Plasmid 68 (2012) 149–158

Contents lists available at SciVerse ScienceDirect

Plasmid

journal homepage: www.elsevier.com/locate/yplas

Review

Rhizobial extrachromosomal replicon variability, stability and expression in natural niches

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ARTICLE INFO

Article history:
Received 30 March 2012
Accepted 6 July 2012
Available online 16 July 2012
Communicated by Eva Top

Keywords:
Plasmids
Plasmid instability
Symbiotic plasmids
Rhizobium
Sinorhizobium
Ensifer

ABSTRACT

In bacteria, niche adaptation may be determined by mobile extrachromosomal elements. A remarkable characteristic of *Rhizobium* and *Ensifer* (*Sinorhizobium*) but also of *Agrobacterium* species is that almost half of the genome is contained in several large extrachromosomal replicons (ERs). They encode a plethora of functions, some of them required for bacterial survival, niche adaptation, plasmid transfer or stability. In spite of this, plasmid loss is common in rhizobia upon subculturing. Rhizobial gene-expression studies in plant rhizospheres with novel results from transcriptomic analysis of *Rhizobium phaseoli* in maize and *Phaseolus vulgaris* roots highlight the role of ERs in natural niches and allowed the identification of common extrachromosomal genes expressed in association with plant rootlets and the replicons involved.

Contents

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0147-619X
http://dx.doi.org/10.1016/j.plasmid.2012.07.002

1. Introduction

Rhizobia is a generic name to refer to several genera of α and β-Proteobacteria. Rhizobia are successful legume and non-legume rhizosphere colonizers and form nitrogen fixing nodules in legumes. Rhizobia inhabit the soil and other niches such as seeds (López-López et al., 2010; Pérez-Ramírez et al., 1998) or inside plant tissues as endophytes (Chaintreuil et al., 2000; Gutiérrez-Zamora and Martínez-Romero, 2001; Reiter et al., 2003; Yanni et al., 1997). Legumes that establish symbiosis with rhizobia can colonize nitrogen poor environments, may enrich the soil or require less chemical nitrogen fertilizers as agricultural crops.

In rhizobial research, an outstanding discovery was that symbiosis abilities resided in plasmids that could be lost or transferred among bacteria (Bánfalvi et al., 1981; Hooykaas et al., 1982; Johnston et al., 1978; Nuti et al., 1977; Nuti et al., 1979; Rosenberg et al., 1982; Sutton, 1974; Tshitinge et al., 1975; Zurkowski, 1982; Zurkowski and Lorkiewic, 1976). Symbiotic plasmids are found in *Rhizobium*, *Ensifer* = *Sinorhizobium*, in few *Mesorhizobium* species, in the β-Protobacterium *Cupriavidus taiwanensis* that forms nodules in *Mimosa* species (Amadou et al., 2008) and in *Burkholderia* sp. CCGE 1002 isolated from a nodule of *Mimosa occidentalis* collected in Tepic, Mexico (genome NCBI ID 640511). However symbiotic plasmids are not found in *Bradyrhizobium* (Cytryn et al., 2008; Hahn and Hennecke, 1987; Haugland and Verma, 1981), in *Azorhizobium caulinodans* (Lee et al., 2008) or in most *Mesorhizobium* strains (Wang et al., 1999; Xu and Murooka, 1995; Zou et al., 1997). Nitrogen fixation occurring in nodules may be considered as an ecological service. Genes involved in this process (*nif* genes) are plasmid encoded in *Rhizobium, Ensifer* (*Sinorhizobium*), few *Mesorhizobium* species, *Burkholderia* and *Cupriavidus* strains but located in chromosomes in many bacteria (reviewed in Ormeño-Orrillo et al., in press). In rhizobia, symbiosis variants (symbiovars) are recognized on the basis of host specificity and effectiveness (nitrogen fixation) mainly determined by symbiotic plasmids or islands (Rogel et al., 2011). Reviews on symbiotic plasmids (Romero and Brom, 2004) and on the bacterial and plant functions required during the symbiotic process have been published (Oldroyd et al., 2011; Peix et al., 2010).

