators (Big Dye, Perkin-Elmer/Applied Biosystems, Foster City CA) and run in an Applied Biosystems ABI Prism 3730XL automated DNA sequencer. Chromatograms were analysed using CHROMAS 2.23 software (Technelysium Pty Ltd, Tewantin, Australia).

Results

Plant collection and nodule examination

The root systems of all field-collected species bore tubercular structures that varied in number and shape (Fig. 1). Root nodules on *T. purpureus* and *P. bituminosa* were typically round and determinate, and very large in the case of the latter species, whereas the root nodules on the other legumes had elongated-indeterminate to irregular shapes. Multi-lobed nodules were present on roots of *H. spinosissimum* often varying in character according to the site of isolation.

Figure 1i depicts examples nodules for this case, all belonging to a vigorous plant specimen thriving in a sandy scree near Castelsardo and endowed with a single nodular cluster with a noticeable maximum diameter larger than 3.5 cm. In the more clayish soil of Giòscari, however, the same plant tended to form unilobed nodules.

Nodule microscopy

A minimum of three nodules per plant species were inspected under optical as well as electron microscopy. Standard nodule histology techniques involving toluidine-stained thin sections and transmission electron microscopy (TEM) ultra-thin sections indicated that these root-borne hypertrophy structures from different host plants are consistent with the anatomy of genuine legume nodules, displaying an uninfected cortex, a peripheral vascular system, and a central tissue of host cells infected to various degrees with bacteria (Fig. 2a–h). TEM confirmed the presence of bacteria within the lumen of walled infection threads that crossed the walls of adjacent host cells, and provided evidence that infected host cells contained bacteroid-like cells of various sizes and shapes, including elongated rods (Fig. 3a and b), clubs (Fig. 3a and h), lobed cells (Fig. 3c–e and h), ellipsoids (Fig. 3b), coccobacilli (Fig. 3f), and spheres (Fig. 3g and h). Intracellular bacteria contained intracellular inclusions that were either electron-opaque (Fig. 3b) or electron-transparent (Fig. 3c and h), resembling polyphosphate or polyhydroxybutyrate storage granules, respectively.

We also tested two other novel double-staining procedures not previously used on root nodules (Aniline Blue Black/Basic Fuchsin and Astra Blue/Basic Fuchsin, Fig. 4a and b, respectively). Nodules present on 70-day-old plants of *H. spinosissimum* cultivated in a growth cabinet in Leonard jars filled with original soil from Giòscari were used. The double stains worked well by differentially staining structures such as cell walls, infection threads and bacteria. Basic Fuchsin stained the endosymbiotic bacteria pale red (Fig. 4b), whereas Astra Blue added contrast with blue staining of mucilages, including the lumen of infection threads and the pecto-cellulosic plant cell walls. Aniline Blue Black (Fig. 4a), a protein stain, was particularly suitable for staining bacteria within the plant cells whose cell walls were stained red by Basic Fuchsin. Compared with Toluidine Blue, these stains increased the contrast, providing more detail and improving the visualization of infection threads, which appear in colours different from those of the bacteroid tissue. Moreover, the physiological state of the plant nuclei appears to be proportional to the intensity of the blue colour upon Aniline Blue Black/Basic Fuchsin staining. Thus both procedures can be recommended in general histological studies of legume nodulation and other plant–microorganism interactions.

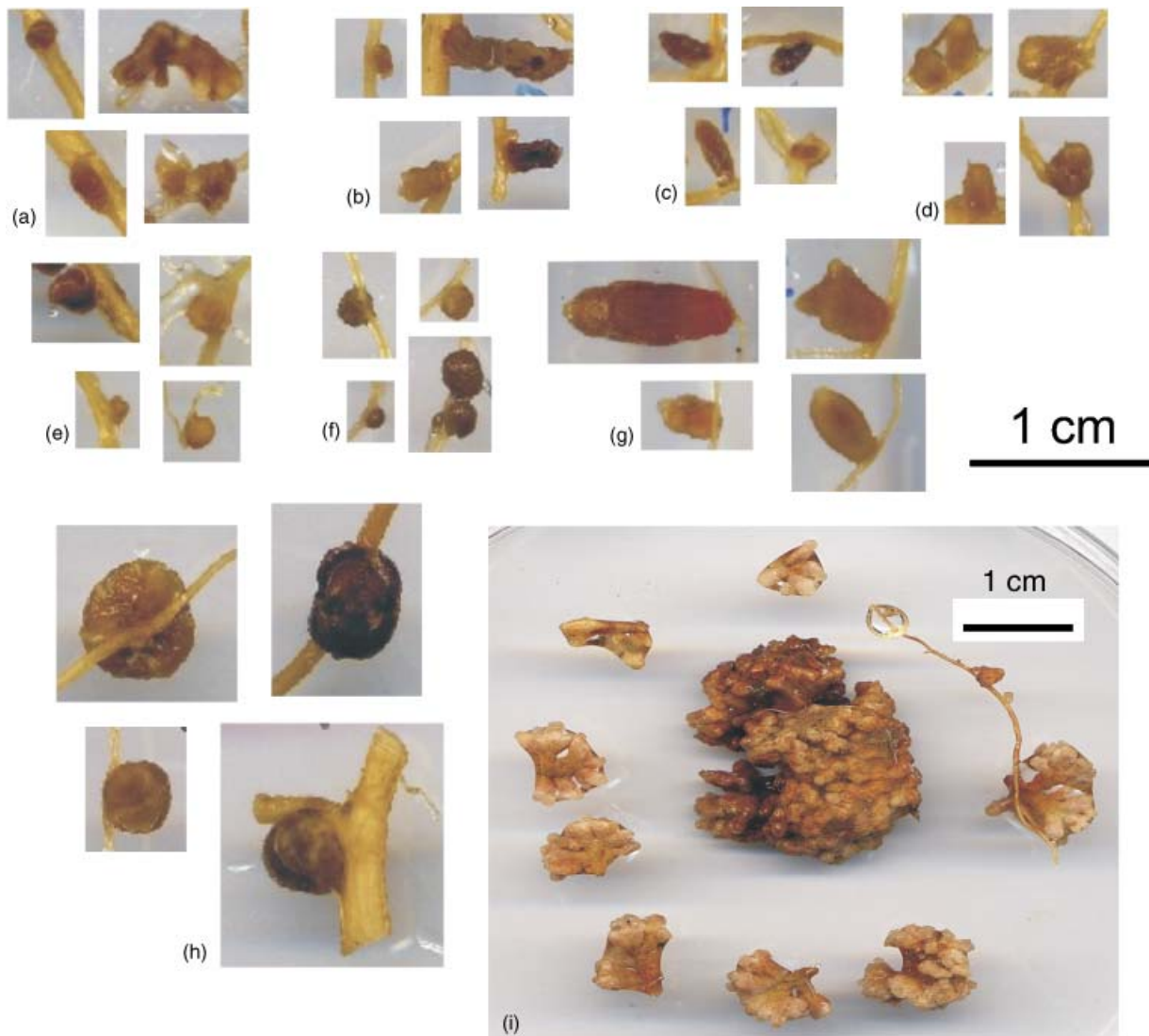


Fig. 1. Examples of excised root nodules of the various plants included in this study. (a) *Hedysarum glomeratum*; (b) *Hedysarum spinosissimum*; (c) *Hippocrepis unisiliquosa*; (d) *Ornithopus compressus*; (e) *Ornithopus pinnatus*; (f) *Tetragonolobus purpureus*; (g) *Scorpiurus muricatus*; (h) *Psoralea bituminosa*. (i) Clustered root nodules of a healthy *Hedysarum spinosissimum* plant collected in Castelsardo.