Methods to visualize rhizobial plasmids (Eckhardt, 1978; Hirsch et al., 1980; Hynes and McGregor, 1990) were pivotal to the study of their diverse patterns, their stability and for the determination of the plasmid location of symbiosis significant genes. In addition to symbiotic plasmids, different large plasmids or extrachromosomal replicons (ER) are found in nodule forming bacteria. However, only 23% of *Bradyrhizobium japonicum* and *B. elkanii* strains from different geographical regions contained plasmids (Cytryn et al., 2008). The role of plasmids in the Rhizobiaceae focusing on interbacterial and transkingdom interactions was recently reviewed (Pappas and Cevallos, 2011). Different types of ER have been described, such as chromids (Harrison et al., 2010) as well as secondary chromosomes (Slater et al., 2009). Housekeeping and ribosomal genes that are relocated to plasmids may make them look like secondary chromosomes. ER that encode housekeeping or essential functions, stably maintained in bacteria and having a GC content similar to that of the chromosome, have been designated chromids and have been identified from genomic data in several rhizobial strains (Harrison et al., 2010). The definition of essential functions encoded in ER must be reviewed because genes may only be conditionally essential on some media or conditions. For example, a plasmid may be cured in the laboratory and thus be considered non essential but may be essential in soil or in the rhizosphere. On the other hand, use of the curing plasmid strategy to recognize essential genes may lead to erroneous conclusions if essential genes move to other replicons during the plasmid elimination (curing) process and selection of survivors. Genome sequence analysis of cured strains would reveal such events.

2. Extrachromosomal replicons in rhizobia, a substantial proportion of their genomes

We will focus mainly on *Rhizobium* with only some references on *Ensifer* and the related *Agrobacterium* genus that includes species forming tumors in plants. A remarkable characteristic of *Rhizobium*, *Ensifer* but also of *Agrobacterium* species is the large amount of genomic DNA contained in ER. From 30% to almost 50% of the genome may be extrachromosomal in symbiotic or pathogenic strains (Table 1). Agrobacterial plasmids were reviewed in Suzuki et al. (2009). Although ER may represent a burden for bacterial growth in some cases, this is not the case with rhizobial plasmids. On the contrary, they are important for bacterial physiology as has been shown for *Rhizobium etli* CFN 42 in which strains cured of most of the plasmids had larger duplication times (Brom et al., 1992). Furthermore, ER may contribute significantly to the phenotype and to the bacterial pangenome, the whole species genome.

Most rhizobial ERs are large and in low copy number. Rhizobial strains have several ERs (Table 1 in Romero and Brom, 2004), up to 11 in *R. leguminosarum*. Agrobacteria, *R. galegae*, *R. phaseoli*, *R. tropici* and *R. gallicum* seem to have fewer, 2–4. In rhizobia and in other α-Proteobacteria most ERs have *repABC* replication systems (Cervantes-Rivera et al., 2011; Pappas and Cevallos, 2011). A 7.2 kb plasmid with rolling circle replication was described in an *E. meliloti* strain but small size plasmids are uncommon in rhizobia

Table 1
Size and percent of extrachromosomal genome in rhizobia and related strains with completely sequenced genomes.

Strain	Genome size (Mb)	Percent in extrachromosomal replicons (%)
Rhizobium tropici CIAT 899	6.69	42.6
Rhizobium etli CFN 42	6.53	32.9
Rhizobium phaseoli CIAT 652	6.44	30.1
Rhizobium phaseoli Ch24-10	6.63	32.0
Rhizobium leguminosarum 3841	7.79	34.5
Rhizobium leguminosarum WSM1325	7.45	35.6
Rhizobium leguminosarum WSM2304	6.87	34.0
Rhizobium rhizogenes (*Agrobacterium radiobacter*) K84	7.31	44.7
Agrobacterium tumefaciens C58	5.65	50.0[a]
Agrobacterium vitis S4	6.31	41.0[a]
Ensifer meliloti (*Sinorhizobium meliloti*)1021	6.80	44.9
Ensifer sp. NGR234 (*Sinorhizobium* sp.)	6.90	43.0
Ensifer medicae WSM419 (*Sinorhizobium medicae*)	6.82	44.5

[a] Including the secondary chromosome that has ribosomal genes but an origin of replication typical of plasmids.

(Barran et al., 2001). ER sizes in *Rhizobium* and *Ensifer* (*Sinorhizobium*) are in the range of 45 kb to around 2.5 Mb.

3. Rhizobial hypervariable genome is in extrachromosomal elements

Chromosomes are more conserved than ER both at the gene sequence and synteny levels (Guerrero et al., 2005). Plasmid patterns are different even within a single rhizobial species (Rosenblueth and Martínez Romero, 2004; Wang et al., 1999). This is particularly evident among *R. etli* and *R. leguminosarum* strains but less variability has been observed in *R. tropici*, *R. phaseoli* or *Ensifer* plasmid profiles (not shown). Plasmid pattern differences suggest that rhizobia may thrive in different environments.

Plasmid gene content variation has been revealed from genomic projects and mosaicism seems to be a common characteristic of plasmids (Cervantes et al., 2011) and symbiotic plasmids (Freiberg et al., 1997; González et al., 2003). Recombination was evidenced with a PCR approach in *Rhizobium etli* plasmids (Flores et al., 2005). Plasmids seem to be prone to pick up novel genes or to suffer deletions. How are plasmids assembled or disassembled? Once a successful plasmid is arranged it may be stably maintained even in distinct chromosomal backgrounds over time (Crossman et al., 2008).

Duplicated copies from chromosomal genes have been allocated to plasmids. In *R. tropici* and in *R. leucaenae* a duplicated citrate synthase gene is found in the symbiotic plasmid, conditioning nodulation (Pardo et al., 1994) and differentially regulated from the chromosomal copy (Hernández-Lucas et al., 1995). Glucosamine synthase (*nodM*) duplicated genes in plasmids (Marie et al., 1992), are needed to provide additional substrates for Nod factor production.

ER may integrate into chromosomes (Guo et al., 2003), rearrange (Brom et al., 1991; Flores et al., 1988, 2000; Soberón-Chávez et al., 1986; Zhang et al., 2001) or form cointegrates with other plasmids (Brom et al., 2004; Cervantes et al., 2011; Guo et al., 2003; Mavingui et al., 2002). Fragments of plasmids may be amplified and in some cases this leads to enhanced nodulation (Mavingui et al., 1997, 1998; Romero et al., 1991; Romero et al., 1995). Extrachromosomal location of genes is not universal and fixed in strains because some genes may be in chromosomes and in other cases in extrachromosomal elements (Crossman et al., 2008 and Fig. 1). There are clues that indicate that some plasmids may be chimeras resulting from the fusion of different plasmids (Cervantes et al., 2011; Ormeño-Orrillo et al., unpublished). Plasmid co-integrates may excise correctly or incorrectly. Plasmids seem to be more dynamic than chromosomes and equivalent genes found among distinct ER in related species are evidence of extensive plasmid rearrangements (Fig. 1, Fig. 6 in Crossman et al., 2008).

4. Instability and stability of extrachromosomal elements

Plasmid instability has been known for a long time and it has been recommended to avoid the practice of single colony isolation when purifying rhizobia especially for inoculant production as they may lose relevant plasmids (Weaver and Wright, 1987). Absence of symbiotic plasmids is remarkable as rhizobial natural populations without symbiotic plasmids lose their access to legume nodules, however *R. etli* strains lacking Sym plasmids seem to be very successful rhizospheric or endophytic colonizers (López-López et al., 2010; Segovia et al., 1991). As plasmids encode carbon assimilation genes, rhizobia may change phenotype in one step when losing or gaining plasmids. After *R. etli* CFN42 was resequenced to test Illumina sequencing facilities at UNAM, it was evident that plasmid pReCFN42a was lost in the cultured cells grown to extract DNA (González and Lozano, personal communication) while the original stock maintained the whole set of plasmids. Some rhizobial strains when subcultured in the lab were prone to lose their plasmids (Weaver et al., 1990). A Tn5 had to be inserted in CFN 23 symbiotic plasmid to exert a selective pressure to maintain the plasmid in this *Rhizobium* strain (Soberón-Chávez and Nájera, 1989).

Instability has also been observed in *Burkholderia* strain CCGE 1001 isolated in our laboratory from a nodule of a *Mimosa affinis* plant grown in soils from Acayuca, Veracruz.