Bacteria isolation from nodules

In total, 79 nodules from the 2004 campaign and 25 nodules from the 2005 campaign were evaluated. The surface-sterilization treatment was generally efficient, as in most cases no colonies developed on the PCA plates upon which nodules were rolled prior to sectioning. In about 10% of the cases where surface sterilization was not achieved, the squashed nodule samples were not considered further. A total of 161 bacterial colonies were isolated from inside the surface-sterilized nodules from the eight plant species harvested in Sardinia and grouped by ARDRA. Partial sequencing (700–800 nucleotides) of the corresponding amplified

16S rRNA gene revealed at least 12 broad lineages, encompassing a diversity represented by several taxa as defined by GenBank database similarities. The ranked abundance of the various bacteria in the nodules is summarized in Table 1. Quite unexpectedly, rhizobial lineages were rarely found, amounting to only single-colony occurrences in four nodule squashes that also yielded various other taxa. The most common result (27%) from plating nodule squashes was no development of microbial growth on YMA, BIII or PCA plates. When growth did occur, fewer than ten colonies developed. This contrasts with typical results obtained using cultivated legumes, for which the rhizobial occupants rescued by such techniques normally form a profuse lawn along

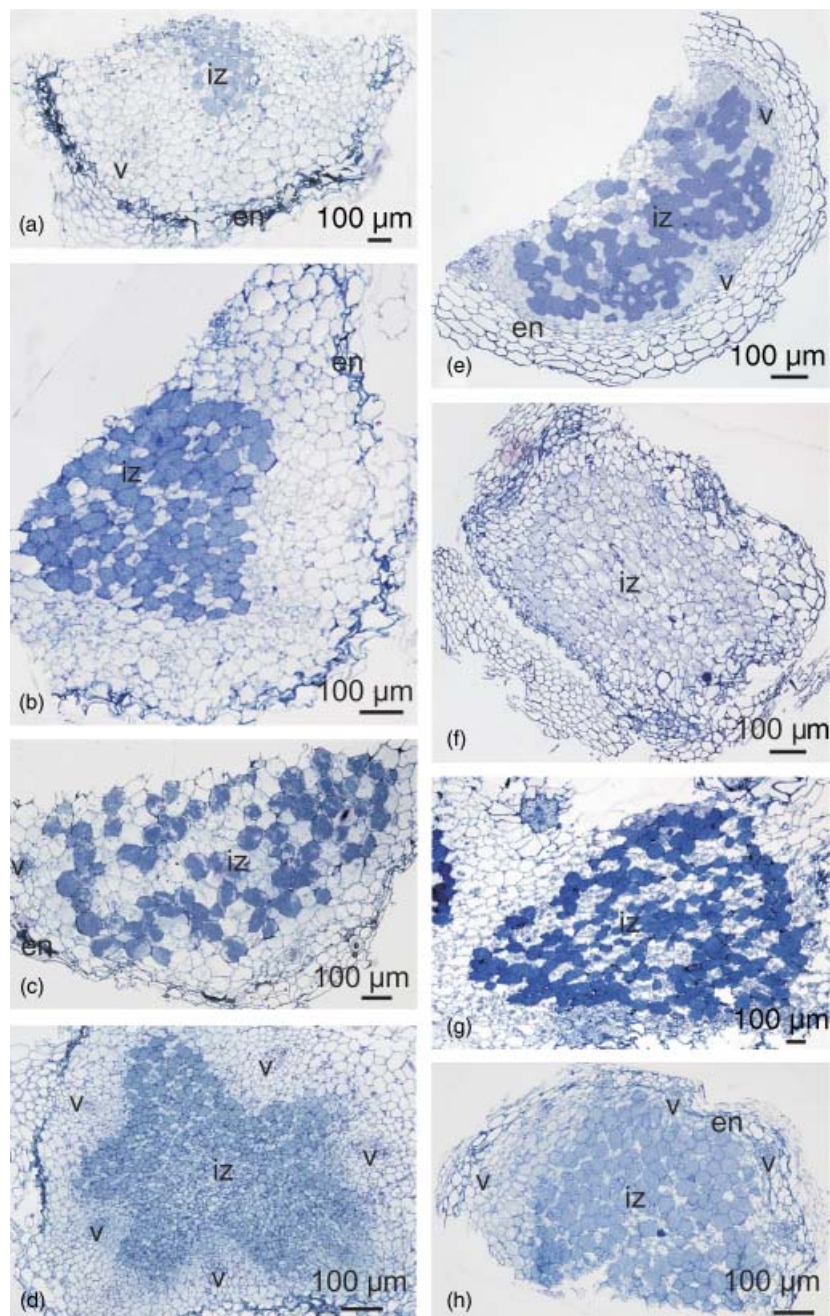


Fig. 2. Structure of the legume nodules. (a–h) Bright-field micrographs of sections stained with 1% Toluidine Blue. (a, d) *Ornithopus compressus*; (b) *Ornithopus pinnatus*; (c) *Tetragonolobus purpureus*; (e) *Hedysarum spinosissimum*; (f) *Hedysarum glomeratum*; (g) *Psoralea bituminosa*; (h) *Scorpiurus muricatus*. All examined nodules present a cortex containing vascular elements (v), an endodermis (en) and a central infected zone (iz). The reason why nodule halves rather than whole nodules were processed is that the complementary halves were used to isolate the hosted bacteria and enable a direct comparison between cultured taxa and microscopy.

most of the streak length on these plating media, indicating their abundance and culturability. In the present study, only nodules from *H. spinosissimum*, *T. purpureus*, and, from our previous Algerian campaign (Benhizia *et al.*, 2004a, b), *H. spinosissimum* ssp. *capitatum*, *H. pallidum* and *H. carnosum* yielded a dense lawn of confluent bacterial colonies, but none of these were rhizobia. As controls in our experiments we often included nodules from the cultivated legume *Hedysarum coronarium*, which, by contrast, always yielded a fully culturable load of *Rhizobium sullae*, ruling out the

possibility that the results observed with the other legumes could have arisen from a general fault in the surface sterilization procedure.

Nodulation tests

The absence of a consistent culturable rhizobial occupant revealed by the standard procedure used for the isolation of legume nodule symbionts confirmed our previous findings (Benhizia *et al.*, 2004a, b). We next checked whether the

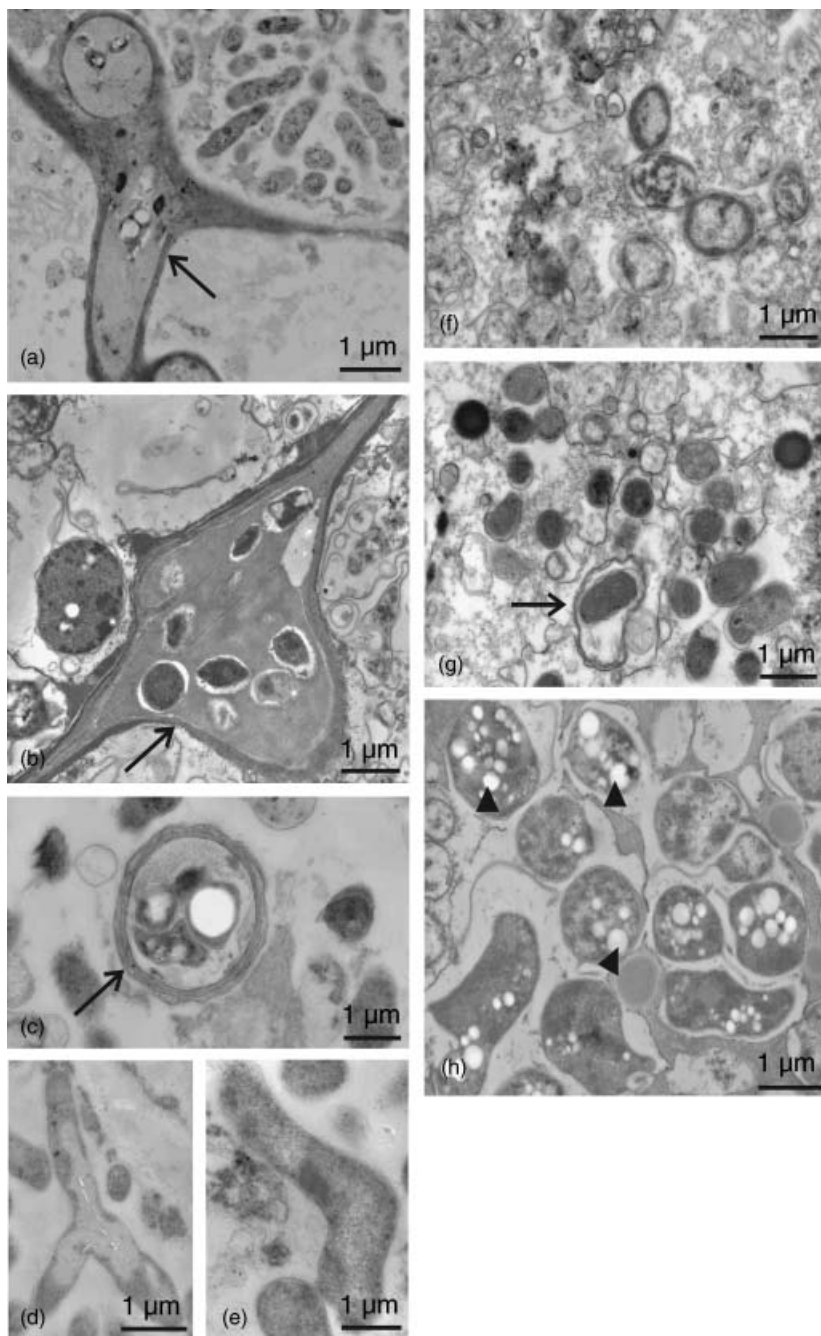


Fig. 3. Transmission electron micrographs of the infection zone in *Ornithopus compressus* and *Hedysarum spinosissimum* nodules. Arrows indicate (a) longitudinal- and (b, c) cross-sections of bacteria-containing infection threads (a, b) between and (c) inside a *Ornithopus compressus* host cell. (d, e) Endosymbiotic bacteroids of various shapes in *O. compressus* nodules. (f, g) *Hedysarum spinosissimum* micrographs from the same infected cell showing endosymbionts having different shapes and electron density. In (g) peri-bacteroid membranes surround the symbiotic bacteroids (arrow). (h) Rod-shaped bacteria containing granules of β -hydroxybutyrate (arrowheads).