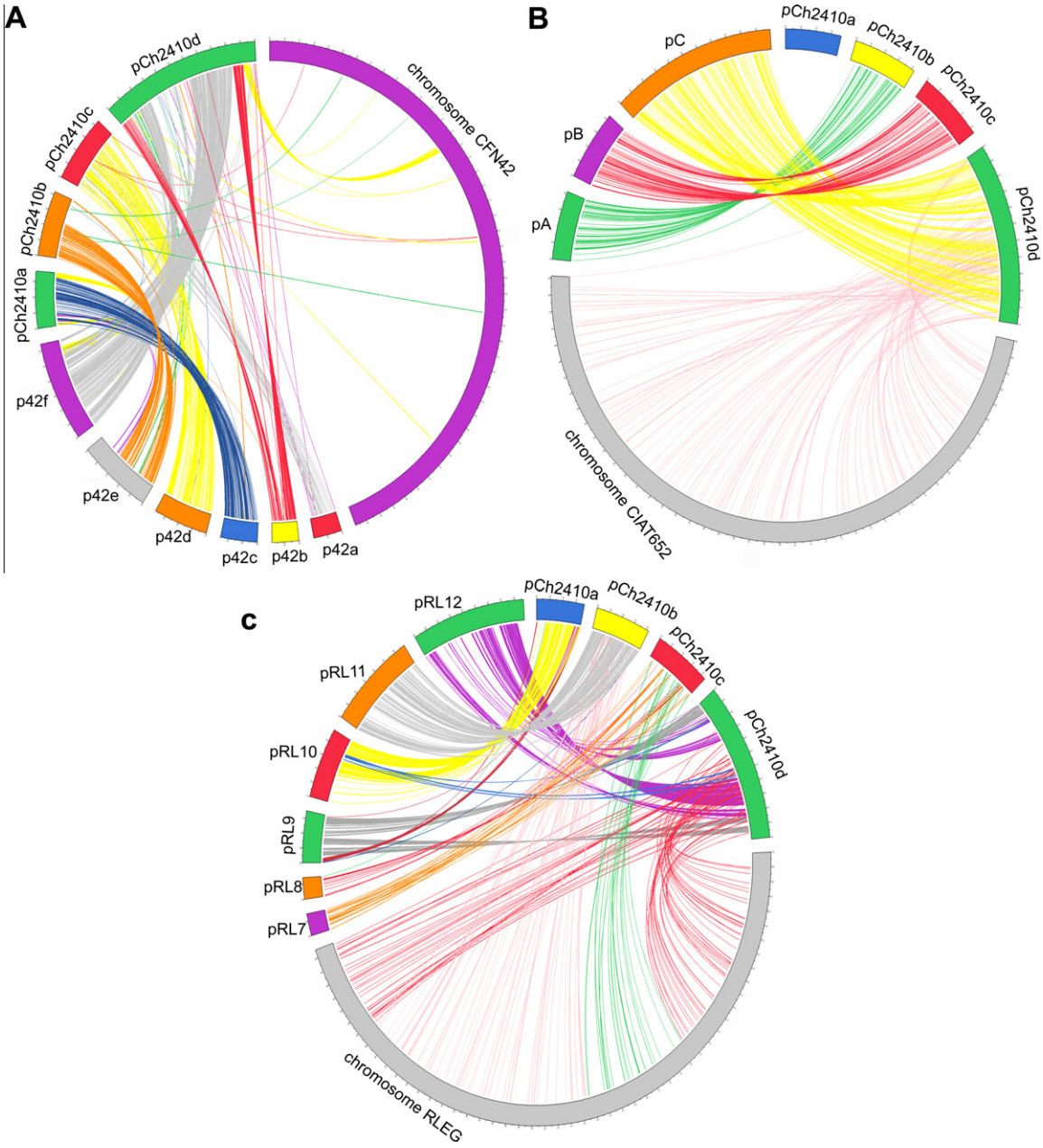

Fig. 1. Comparison using satsumasinteny of *R. phaseoli* Ch24-10 extrachromosomal replicons (ERs) to (A) *R. etli* CFN42, (B) *R. phaseoli* CIAT 652 and (C) *R. leguminosarum* 3841 (RLEG) chromosomes and ERs.

Upon subculturing this strain lost its symbiotic plasmid as evidenced from the whole genome analysis (NCBI ID 640510). The original strain is still capable of nodulating *Phaseolus vulgaris* and mimosa plants (unpublished). In another case, when we analyzed the transcripts from *R. phaseoli* strain Ch24-10 (see below) there were none corresponding to a 370 kb plasmid (the smallest, non-symbiotic plasmid) that was revealed in the whole genome analysis of the same strain (López-Guerrero et al., in press). We supposed that the plasmid was lost upon subculturing as the original stock has all plasmids. Our analysis of the published genome of *R. phaseoli* CNPAF512 (Fauvart et al., 2011) revealed sequences corresponding to the 370 kb plasmid from Ch24-10, however these were not found (Fig. 1B) in the published genome of another *R. phaseoli* strain, CIAT 652 (González et al., 2010). This shows that this plasmid is not homogenously conserved among *R. phaseoli* strains. It is worth mentioning that *R. phaseoli* CIAT652 is a very efficient *P. vulgaris* symbiont in spite of lacking this plasmid.

ER maintenance seems to be forced when carrying genes required for growth or survival. This is illustrated in *R. etli*

CFN 42 with pReCFN42e carrying genes needed for growth or optimal growth in rich medium such as those encoding a sensor histidine kinase/ response regulator hybrid protein and a hypothetical protein with a winged helix-turn-helix motif (Landeta et al., 2011) in addition to containing some of the genes for cobalamin biosynthesis. Both genes encoding the sensor histidine kinase/ response regulator hybrid and the hypothetical protein with a winged helix-turn-helix motif are found in *R. leguminosarum* sv. viciae 3841 (in chromid PRL11) and in sv. trifolii strains 1325 and 2304 plasmids as well as in an *R. phaseoli* CIAT 652 plasmid (pRp652a) that corresponds to pReCFN42e.

Toxin–antitoxin genes were discovered as plasmid stabilizers (Jensen and Gerdes, 1995; Ogura and Hiraga, 1983) and have been identified in many bacteria (Pandey and Gerdes, 2005; Van Melderen et al., 2009). Toxin–antitoxin genes have been found in the symbiotic plasmid of *Ensifer* sp. NGR234 (Falla and Chopra, 1999). Antitoxins are more unstable than toxins so when the antitoxin is missing due to plasmid loss, the toxin inhibits cell growth and leads to death (Jensen and Gerdes, 1995). Bacterial genetic mechanisms to ensure plasmid maintenance both in symbionts and pathogens have been reviewed (Sengupta and Austin, 2011).

5. Extrachromosomal replicons involved in plant–rhizobium interactions

ERs in addition to the symbiotic plasmids have roles in symbiosis with legumes (Hynes and McGregor, 1990). Curing of a cryptic plasmid in *Ensifer* (*Sinorhizobium*) *meliloti* led to a more efficient symbiosis in alfalfa (Velázquez et al., 1995). In *R. leguminosarum* an exogenous RP4 plasmid decreased symbiotic effectiveness (O'Connell et al., 1998). Enhanced nodulation competitiveness was recorded in *R. etli* strains that gained an *R. leucaenae* (185 kb) plasmid (Martínez-Romero and Rosenblueth, 1990). *A. tumefaciens* transconjugants that in addition to carrying the *nod-nif* plasmid had a 200 kb plasmid from *R. leucaenae* fixed more nitrogen than that with only the symbiotic plasmid (Martínez et al., 1987).

Non symbiotic plasmids participate in rhizobial interactions with plants (Brom et al., 2000; Chen et al., 2000; Hynes and McGregor, 1990; Pappas and Cevallos, 2011). Some *R. leguminosarum* strains capable of associating with rice promoted its growth and alleviated N deficiencies (Yanni et al., 1997), but others from clover inhibited rice root growth. Rice inhibition or promotion is plasmid dependent in *R. leguminosarum* (Perrine et al., 2001) and in *E. meliloti* (Perrine et al., 2005). Derivatives of *R. leguminosarum* sv. trifolii W14–12 lacking two plasmids were unable to grow in soil (Moënne-Loccoz and Weaver, 1995a) and different plasmids were found to contribute to growth in the clover rhizosphere (Moënne-Loccoz and Weaver, 1995b) or in saprophytic life (Moënne-Loccoz et al., 1995). The most competitive maize colonizing *R. phaseoli* strains had the most common plasmid pattern observed among many rhizospheric strains analyzed (Rosenblueth and Martínez Romero, 2004). In *R. leguminosarum* sv. viciae, a plasmid contains several genes needed and expressed by bacterial cells when colonizing the pea rhizosphere (Ramachandran et al., 2011). Similarly we found that extrachromosomal genes were expressed in *R. phaseoli* strain Ch24-10 (Rosenblueth and Martínez Romero, 2004) associated with maize and *P. vulgaris* (common bean) roots (see Section 7).