various bacterial isolates could induce nodule formation on their hosts under gnotobiotic conditions. A series of nodulation tests was performed in growth cabinets using sterilized sand/vermiculite in Leonard jars, with the cultivation period extended up to two months. Five plant species, propagated from surface-sterilized seeds, were tested, including all *Hedysarum* species used here and in our previous studies, plus *Ornithopus compressus*, which was tested for the first time. Tests were repeated four times in two laboratories

(Padova and Constantine). A total of 24 distinct purified isolates, encompassing all the diverse taxa in Table 1 and including 10 strains of *Gammaproteobacteria* from the previous study (Benhizia *et al.*, 2004a, b), were tested either alone or in mixed inocula containing up to 10 strains. These inocula included the strains whose 16S rRNA gene had high similarity to *Mesorhizobium* and *Rhizobium*. No nodules were produced in any of these gnotobiotic plant tests, including those inoculated with the above isolates related

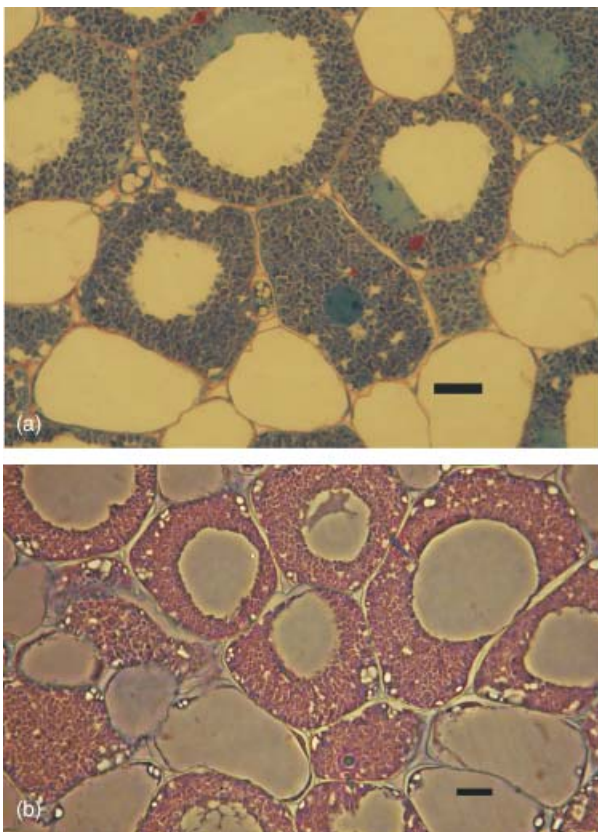


Fig. 4. (a) Optical microscopy of Aniline Blue Black/Basic Fuchsin double-stained thin sections from a *Hedysarum glomeratum* nodule. Plant cell walls and bacterial infection threads (thick round cross-sections) stain red. Plant nuclei stain from dark to light blue in relation to cell physiological status. Residual plant cytoplasm stains very pale blue. Bacteroids filling partially or totally plant cells stain dark grey to brown. Vacuoles (large central cell lumens) and starch granules (clumped masses in small interstitial cells) remain white. Scale bar: 10 μm . (b) Optical microscopy of Basic Fuchsin/Astra Blue double-stained thin sections from a *H. glomeratum* nodule. Plant cell walls stain greenish blue, bacterial infection threads stain blue (pointed lozenge shape between two plant cells in the upper right of the image). Vegetative bacteria inside infection threads stain purple. Bacteroids stain pale fuchsia. Vacuoles do not stain, and starch granules appear refractile white. Scale bar: 10 μm .

to rhizobia. Occasionally, shovel-like swellings similar to those that develop in *sulla*, *H. coronarium* (Squartini *et al.*, 1993), developed on *H. spinosissimum* roots, but these are known to be modified short lateral roots that form independently of bacteria (A. Squartini & F. Dazzo, unpublished data).

Direct 16S PCR from nodules

Because the negative nodulation results indicated that the various bacterial occupants did not induce the nodules from which they were isolated, we performed direct PCR from the squashed nodule samples, targeting 16S rRNA gene with

bacterial primers to test the hypothesis that the true rhizobial occupants may have lost their culturability within the nodules. The results (Table 2) clearly showed that, indeed, most of these nodules actually contain a dominant amount of rhizobial DNA, sufficient in most cases to overwhelm and outcompete in the PCR amplification the heterologous DNA of the other nonrhizobial occupants and produce a clean sequence chromatogram upon nucleotide sequencing of the amplicon. In essence, this approach is not intended to examine the diversity of bacteria in nodules but rather to reveal which is the most abundant species within them. The bacterial rRNA gene from some of the nodules gave a mixed but still readable sequence, with a major template series of peaks superimposed over a rather high background (data not shown), further confirming that rhizobia are dominant but not alone within the nodule. In the case of nodules of *H. spinosissimum*, *T. purpureus*, and the Algerian *Hedysarum*, for which the nonrhizobial occupants produced the most profuse growth on plates (rather than sparse colonies), the direct PCR also resulted in the dominant amplification of the gammaproteobacterial occupant. The only case for which the 16S rRNA gene sequence from the whole nodule squash matched the same taxon as the corresponding culturable isolate was the *Mesorhizobium* sporadically found in *T. purpureus*.

Cultivation of wild plant species from seed in their natural soil

The above studies were performed on flowering plants collected during April 2004 and 2005. To test whether the rhizobia eventually lose culturability within the nodules or during a possible late-seasonal physiological stress of the plants, a growth chamber test was set up using seedlings of *H. spinosissimum* and *H. glomeratum* derived from natural seeds collected in July–August. Seeds were surface-sterilized, germinated on PCA plates, and transplanted into Leonard jars filled with Sardinian soil from two sites where the two species naturally occur (one was a compact soil from Giòscari, and the other was a sandy soil from an erosive hill near the coastal city of Castelsardo). Plants were harvested as early as 40 days after germination (early three-leaf stage) in order to obtain young nodules induced and invaded by their natural microbial partners before exposure to the stress of mature plant senescence. Both hosts formed two to five nodules in both soils. Ten nodules were analysed, but even in this case the same situation as observed with all the field-collected plants was confirmed. That is, three nodule squashes did not yield colonies, and the other seven produced one to five colonies per nodule. The identity of these culturable taxa revealed 99% similarity to *Bacillus simplex* (DQ457600) from five nodules, 98% similarity to *Bacillus megaterium* (DQ457599) from two nodules, and one single

Table 1. Number of nodules in which members of the above phylogenetic groups (identified by sequence homology to database) were found in the various plants upon colony culturing and 16S rRNA gene sequencing

Host plant	<i>Hedysarum spinosissimum</i>	<i>Hedysarum glomeratum</i>	<i>Tetragonolobus purpureus</i>	<i>Hippocrepis unisiliquosa</i>	<i>Scorpiurus muricatus</i>	<i>Psoralea bituminosa</i>	<i>Ornithopus compressus</i>	<i>Ornithopus pinnatus</i>	Total
No growth	0	3	0	9	5	4	1	2	24
<i>Enterobacteriaceae</i> *8		1	1	0	0	0	8	4	22
<i>Bacillus</i> †	2	2	2	1	5	0	0	1	13
<i>Pseudomonas</i> ‡	6	0	0	0	0	0	6	0	12
<i>Curtobacterium</i> §	0	0	0	0	0	0	0	4	4
<i>Rhizobium</i> ¶	2	0	0	0	0	0	0	1	3
<i>Staphylococcus</i>	0	1	2	0	0	0	0	0	3
<i>Xanthomonas</i> **	2	0	0	0	0	0	0	0	2
<i>Microbacterium</i> ††	0	0	0	0	0	0	0	1	1
<i>Arthrobacter</i> ‡‡	0	0	0	0	0	1	0	0	1
<i>Mesorhizobium</i> §§	0	0	1	0	0	0	0	0	1
<i>Agrobacterium</i> ¶¶	1	0	0	0	0	0	0	0	1
<i>Ralstonia</i>	0	1	0	0	0	0	0	0	1
<i>Thiobacillus</i> ***	0	0	0	0	1	0	0	0	1

The following numbered notations specify which taxa present in the GenBank database share the highest homologies with the sequences obtained. The accession number in parenthesis refers to the novel deposited sequences.