6. Extrachromosomal genes associated with rhizobial environmental adaptation

There is a functional bias in extrachromosomal genes, the ERs tend to contain genes implicated in processes like chemotaxis (Yost et al., 1998) and transport, and they are enriched in elements of external origin (Crossman et al., 2008). Some plasmids, megaplasmids or chromids encode many carbon assimilation genes (Baldani et al., 1992; Oresnik et al., 1998); vitamins like biotin, thiamine or pantothenate (Finan et al., 1986; Miranda-Ríos et al., 1997; Streit et al., 1996; Villaseñor et al., 2011), bacteriocin (Oresnik et al., 1999; Venter et al., 2001), melanin (Hynes et al., 1988) or autoinducer (Schripsema et al., 1996) biosynthetic pathways; and may encode chaperons and modification-restriction systems (Rochepeau et al., 1997). Quorum sensing systems that regulate plasmid transfer or expression of genes in plants may be plasmid encoded in rhizobia (Cubo et al., 1992; Edwards et al., 2009; Lithgow et al., 2000). Reviews on gene functions of plasmids (García-de los Santos and Brom, 1996; Mercado-Blanco and Toro, 1996; Pappas and Cevallos, 2011) and of megaplasmids from *Ensifer* sp. NGR234 (Mavingui, 2009) and *E. meliloti* (Barloy-Hubler and Jebbar, 2009) have been published. Only some functions that we considered important for plant niche colonization will be reviewed here.

6.1. Transporters in ERs

In megaplasmid pSymA but especially in pSymB of *Ensifer meliloti* 1021 there are large numbers of transporters (Mauchline et al., 2006) that may allow the bacteria to use different soil nutrients or root exudates. They are inducible by a large number of substrates (Mauchline et al., 2006). Plasmids in *R. etli*, *R. tropici*, *R. leucaenae* and *R. gallicum* sv. phaseoli carry *teu* genes that code for putative sugar ABC transporters involved in the uptake of molecules found in *P. vulgaris* and siratro exudates (Rosenblueth et al., 1998). Four of six quaternary amine transporters that were characterized are located in chromids pRL10 and pRL12 in *R. leguminosarum* 3841 (Fox et al., 2008).

6.2. Catabolism

In *E. meliloti putA* genes (for proline catabolism) are involved in rhizobial competitiveness (Van Dillewijn et al., 2002), *putA* is in the chromosome in *E. meliloti* and in *Ensifer* sp. NGR234. *putA* genes are in ER in *R. etli*, *R. phaseoli* and *R. leguminosarum*.

Rhamnose catabolic genes are plasmid borne and inducible (Oresnik et al., 1998). Transport and catabolism of erythriol is plasmid dependent (Geddes et al., 2010; Yost et al., 2006). *R. leguminosarum* mutants in glycerol catabo-

lism have diminished competitiveness. Glycerol uptake and catabolism is plasmid encoded (Ding et al., 2012).

Calystegine catabolism genes are plasmid borne in *E. meliloti* (Guntli et al., 1999; Tepfer et al., 1988). These genes participate in bacterial competitive colonization of non legume rhizozpheres such as those from morning glory plants. Mimosine catabolism genes are also plasmid borne (Borthakur et al., 2003). Opine uptake and catabolism genes reside in the symbiotic megaplasmid a in *E. meliloti* (Murphy et al., 1987). There are also opine catabolizing plasmids in agrobacteria (Bruce et al., 1990).

6.3. Surface polysaccharides

Different surface polysaccharides are needed in rhizobial attachment to roots (Downie, 2010) and genes for their biosynthesis are located in different bacterial replicons. Some lipopolysaccharide (LPS) biosynthetic genes have been found in *R. etli* plasmids (García-de los Santos and Brom, 1997). Biosynthetic genes for exopolysaccharides reside in megaplasmid b in *E. meliloti* (Finan et al., 1986) and also in megaplasmids of other rhizobia (Skorupska et al., 2006). Megaplasmid a of *Ensifer* sp. NGR234 encodes flavonoid-inducible genes required for the biosynthesis of a rhamnose-rich LPS produced only inside nodules and that is required for symbiosis (Broughton et al., 2006).