*Including: *Enterobacter agglomerans* 97–100% (DQ457576), *Pantoea ananatis* 98% (DQ457592), *Erwinia persicina* 100% (DQ457577).

†Including: *Bacillus simplex* 100% (DQ457586), *Bacillus megaterium* 99% (DQ457585), *Paenibacillus* sp. 98% (DQ457593).

‡Including: *Pseudomonas* sp. 98–100% (DQ457590), *P. lini* 99% (DQ457578), *P. viridiflava* 100% (DQ457579).

§Including *Curtobacterium flaccumfaciens* 100% (DQ457589), *C. herbarum* 99% (DQ457575).

¶Including: *Rhizobium* sp. ORS1466 100% (DQ457594), ORS1465 96% (DQ457595).

||Including *Staphylococcus epidermidis* 100% (DQ457587), *S. pasteurii* 100% (DQ457583).

***Xanthomonas translucens* 100% (DQ457591).

††*Microbacterium* sp. 99% (DQ457581).

‡‡*Arthrobacter* sp. 99% (DQ457580).

§§*Mesorhizobium loti* 100% (DQ457584).

¶¶*Agrobacterium* sp. SDW052 99% (DQ457596).

|||*Ralstonia pickettii* 100% (DQ457588).

****Thiobacillus* sp. 99% (DQ457582). The total number of nodules refers to the occurrence of each bacterial species and is therefore not coincident with the absolute number of nodules examined, as in some instances multiple microbial taxa were isolated from the same nodule. No remarkable differences in the occurrence of taxa were observed comparing the 2 years of isolation.

colony with 99% similarity to *Thiobacillus* sp. (DQ457598). Nevertheless, the direct PCR of bacterial 16S rRNA genes from the nodule squashes gave the same sequence with 99% identity to *Mesorhizobium* sp. strain H-4 (AF279889). In terms of endophyte dynamics, considering that, as in *H. coronarium*, nodules are first observed one month after seedling inoculation, the nodules examined in this case represent early phases of their ontogenesis and confirm that endophytic invasion of the nodules has already started at this stage.

Some of the *H. glomeratum* plants grown in soil from Giòscari were kept for up to three months in the growth cabinet. An analysis of seven nodules from these plants yielded culturable colonies with 100% similarity to *Staphylococcus aureus* (DQ457597), 100% to *Rothia mucillaginosa* (DQ457602), and, in the case of one nodule, an isolate (DQ457601) with 99% identity to *Mesorhizobium* sp. strain H-4 (AF279889), whose identity completely matches the one consistently obtained by direct PCR from nodule

squashes of this legume species, indicating that in this case a sporadic event of culturability of the rhizobial occupant was possible (the above GenBank accession numbers refer to the matching sequences). A subsequent nodulation test, this time under sterile conditions, on the same host plant *H. glomeratum* was run with this cultured strain, which proved able to reinduce the abundant formation of nodules and sustain plant growth, as indicated by the green foliage and healthy aspect. However, attempts at reisolating the inoculated strain led again to the usual scenario: most nodules, from individual plants, did not yield any growth. Only from one nodule could we recover three single colonies, whose ARDRA profile confirmed the identity of the inoculated *Mesorhizobium*.

Acetylene reduction assays

The nitrogen-fixing functionality of nodules was tested by means of the acetylene reduction activity test. Nodules from

Table 2. Results of nucleotide sequencing by direct 16S rRNA gene PCR amplification from the nodule squash

Plant host	Number of nodules	GenBank code	Best-match homologies to GenBank taxa, found with direct 16S PCR of individual nodules
Sardinia, Italy			
<i>Hedysarum spinosissimum</i>	5	DQ457614	(<i>Mesorhizobium</i> sp. (<i>H. spinosissimum</i>) H-4 (AF279889) 99%
	1	DQ457613	<i>Rhizobium</i> sp. (AY278884) 99%
	1	DQ457612	<i>Pantoea ananatis</i> (AY173021) 96%
<i>Hedysarum glomeratum</i>	2	DQ457611	(<i>Mesorhizobium</i> sp. H-4 (AF279889) 99%
<i>Hippocrepis unisiliquosa</i>	2	DQ457615	(<i>Mesorhizobium</i> sp. H-4 (AF279889) 100%
	2	–	not amplifiable
<i>Scorpiurus muricatus</i>	6	DQ457619	<i>Mesorhizobium</i> sp. (<i>Argyrobium uniflorum</i>) (AY500256) 99%
<i>Tetragonolobus purpureus</i>	2	DQ457620	<i>Mesorhizobium</i> sp. (<i>Argyrobium uniflorum</i>) (AY500256) 99%
	1	DQ457621	<i>Pseudomonas</i> sp. KD (AY456697) 98%
<i>Ornithopus compressus</i>	3	DQ457616	<i>Bradyrhizobium</i> sp. Spain 5 (AF461195) 100%
<i>Ornithopus pinnatus</i>	3	DQ457617	<i>Bradyrhizobium</i> sp. Spain 6 (AF461196) 98%
<i>Psoralea bituminosa</i>	2	DQ457618	<i>Mesorhizobium chacoense</i> (AJ278249) 98%
Algeria			
<i>Hedysarum</i> sp. ssp. <i>capitatum</i>	4	DQ457608	(<i>Mesorhizobium</i> sp. (<i>H. spinosissimum</i>) H-4 (AF279889) 100%
	2	DQ457609	<i>Pseudomonas</i> sp. TM7.1 (<i>Tuber magnatum</i>) (DQ279324) 100%
	1	DQ457610	<i>Pantoea ananatis</i> (AY173021) 98%
<i>Hedysarum pallidum</i>	2	DQ457606	<i>Pantoea ananatis</i> (AY173021) 98%
	2*	–	Not amplifiable
	1	DQ457607	<i>Pseudomonas</i> sp. CLb01 (AY574283) 99%
<i>Hedysarum carnosum</i>	2	DQ457604	<i>Pantoea ananatis</i> (AY173021) 98%
	1	DQ457603	<i>Enterobacter hormaechei</i> (AJ853889) 98%
<i>Hedysarum naudinianum</i>	4	–	Not amplifiable
	1	DQ457605	<i>Pseudomonas</i> sp. CLb01 (AY574283) 99%

The number of nodules from which each given result was obtained is indicated, along with the GenBank accession number of a representative sequence from that group. The percentage identity with database sequences is indicated. * Both nodules of *Hedysarum pallidum* from which no amplicons could be obtained are from plants collected in a mine site contaminated with high levels of antimony.

40-day-old plants grown in the growth chamber were assayed. Species tested included *H. glomeratum* grown in two types of soil (Giòscari or Castelsardo), *S. muricatus* grown in soil from Giòscari, and *H. glomeratum* grown in sterile conditions in vermiculite mix inoculated with the above-described single culturable strain of *Mesorhizobium*. As a control, *H. coronarium* of the same age inoculated in vermiculite with *R. sulae* type strain IS123 was used. Data indicated that the nodules from each of the combinations reduced acetylene to an extent not significantly different from that displayed by the *sulla* positive control. Values observed ranged between 2 and 10 nanomoles mg⁻¹ of nodule dry weight per hour. Attempts to cultivate rhizobia from other nodules from the same roots, not used for the ARA tests, yielded the usual nonculturability result. This analysis rules out the possibility that the nonrecoverable state of the rhizobia could correlate with a generally inefficient (fix-minus) phenotype of the nodules.

Variations tested in the isolation procedure

The hypothesis of a general problem resulting in too harsh a sterilization method was investigated. Using nodules either

collected in nature or developed in the growth cabinet, we tested various alternatives among the standard methods used for rhizobia (Vincent, 1970; Jordan, 1984; Somasegaran & Holben, 1985). However, using either hypochlorite or mercuric chloride-based procedures on nodules from *H. glomeratum* or *H. spinosissimum* did not alter the outcome. A lower concentration of NaClO (3%) was also tested, yielding the usual results. However, the same protocols used on positive-control nodules of *sulla* (*H. coronarium*) always enabled full recovery of *R. sulae* as ascertained by ARDRA and 16S rRNA gene sequencing. In light of possible osmotic damage or salinity impact we checked both saline solution and distilled water as alternatives for resuspending bacteria from nodule squashes, and, to dilute bacterial inhibitors that might be present, we performed serial dilutions prior to plating. None of these measures succeeded in solving the problem. We also considered the possible sensitivity to acidic pH for the rhizobia of our wild legumes, as indicated for certain strains of *Sinorhizobium meliloti* (Howieson *et al.*, 1988) and *Bradyrhizobia* from *Arachis* (Macciò *et al.*, 2002). In these species, colony development of rhizobia is halted by the acidification resulting from their own metabolism. The problem is often coupled with the need for calcium

(Howieson *et al.*, 1992), and we addressed it with media reported to circumvent the inhibition such as YMA containing calcium carbonate (Jordan, 1984) or media featuring the phosphate buffer (Howieson *et al.*, 1988; Nandasena *et al.*, 2001). However, none of these relieved the nonculturability phenomenon.

Exploring the possibility of a strict seasonal dependence of rhizobium viability, we collected *H. spinosissimum* in early February 2006 (in the Algerian site of Constantine), while still in its youngest recognizable stage, consisting of the newly emerged 3–4 cm tall plantlet displaying the first composite leaf and an average of two to three root nodules. The results (no culturable rhizobia) were no different from the ones observed in spring isolations. We again included, as a positive control, nodules from a spontaneous stand of *H. coronarium* collected on the same day in a nearby location, which produced, as expected, regularly growing streaks of *R. sulae*. Nevertheless, a possibility still exists that rhizobia from these hosts, for inherent physiological reasons, are particularly sensitive to all surface sterilization procedures so far used for the isolation of nodule symbionts.

Presence of endophytic bacteria in other parts of the plants

In order to test whether internal colonization of the non-rhizobial species in these legumes is limited to nodules (as opposed to a systemic plant invasion), specimens of *H. spinosissimum*, *T. purpureus*, *P. bituminosa* and *S. muricatus* collected between April and May 2005 in two locations in Sardinia were examined for endophytic colonization in primary and secondary root segments and in

stems. For comparison, we examined the cultivated legume *sulla* (*H. coronarium*), whose nodules consistently yield their culturable, well-characterized rhizobial symbiont previously described by our group as the novel species *R. sulae* (Squartini *et al.*, 2002). Surface sterilization was confirmed by sterility control tests. The majority of plant species, including *sulla*, were found to contain an internal community of culturable bacteria within all parts examined. The number of CFUs on PCA plates (Table 3) reached values typically above 10^5 g^{-1} of root tissue, consistent with the numbers found by other authors for plant endophytism. The highest value was found in a primary root of *H. spinosissimum*, which yielded nearly 10^7 CFU g^{-1} of fresh weight. In our study, stems of the wild species generally harboured populations that were one to two orders of magnitude smaller than those found in the roots, with the exception of *H. coronarium*, the cultivated control, in whose stems nonrhizobial culturable endophyte densities were as high as in roots. Plants of *Scorpiurus muricatus* were the only case from which no culturable microbial community was recoverable on PCA plates. It should be recalled that nodules of this host were also often devoid of a culturable biota (Table 1).

Direct PCR was also performed on dominant bacterial 16S rRNA genes within macerated tissues of previously surface-sterilized root and stem segments. In about 75% of the cases (consistent with levels exceeding 1×10^4 CFU g^{-1}), the macerates produced a gel-visible amplicon from which a clean sequence could be obtained, enabling direct assay and determination of the dominant endophytes in these plant tissues. Such test revealed that macerates of *H. spinosissimum* and *H. coronarium* roots and *T. purpureus* stems

Table 3. Mean values of PCA-culturable colony forming units obtained from surface-sterilized portions of plants harvested in nature or germinated and grown in cabinet conditions

Plant and parts tested	Mean number of CFUs g^{-1} fresh weight
Field-collected plants (late spring)	
<i>Hedysarum spinosissimum</i> , primary roots	8.9×10^4 (Max. 1.1×10^7)
<i>Hedysarum spinosissimum</i> , secondary roots	4.3×10^5
<i>Hedysarum spinosissimum</i> , stems	2.1×10^3
<i>Hedysarum coronarium</i> , primary roots	2.5×10^5
<i>Hedysarum coronarium</i> , stems	4.1×10^5
<i>Psoralea bituminosa</i> , primary roots	3.6×10^5
<i>Psoralea bituminosa</i> , stems	0.1×10^2
<i>Tetragonolobus purpureus</i> , primary roots	5.0×10^3
<i>Tetragonolobus purpureus</i> , stems	4.1×10^2
<i>Scorpiurus muricatus</i> , primary roots	0
<i>Scorpiurus muricatus</i> , stems	0
Plants grown from surface-sterilized seeds, transplanted in natural soil, in a growth chamber	
<i>Hedysarum spinosissimum</i> , 5-day-old seedlings roots (before transplant)	0
<i>Hedysarum spinosissimum</i> , 40-day-old primary roots	0.3×10^2

Values are the average of three to six plant specimens per species. SDs (not shown) ranged in all cases within 10% of the mean value, with the exception of primary roots of *Hedysarum spinosissimum*, for which a single specimen with a particularly high value (1.1×10^7) was recorded.

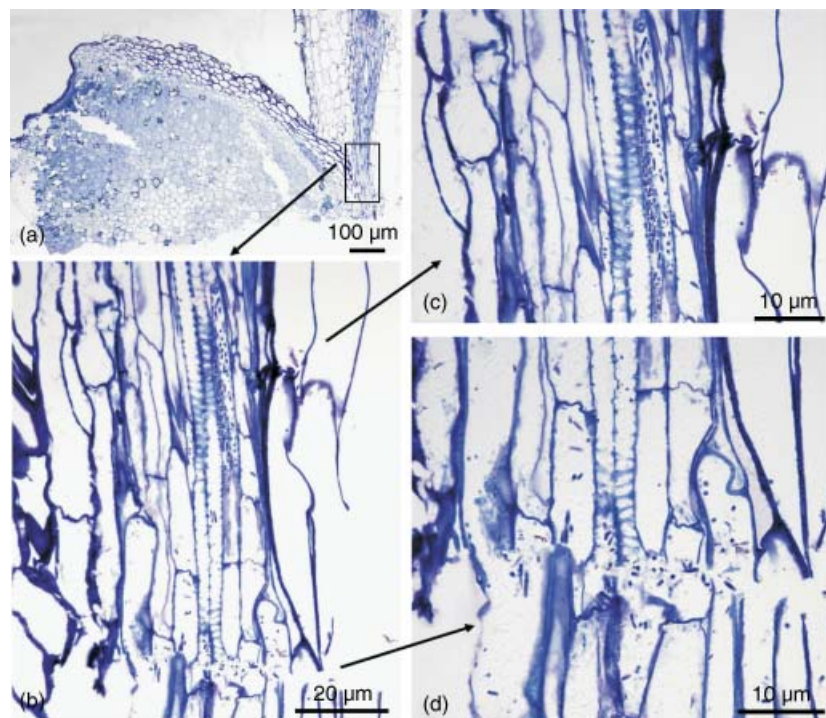


Fig. 5. Toluidine-stained longitudinal sections from a nodule and root of *Hedysarum naudinianum*. (a) Sections were taken from the junction between a nodule and the primary root. (b) High magnification of the square in (a). (c, d) At higher magnification, bacteria-like rods are clearly visible in the vascular elements of the primary root.

contain a recurring endophyte having 98% similarity to *Pseudomonas* sp. K94.14 (AY456697).

By varying the sampling date after planting in this same experiment, it was possible to ascertain how early the general endophytes establish in the plant and whether they are seed-borne. Roots of the young seedlings of *H. spinosissimum* were devoid of culturable endophytes, whereas surface-sterilized roots of 40-day-old plants harboured bacterial endophytes that were orders of magnitude lower than what we recorded at flowering stage under natural conditions (Table 3).