6.4. Hormone biosynthesis and protein secretion

Upon inspection of reported genomes we found genes that seem to be involved in gibberellin biosynthesis located in the symbiotic plasmids of *E. fredii* HH103 and *Ensifer sp.* NGR234, *R. etli* CFN42, *R. phaseoli* CIAT 652, *R. tropici* CIAT 899 and in the symbiosis islands of *B. japonicum* USDA 6, *Mesorhizobium loti* R7A, and *M. huakuii* MAFF303099. These genes were originally described in *Bradyrhizobium japonicum* USDA 110 (Morrone et al., 2009) and are not present in the reported genomes of *E. meliloti* and *R. leguminosarum* strains. Gibberellins have diverse effects on plants and its balance in relation to auxins affects plant growth (Brian, 2008). Rhizobial mutants in these genes have not been tested in their hosts. ACC deaminases that modulate ethylene levels are encoded in symbiosis islands in mesorhizobial strains R7A and MAFF303099 (Conforte et al., 2010) and in the symbiotic plasmid of *R. tropici* (Ormeño-Orrillo et al., unpublished). Genes for different auxin biosynthetic pathways are plasmidic in NGR234 (Theunis et al., 2004) and in *R. tropici* CIAT 899 and they are flavonoid inducible (Theunis et al., 2004; Ormeño-Orrillo et al., unpublished).

Rhizobia use different types of secretion systems (excellently reviewed in Downie, 2010). Type III secretion systems (T3SS) are found in several *Rhizobium* and *Ensifer* strains (Marie et al., 2001), these genes are in the symbiotic plasmid in *Ensifer* sp. strain NGR234 and mutants in this system have altered plant specificity. NGR234 T3SS genes are inducible and expressed in the presence of flavonoids (Viprey et al., 1998). A T3SS cluster is also present in the phaseoli symbiotic plasmid (González et al. 2006). Genes coding for Type 1 and 5 secretion systems are found in megaplasmids in *R. tropici* (Ormeño-Orrillo et al., unpublished).

6.5. Other functions

In *R. etli*, genes to tolerate polyphenols are plasmid borne (García-de los Santos et al., 2008). The only *R. etli* CFN42 catalase is located in a large ER (pReCFN42f) and is required for bacterial survival in polyphenol rich medium (García-de los Santos et al., 2008). The same replicon carries *nirK* and *norCB* genes for nitrite reduction involved in nitrite detoxification but not in nitrite respiration (Gómez-Hernández et al., 2011). Genes that encode efflux pumps (inducible with bean exudates) that eliminate plant toxic molecules or antibiotics are located in pReCFN42b (184 kb) (González-Pasayo and Martínez-Romero, 2000). The same replicon carries genes for thiamine biosynthesis (Miranda-Ríos et al., 1997).

7. Transcriptional profiling of rhizobial ER in natural niches such as the root environment

Are there rhizobial genomic islands or plasmids that are preferentially expressed in the environment? Many stress induced genes that could play a role in the environment are extrachromosomal in *R. etli* CFN42 (Ramírez, unpublished). Expression of symbiosis genes dependent on plant hosts and the molecules and conditions required for gene expression have been well studied and have been extensively reviewed (Cooper, 2004; Le Strange et al., 1990; Maj et al., 2010; Masson-Boivin et al., 2009). Rhizobial genes expressed under stress (Vercruysse et al., 2011), in presence of flavonoids (Perret et al., 1999; Zhang and Cheng, 2006) or in nodules have been reported (Barnett et al., 2004; Chang et al., 2007; Karunakaran et al., 2009; Tsukada et al., 2009) but less is known on genes expressed in soil or in the rhizosphere. Mutations in the *cin* and *rhi* quorum sensing systems affect rhizospheric growth (Cubo et al., 1992; Edwards et al., 2009).

7.1. Rhizobium leguminosarum ER rhizospheric expression

A microarray based approach to study *R. leguminosarum* gene expression in pea, alfalfa or sugar beet rhizospheres showed that many of the genes preferentially expressed in *R. leguminosarum* 3841 when inhabiting the pea rhizosphere are encoded in the conjugative 147 kb plasmid pRL8 (Ramachandran et al., 2011). From pRL8, 11 or 21 genes (depending on the threshold considered) were up regulated in pea and only 3 or 2 in alfalfa or sugar beet rhizospheres. Pea induced genes represented around 15% of all genes on pRL8. In total 138 genes were specifically up regulated in 7 day old pea plants and 106 genes were up regulated in all rhizospheres, 70 of those were hypothetical. Among genes expressed were those encoding phenylanine and tyrosine catabolism, dicarboxylate transport, *rhiABC*, *rhiI*, *cinI*, protocatechuate and shikimate uptake, xanthine, formate and other dehydrogenases, as well as some *nod* genes (Ramachandran et al., 2011).