We also examined the relative abundance and location of endophytes *in situ* by plant microscopy. Figure 5, a toluidine-stained thin section, shows a junction between a nodule and the primary root of *H. naudinianum*, with short rod-shaped bacteria that gained access to the vascular tissue near the nodule. This suggests that an endophytic infection process is used to enter the plant, unlike that used by rhizobia. Stem invasion by bacteria in *H. spinosissimum* was investigated by epifluorescence microscopy upon staining thin sections with DNA-staining propidium-iodide, and indicated the presence of sparse bacteria in intracellular positions (data not shown). Finally, immunofluorescence microscopy was used to locate *Pantoea agglomerans* as the most abundant enterobacterial endophyte within *H. spinosissimum* nodules based on direct PCR results. Toluidine blue was used to quench root autofluorescence. One thousand plant nodule cells were examined. Of these, about 100 (10%) contained immunofluorescent-positive short rod-

shaped bacteria (Fig. 6). No immunofluorescent bacteria were found in *c*. 1500 plant cells examined in the negative-control plant sections. A nodule section of low magnification is presented in Fig. 6a, showing the typical central zone containing infected host cells with or without a vacuole in their centre. Higher magnification reveals plant cells containing isolated immunofluorescing rods (Fig. 6b–d). These results provide evidence that enterobacterial endophytes are not restricted to vascular bundles or outer cortical regions in legume nodules but can also occupy the effective bacteroid tissue.

Discussion

The data presented here emphasize two important aspects of rhizobiology, namely that root nodules of some Mediterranean legumes harbour prevalingly nonculturable rhizobia, and that these same nodules are colonized internally by an array of culturable nonrhizobial endophytes. The microscopical analysis is supportive of this indication. Although the rhizobial endosymbiont exhibits various transition stages from vegetative to bacteroids, the striking variation in morphology of the bacteria within nodules is in line with the presence of other nonrhizobial taxa. Their occurrence is collectively supported by several lines of microscopical evidence. First, there appears to be more variation in bacterial morphotypes within a given host nodule cell than is typical for rhizobia (Fig. 3a–g and h). Second, the degree of electron opacity varied dramatically among some

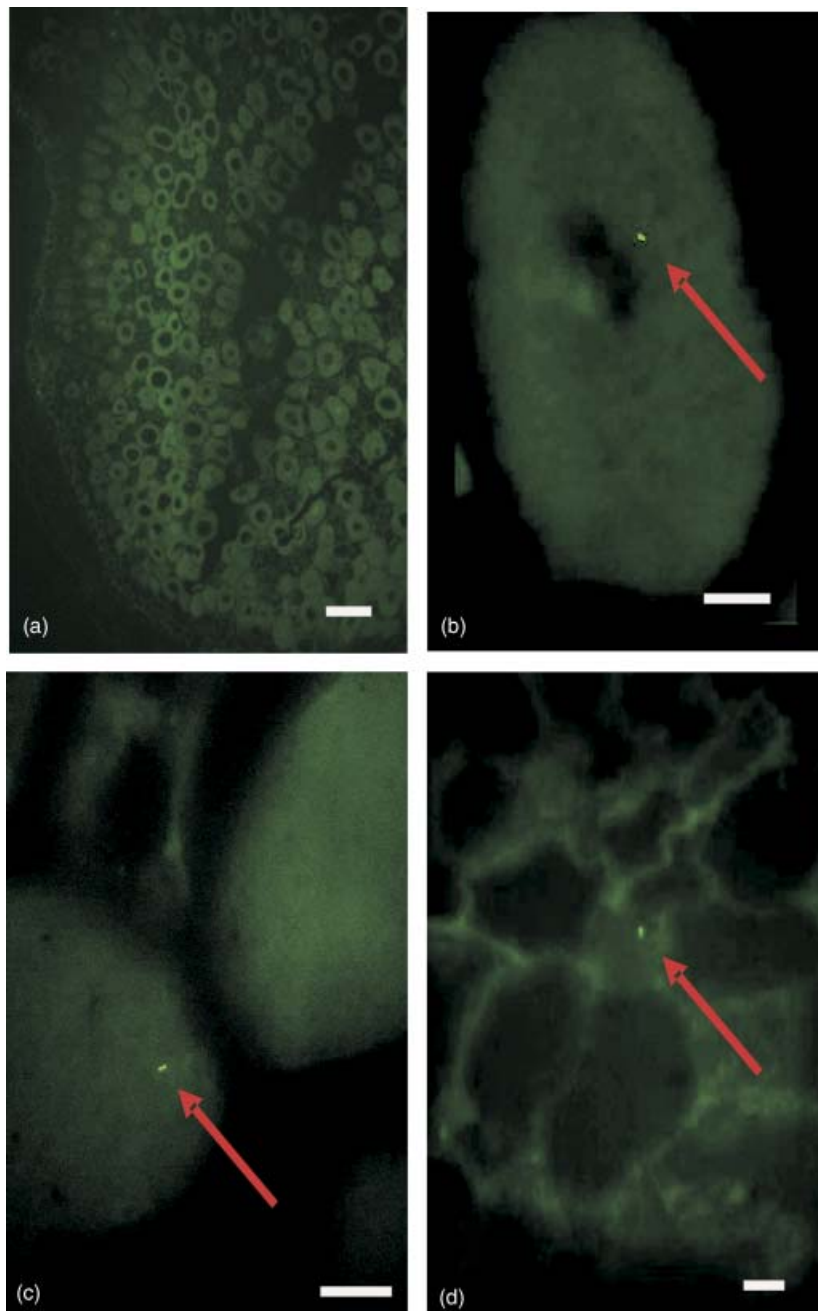


Fig. 6. Antibody-mediated fluorescent microscopy immunolocalization of *Enterobacter* on thin sections of a *Hedysarum spinosissimum* nodule. (a) Low-magnification image of the nodule showing the background level of fluorescence. (b–d) Higher-magnification examples of single isolated fluorescent antigen-bearing vegetative rods (indicated by arrows) among dark masses of nonfluorescing bacteroids. Scale bars: (a) 0.1 mm; (b–d) 10 μ m.

intracellular bacteria within a given infected host cell, more so than the variation typically exhibited by rhizobia *in planta* (Fig. 3f and g). Third, peribacteroid membranes enclosed intracellular bacteria in some cases, whereas these signature ultrastructural features of the rhizobial endosymbiotic state were notably absent in other cases (compare, for example, Fig. 3g and h). Fourth, the immunolocalization with an *Enterobacter*-specific antibody points towards its coexistence in the bacteroid tissue.

By introducing a direct PCR analysis of nodule endophytes, the problem of nonculturability can be bypassed, and in most cases this approach reveals the putative rhizobial aetiological agent that resides in each nodule. Recovery of the rhizobial occupants to the culturable state could not be achieved under the conditions used. This phenomenon of nonculturable rhizobia within nodules appears to be commonplace, as we tested eleven legume species, including annuals and perennials, belonging to six genera distributed

in three tribes (*Hedysareae*, *Psoraleae* and *Loteae*). The condition appears widespread in both continents facing the Mediterranean Sea. Parallel studies of nodules from cultivated legumes in the same area, such as clover (Mateos *et al.*, 1992), pea (Corich *et al.*, 2001), and sulla (*H. coronarium*), routinely yielded fully culturable rhizobia, implying that physiological hindrances imposed by climatic or habitat factors could not explain why rhizobia lost their culturability within nodules of the legumes investigated in the present study. A question that arose was whether rhizobia could ever be cultured from nodules of these legumes. This was the case for *H. spinosissimum*, from which in Israel, Kishinevsky *et al.* (1996, 2003), using standard methods, isolated strains whose 16S rRNA gene sequence clusters in the *Mesorhizobium* branch with 99% identity to the sequence that we obtained by direct PCR from the unculturable occupant of nodules of the same species. To this we can add the single isolate from one of our *T. purpureus* nodules (Table 1). Thus, culturability appears to be an exception rather than the usual state. It should be noted, however, that the isolation of unexpected taxa from nodules can often be disregarded as the result of an inefficient sterilization procedure. While presenting preliminary results of these findings at a Nitrogen Fixation meeting (Benhizia *et al.*, 2004a) and shortly afterwards, we received three independent personal communications (Marta Laranjo, Therese Atallah, and Ines Soares) sharing the unpublished experience of having found (in Portugal, Lebanon and Uzbekistan, respectively) also *Enterobacteriaceae* or *Pseudomonadaceae* instead of rhizobia from nodules of several different wild legumes, including some of the ones investigated in our project. Further evidence of this phenomenon is the above-quoted report from Zakhia *et al.* (2006). Other studies also suggest the same pattern. Brundu *et al.* (2004) examined 15 wild species of *Medicago* in Sardinia and isolated 125 strains from nodules: only 29 were able to re-nodulate their host in gnotobiotic culture (as expected from a *Sinorhizobium* partner); the remaining 94 nodule isolates were saprophytes. Ben Romdhane *et al.* (2005) used a direct PCR protocol to study the symbionts of *Acacia tortilis* in Tunisia: 25.8% of their nodule-associated bacteria were not identified as rhizobia.

Our data showed that the diverse nodule endophytes are most often represented by a few CFUs, allowing identification of the unculturable rhizobium by direct nodule PCR. In nodules of some host species (*H. spinosissimum*, *T. purpureus*), however, the nonrhizobial occupant is sometimes abundant ($\geq 5 \times 10^5$ CFU nodule⁻¹), yielding a dominant 16S rRNA gene sequence. In other cases, represented by four *Hedysarum* species examined in Algeria, the direct PCR analysis of nodules revealed nonrhizobial sequences or no amplifiable DNA, raising doubts about the presence of rhizobia at all. Interestingly, the culturable inhabitants of

nodules (*Pseudomonas*, *Enterobacter/Pantoea* and others) consistently produce a uniform lawn of growth instead of isolated colonies on plates. Our earlier hypothesis (Benhizia *et al.*, 2004b), that the gammaproteobacterial nodule occupants may represent the nitrogen-fixing symbiont replacing rhizobia for those legumes, seems unlikely in light of what we found here, implying that the rhizobia microsymbiont in nodules of Algerian *Hedysarum* species could be quantitatively overwhelmed and masked by endophytes in ways that prevent their detection even by PCR. The inability of pure cultures of the nonrhizobial occupants to nodulate the legume under microbiologically controlled conditions precludes the hypothesis of their involvement in that type of symbiosis. Moreover, dedicated microarray analysis using oligonucleotides for *nodC* and *nifH* genes (Bontemps *et al.*, 2005) on two gammaproteobacterial strains isolated from *H. pallidum* and *H. spinosissimum* (Benhizia *et al.*, 2004b) revealed no detection of these genetic determinants for nodulation or nitrogen fixation. However, the abundance of these bacterial endophytes within perfectly healthy plants suggests that other beneficial interactions may be operative. In retrospect, we can hypothesize that the different bacterial taxa found by Zakhia *et al.* (2006) in nodules of legumes, some of which had previously been reported to yield rhizobia (Zakhia *et al.*, 2004), might also represent endophytes growing from nodules whose rhizobia were not easy to culture as a result of the same phenomenon observed in this paper.

In addition to the early demonstration of endophytes within legume nodules, the main issue is the unexpected nonrecoverable state that affects rhizobia in these plants. Having observed the phenomenon also when reisolating from nodules originated in sterile vermiculite (after inoculating *H. glomeratum* seedlings with the only culturable strain that we obtained), it can be concluded that the phenomenon is caused by the plant itself and not by other possible environmental factors existing in the soils of origin. The acetylene reduction test on *H. glomeratum* and *S. muricatus* showed that nodules do possess nitrogenase activity comparable to that of sulla. Our current work aims to investigate the physiological status of the rhizobia within those nodules, using stains reporting membrane-integrity and respiratory activity to assess whether they are alive and metabolically active. If rhizobia loose viability in these legume root nodules, it would be difficult to explain their persistence in the soils at levels that do not limit legume nodulation, unless they could occupy other niches that permit their multiplication, for example as cereal root endophytes (Yanni *et al.*, 1997). On the other hand, there are reports of rhizobia such as *R. leguminosarum* (Alexander *et al.*, 1999) and *R. sulae* (Toffanin *et al.*, 2000) that enter the 'viable but not culturable' (VBNC) state, although the involvement of host legumes in this syndrome has not yet been established. It is possible that the diverse endophytes

coinhabiting the nodule may produce antagonistic compounds negatively affecting rhizobia, and/or may trigger a systemic host defence response resulting in the production of inhibitory compounds such as salicylic acid (De Meyer *et al.*, 1999) to which the rhizobial symbionts might have a differential sensitivity. Indeed, some nonpathogenic bacteria can themselves produce sufficient levels of salicylic acid to generate reactive oxygen species (De Meyer *et al.*, 1999; Audenaert *et al.*, 2002). We also plan to examine if these undomesticated legumes differ from those cultivated in agriculture in their production of metabolites that inhibit rhizobia *in planta* and/or when carried-over with rhizobia to culture on plating media. Investigating these aspects will provide a better insight into the microbial interactions occurring in native and introduced wild legume plants and will lead to a better understanding of their nitrogen-fixing symbioses.

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References

- Alexander E, Pham D & Steck TR (1999) The viable but nonculturable condition is induced by copper in *Agrobacterium tumefaciens* and *Rhizobium leguminosarum*. *Appl Environ Microbiol* **65**: 3754–3756.
- Audenaert K, Pattery T, Cornelis P & Höfte M (2002) Induction of systemic resistance to *Botrytis cinerea* in tomato by *Pseudomonas aeruginosa* 7NSK2: role of salicylic acid, pyochelin, and pyocyanin. *Mol Plant-Microbe Interact* **15**: 1147–1156.
- Bai Y, Zhou X & Smith D (2003) Enhanced soybean plant growth due to coinoculation of *Bacillus* strains with *Bradyrhizobium japonicum*. *Crop Sci* **43**: 1774–1781.
- Benhizia Y, Benhizia H, Benguedouar A, Muresu R, Giacomini A & Squartini A (2004a) The root nodules of *Hedysarum carnosum*, *H. spinosissimum* subsp. *capitatum*, and *Hedysarum pallidum* (Leguminosae) host Gammaproteobacteria. 6th European Nitrogen Fixation Conference, Toulouse, July 24–27th 2004.
- Benhizia Y, Benhizia H, Benguedouar A, Muresu R, Giacomini A & Squartini A (2004b) *Gammaproteobacteria* can nodulate legumes of the genus *Hedysarum*. *Syst Appl Microbiol* **27**: 462–468.
- Ben Romdhane S, Nasr H, Samba-Mbaye R, Neyra M & Ghorbal MH (2005) Diversity of *Acacia tortilis* rhizobia revealed by PCR/RFLP on crushed root nodules in Tunisia. *Ann Microbiol* **55**: 249–258.
- Bontemps C, Golfier G, Gris-Liebe C, Carrere S, Talini L & Boivin-Masson C (2005) Microarray-based detection and typing of the *Rhizobium* nodulation gene *nodC*: potential of DNA arrays to diagnose biological functions of interest. *Appl Environ Microbiol* **71**: 8042–8048.
- Brundu G, Camarda I, Caredda M, Garau G, Maltoni S & Deiana P (2004) A contribution to the study of the distribution of *Medicago*–*Rhizobium* symbiosis in Sardinia (Italy). *Agr Med* **134**: 33–48.
- Chen WM, Laevens S, Lee TM, Coenye T, de Vos P, Mergeay M & Vandamme P (2001) *Ralstonia taiwanensis* sp. nov., isolated from root nodules of *Mimosa* species and sputum of a cystic fibrosis patient. *Int J Syst Evol Microbiol* **51**: 1729–1735.
- Chen WM, de Faria SM, Stralioetto R *et al.* (2005) Proof that *Burkholderia* strains form effective symbioses with legumes: a study of novel *Mimosa*-nodulating strains from South America. *Appl Environ Microbiol* **71**: 7461–7471.
- Corich V, Giacomini A, Carlot M, Simon R, Tichy H, Squartini A & Nuti MP (2001) Comparative strain-typing of *Rhizobium leguminosarum* bv. *viciae* natural populations. *Can J Microbiol* **47**: 580–584.
- Dazzo FB (1982) Leguminous root nodules. *Experimental Microbial Ecology* (Burns R & Slater J, eds), pp. 431–446. Blackwell Scientific Publications, Oxford.
- De Lajudie P, Willems A, Nick G, Mohamed TS, Torck U, Filai-Maltouf A, Kersters K, Dreyfus B, Lindström K & Gillis M (1999) *Agrobacterium* bv. 1 strains isolated from nodules of tropical legumes. *Syst Appl Microbiol* **22**: 119–132.
- De Meyer G, Capiou K, Audenaert K, Buchala A, Metraux JP & Höfte M (1999) Nanogram amounts of salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 activate the systemic acquired resistance pathway in bean. *Mol Plant-Micro Interact* **12**: 450–458.
- Dong Y, Iniguez AL, Ahmer BMM & Triplett EW (2003) Kinetics and strain specificity of rhizosphere and endophytic colonization by enteric bacteria on seedlings of *Medicago sativa* and *Medicago truncatula*. *Appl Environ Microbiol* **69**: 1783–1790.
- Elliott GN, Chen WM, Chou JH *et al.* (2007) *Burkholderia phymatum* is a highly effective nitrogen-fixing symbiont of *Mimosa* spp. and fixes nitrogen *ex planta*. *New Phytol* **173**: 168–180.