7.2. Rhizobium phaseoli ER rhizoplane expression

R. phaseoli Ch24-10 was chosen to study gene expression in plant roots because it represents a group of dominant

bacteria in maize rhizosphere (Rosenblueth and Martínez Romero, 2004), is highly competitive to colonize maize and rice and is a very efficient bean symbiont. Bean and maize plants have been grown in association in traditional agriculture for some thousand years and rhizobial gene expression was analyzed in both hosts independently. Upper value tails of bacterial gene transcript distribution in a reported transcriptomic analysis were found to correlate to RNA polymerase occupancy meaning that transcription was occurring in those genes (Vijayan et al., 2011) and, on that basis, highly expressed genes in the Ch24-10 transcriptomic profiling were selected. The 324 extrachromosomal genes highly expressed in maize and/or bean rootlets represented 22% of pRpCh24-10b and 16% of pRpCh24-10d. pSym genes were also expressed in the rhizosphere of maize and bean (representing 13% of the plasmid). Examples of ER genes that were highly expressed in both maize and bean root samples (Supplementary Table S1) are those responsible for proline catabolism, iron uptake, thiamine and gibberellin biosynthesis, a type VI secretion system, oligopeptide or sugar transporters and extrusion pumps as well as polygalacturonase, alpha amylase and Deg protease genes. *teu* genes were not expressed in maize roots in agreement to previous results showing that they are not induced by maize exudates (Rosenblueth et al., 1998). A promoter-less *gusA* gene reporter fused to the polygalacturonase gene was found to be expressed in maize and bean exudates (unpublished) and antibiotic resistance promoter-less genes were found to be expressed in plants when fused to the extrusion pump genes *rmrAB* (González-Pasayo and Martínez-Romero, 2000) or to Deg protease genes (unpublished); this additional evidence is in agreement to the transcriptomic results presented. Furthermore, a radioactive polygalacturonase probe was found to hybridize to the 1 Mb Ch24-10 ER (not shown). Orthologues to previously reported *R. leguminosarum* genes expressed in plant rhizospheres (Ramachandran et al., 2011) were found to be highly expressed in *R. phaseoli* in maize and bean roots (Supplementary Table S1). As in *R. leguminosarum* (Ramachandran et al., 2011), many *R. phaseoli* Ch24-10 highly expressed genes were hypothetical, one of them in common to *R. leguminosarum*.

A comparison of the Ch24-10 transcripts from maize and from bean roots suggested that replicons were differentially expressed depending on the plant host colonized. ER transcripts highly expressed in bean and not in maize roots were found in the Ch24-10 symbiotic plasmid (11 out of 26 bean specific genes) and in a 400 kb ER (pRpCh24-10b with equivalent genes to pReCFN42e), with 9 out of 26 specific genes, while most of the transcripts highly expressed in maize but not in bean (11 out of 14 maize specific genes) were found in pRpCh24-10d, a 1 Mb replicon sharing genes with *R. etli* pReCFN42f. No transcripts could be assigned to a 370 kb plasmid (pRpCh24-10a) as the strain used for the transcriptomic analysis unfortunately lost this plasmid that shares genes with pRL10 and pReCFN42c.

8. Concluding remarks

There is still scarce knowledge of rhizobial genes that are functional in nature, in soil, rhizospheric niches or complex microbial communities. Future studies may provide more data to support that ERs, highly dynamic and variable, determine or condition fitness or survival of rhizobia in the environment. Our data extend the knowledge of root-expressed genes in *Rhizobium* and allowed the identification of some extrachromosomal genes commonly expressed in association with plants such as those for thiamine biosynthesis, oligopeptide, proline betaine, α-galactosidase and other ABC transporters, α-*N*-arabinofuranosidase, *rmrA* (González-Pasayo and Martínez-Romero, 2000) and *nod* genes.

Acknowledgements

To PAPIIT (UNAM) grants IN200709 and IN205412. Martha López-Guerrero was a Ph.D. student at the Programa de Doctorado en Ciencias Biomédicas and had a Consejo Nacional de Ciencia y Tecnología (CONACyT) fellowship. Illumina Sequencing was performed at the Unidad Universitaria de Secuenciación Masiva de DNA (USMDNA) of Universidad Nacional Autónoma de México (UNAM). To Dr. M. Dunn for critically reading the ms.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plasmid.2012.07.002.

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