- Elvira-Recuenco M & van Vuurde JW (2000) Natural incidence of endophytic bacteria in pea cultivars under field conditions. *Can J Microbiol* **46**: 1036–1041.
- Gagné S, Richard C, Rousseau H & Antoun H (1987) Xylem-residing bacteria in alfalfa roots. *Can J Microbiol* **33**: 996–1000.
- Hallmann J, Quadt-Hallmann A, Mahaffee WF & Kloepper JW (1997) Bacterial endophytes in agricultural crops. *Can J Microbiol* **43**: 895–914.
- Howieson JG, Ewing MA & D'Antuono MF (1988) Selection for acid tolerance in *Rhizobium meliloti*. *Plant Soil* **105**: 179–188.
- Howieson JG, Robson AD & Abbott LK (1992) Calcium modifies pH effects on the growth of acid-tolerant and acid-sensitive *Rhizobium meliloti*. *Aust J Agric Res* **43**: 765–772.
- Jordan DC (1984) Family III. *Rhizobiaceae*. *Bergey's Manual of Systematic Bacteriology, Vol. 1* (Krieg NR & Holt JG, eds), pp. 234–256. Williams & Wilkins, Baltimore, MD.
- Kishinevsky BD, Dipanker S & Yang G (1996) Diversity of rhizobia isolated from various *Hedysarum* sp. *Plant Soil* **186**: 21–28.
- Kishinevsky BD, Nandasena KG, Yates RJ, Nemas C & Howieson JG (2003) Phenotypic and genetic diversity among rhizobia isolated from three *Hedysarum* species: *H. spinosissimum*, *H. coronarium* and *H. flexuosum*. *Plant Soil* **251**: 143–153.
- Kraus JE, de Souza HC, Rezende MH, Castro NM, Vecchi C & Luque R (1998) Astra blue and basic fuchsin double staining of plant materials. *Biotech Histochem* **73**: 235–243.
- Kuklinsky-Sobral J, Araujo WL, Mendes R, Geraldi IO, Pizzirani-Kleiner AA & Azevedo JL (2004) Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. *Environ Microbiol* **6**: 1244–1251.
- Kuklinsky-Sobral J, Araujo WL, Mendes R, Pizzirani-Kleiner AA & Azevedo JL (2005) Isolation and characterization of endophytic bacteria from soybean (*Glycine max*) grown in soil treated with glyphosate herbicide. *Plant Soil* **273**: 91–99.
- Macciò D, Fabra A & Castro S (2002) Activity and calcium interaction affect the growth of *Bradyrhizobium* sp. and the attachment to peanut roots. *Soil Biol Biochem* **34**: 201–208.
- Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ & Wade WG (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl Environ Microbiol* **64**: 795–799.
- Mateos P, Jimenez-Zurdo J, Chen JW, Squartini A, Haack S, Martinez-Molina E, Hubbell D & Dazzo F (1992) Cell-associated pectinolytic and cellulolytic enzymes in *Rhizobium leguminosarum* bv. *trifolii*. *Appl Environ Microbiol* **58**: 1816–1822.
- Mhamdi R, Mrabet M, Laguerre G, Tiwari R & Aouani ME (2005) Colonization of *Phaseolus vulgaris* nodules by *Agrobacterium*-like strains. *Can J Microbiol* **51**: 105–111.
- Moulin L, Munive A, Dreyfus B & Boivin-Masson C (2001) Nodulation of legumes by members of the beta-subclass of *Proteobacteria*. *Nature* **411**: 948–950.
- Mrabet M, Mnasri B, Romdhane S, Laguerre G, Aouani ME & Mhamdi R (2006) *Agrobacterium* strains isolated from root nodules of common bean specifically reduce nodulation by *Rhizobium gallicum*. *FEMS Microbiol Ecol* **56**: 304–309.
- Nandasena KG, O'Hara GW, Tiwari RP, Yates RJ & Howieson JG (2001) Phylogenetic relationships of three bacterial strains isolated from the pasture legume *Biserrula pelecinus* L. *Int J Syst Evol Microbiol* **51**: 1983–1986.
- Old K & Nicolson T (1978) The root cortex as part of a microbial continuum. *Microbial Ecology* (Loutit M & Miles J, eds), pp. 291–294. Springer-Verlag, New York.
- Osborn AM, Moore ERB & Timmis KN (2000) An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environ Microbiol* **2**: 39–50.
- Paau AS, Bloch CB & Brill WJ (1980) Developmental fate of *Rhizobium meliloti* bacteroids in alfalfa nodules. *J Bacteriol* **143**: 1480–1490.
- Perotti R (1926) On the limits of biological enquiry in soil science. *Proc Int Soc Soil Sci* **2**: 146–161.
- Philipson MN & Blair ID (1957) Bacteria in clover root tissue. *Can J Microbiol* **3**: 125–129.
- Pignatti S (1982) *Flora d'Italia*. Edagricole, Bologna.
- Rivas R, Velazquez E, Willems A, Vizcaino N, Subba-Rao NS, Mateos PF, Gillis M, Dazzo FB & Martinez-Molina EA (2002) New species of *Devosia* that forms a unique nitrogen-fixing root-nodule symbiosis with the aquatic legume *Neptunia natans* (L.f.) Druce. *Appl Environ Microbiol* **68**: 5217–5222.
- Sattelmacher B (2001) The apoplast and its significance for plant mineral nutrition. *New Phytol* **149**: 167–192.
- Somasegaran P & Holben HJ (1985) *Methods Legume-Rhizobium Technology*. NifTAL Center, Paia, Hawaii.
- Squartini A, Dazzo F, Casella S & Nuti MP (1993) The root nodule symbiosis between *Rhizobium 'hedysari'* and its drought-tolerant host *Hedysarum coronarium*. *Symbiosis* **15**: 227–238.
- Squartini A, Struffi P, Döring H *et al.* (2002) *Rhizobium sullae* sp. nov. (formerly *Rhizobium 'hedysari'*): the root-nodule microsymbiont of *Hedysarum coronarium* L. *Int J Syst Evol Microbiol* **52**: 1267–1276.
- Sturz AV, Christie BR, Matheson BG & Nowak J (1997) Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on growth. *Biol Fertil Soils* **25**: 13–19.
- Sturz AV, Christie BR & Matheson BG (1998) Associations of bacterial endophyte populations from red clover and potato crops with potential for beneficial allelopathy. *Can J Microbiol* **44**: 162–167.
- Sy A, Giraud E, Jourand P *et al.* (2001) Methylophilic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. *J Bacteriol* **183**: 214–220.
- Timmers ACJ, Soupène E, Auriac MC, de Billy F, Vasse J, Boistard P & Truchet G (2000) Saprophytic intracellular rhizobia in alfalfa nodules. *Mol Plant-Microbe Interact* **13**: 1204–1213.
- Toffanin A, Basaglia M, Ciardi C, Vian P, Povoletto S & Casella S (2000) Energy content decrease and viable not culturable (VNC) status induced by oxygen limitation coupled to the

- presence of nitrogen oxides in *Rhizobium "hedysari"*. *Biol Fertil Soils* **31**: 484–488.
- Tokala RK, Strap JL, Jung CM, Crawford DL, Hamby Salove M, Deobald LA, Bailey F & Morra MJ (2002) Novel plant-microbe rhizosphere interaction involving *Streptomyces lydicus* WYEC108 and the pea plant (*Pisum sativum*). *Appl Environ Microbiol* **68**: 2161–2171.
- Vincent JM (1970) *A Manual for the Practical Study of the Root-Nodule Bacteria*, IBP Handbook No. 15. Blackwell Scientific Publications, Oxford.
- Yanni YG, Rizk RY, Corich V et al. (1997) Natural endophytic association between *Rhizobium leguminosarum* bv. *trifolii* and rice roots and assessment of its potential to promote rice growth. *Plant Soil* **194**: 99–114.
- Zakhia F, Jeder H, Domergue O, Willems A, Cleyet-Marel CJ, Gillis M, Dreyfus B & de Lajudie P (2004) Characterisation of wild legume nodulating bacteria (LNB) in the infra-arid zone of Tunisia. *Syst Appl Microbiol* **27**: 380–395.
- Zakhia F, Jeder H, Willems A, Gillis M, Dreyfus B & de Lajudie P (2006) Diverse bacteria associated with root nodules of spontaneous legumes in Tunisia and first report for nifH-like gene within the genera *Microbacterium* and *Starkeya*. *Microbial Ecol* **51**: 375–393.