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Short Communication

Synthetic multi-antibiotic resistant plasmids in plant-associated bacteria from agricultural soils

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ABSTRACT

Objectives: Unlike higher organisms such as domestic animals and cultivated plants, which display a robust reproductive isolation and limited dispersal ability, microbes exhibit an extremely promiscuous gene flow and can rapidly disperse across the planet by multiple ways. Thus, microbial plasmids, including synthetic replicons, containing antibiotic resistance genes are a serious risk to public health. In this short communication, we explored the presence of synthetic elements in alfalfa symbionts (*Ensifer meliloti* strains) from agricultural soils.
Methods: A total of 148 *E. meliloti* isolates from alfalfa plants growing under field conditions were collected from January 2015 to June 2019. Antimicrobial susceptibility testing was performed under laboratory conditions. We identified five kanamycin-resistant *E. meliloti* strains (named K1-K5). Whole genome sequencing analysis and conjugations were used to identify and study the plasmids of K strains.
Results: We found that the genomes of K strains contain ampicillin, kanamycin and tetracycline resistance genes, the reporter gene *lacZ* from *Escherichia coli* and multiple cloning sites. These sequences were found within <58-kb plasmids related to the self-transmissible IncP plasmid RP4 from human pathogen *Pseudomonas aeruginosa*. Conjugation experiments confirmed the ability of K strains to transfer antibiotic resistance via conjugation to the *Pseudomonas* background.
Conclusion: In addition to the traditional analysis of plant growth-promoting factors, the commercial deregulation of putative natural inoculants should also include genomic studies to ensure a reasonable balance between innovation and caution.

1. Introduction

The issue of containment of synthetic constructs and genetically modified organisms (GMOs) to limit their possible health and environmental impacts has been discussed by the research community and the society in general since the development of genetic engineering in the 1970s [1]. Based on these concerns, the commercial applications of genetic engineering have been strongly regulated in both developed and developing countries. Although each country has its own regulatory framework to study and market GMOs, there is a consensus on the vast differences in the risks of the release of genetically modified crops (GMCs) and genetically modified microorganisms (GMMs) in agroecosystems.

Similar to other higher organisms, plant species exhibit robust reproductive barriers to gene flow and a limited ability to disperse and colonize new environments. Thus, GMCs have limited effects on agroecosystems. In addition, the large genetic distance between plants and pathogenic microbes, the use of non-antibiotic-selectable markers for plant transformation, and the transgene expression under plant-specific regulatory elements [2] dramatically reduce the probability of occurrence of horizontal transfer of transgenes (non-reproductive gene transfer) from GMCs to pathogenic bacteria, and thus also reduce the health risks from GMCs. Based on a positive risk-benefit balance and an increased demand of food for an expanding global population, the amount of agricultural land used for GMCs has largely increased from 1.7 to 185.1 million ha in the last two decades (1996-2016) [3]. In

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contrast, due to their small size, stress tolerance, airborne dispersal and metabolic versatility, microorganisms such as bacteria can spread ubiquitously throughout the world in a few months [4]. Thus, the containment of GMMs is an extremely hard activity even in confined small-scale trials [5]. The gene exchange between bacteria is common and extremely promiscuous and horizontal gene transfer (HGT) has been a major contributory factor to the rapid spread of antibiotic resistance and the emergence of increased virulence in pathogenic bacteria in the last decades [6,7]. Thus, the World Health Organization considers horizontal transfer of antibiotic resistance genes in human pathogens as one of the most severe risks to public health in this century [8,9]. In addition, plant growth-promoting genes from beneficial bacteria can become virulence factors via HGT [10], thus expanding the environmental risk of GMMs under unconfined conditions.

In this context, inoculants used to improve plant production are currently restricted to wild-type microbial isolates. However, this should be considered a hypothetical scenario because it is based on the presumption that microbes isolated from agroecosystems are free of synthetic replicons. If this presumption is false, the massive release of GMMs via the unintended use of recombinant inoculants could contribute to the vast dispersion of antibiotic resistance genes. In this short communication, we describe the presence of synthetic conjugative multi-antibiotic resistant plasmids in putative natural alfalfa symbionts from agricultural soils.

2. Materials and methods

The microbial strains used in this study were 148 strains of *Ensifer meliloti* isolated from commercial alfalfa plants collected in the Santa Fe, Cordoba, La Pampa, Chaco, Buenos Aires provinces of Argentina, specifically in fields with a large history (>20 years) of alfalfa production using commercial inoculants. For the isolation of novel strains, alfalfa roots were carefully washed with tap water to remove adhering soil particles, and placed in 50-ml tubes containing 25 ml of sterile distilled water. The tubes were incubated for 1 h with shaking (50 rpm). The water was discarded and roots were washed with a 70% v/v ethanol solution. The roots were then vortexed in this wash for 2 min. The solution was discarded and roots were washed with a 1% v/v sodium hypochlorite solution for 50 min with shaking (50 rpm). After that, the roots were washed two times with saline solution (0.9% w/v NaCl) and then vortexed in the last wash for 1 min. The tubes were centrifuged at 4000 x *g* and 4 °C for 15 min. The supernatants were removed, leaving 1 ml of saline solution. Then, 200 μl was plated in lysogeny broth (LB) medium as a disinfection control. Experiments showing bacteria in this control were discarded. Superficially disinfected nodules were macerated using a mortar in saline solution (200 μl) and aliquots were plated either on LB agar and LB agar containing 300 μg/ml of kanamycin to select kanamycin-sensitive and kanamycin-tolerant rhizobia, respectively.

The 408- and 398-bp fragments of the housekeeping (*aqpZ*) and accessory (*nosZ*) genes were amplified and sequenced by PCR colony assays to confirm that the native rhizobial isolates belonged to *Ensifer meliloti sensu stricto* species (*aqpZ*+) and to select strains (*nosZ*-) unrelated to commercial alfalfa inoculants, respectively [11,12]. Genomic DNA of alfalfa rhizobia was isolated from overnight cultures using the Wizard Genomic DNA Purification Kit (#A1120, Promega, USA). Genome sequencing was performed at Unidad de Genómica, Instituto de Agrobiotecnología y Biología Molecular, CICVyA, Instituto Nacional de Tecnología Agropecuaria, Buenos Aires, Argentina (https://inta.gob.ar). A total of 12.87 million (M) paired-end reads of 32- or 101-bp read length were obtained using Illumina Hiseq1500 technology, resulting in an average of 34-fold genome coverage. The sequencing reads were mapped against a high-diversity synthetic DNA library, which is available in the Geneious software (www.geneious.com). This analysis was developed by setting the following parameters: Mapper: Geneious, Sensitivity: Low Sensitivity/Fastest, Fine Tuning: None (fast/read mapping), Minimum mapping quality: 30, and Map multiple best matches: Randomly. *De novo* assembly of *psyn* plasmids was performed using positive reads of each strain matching to a synthetic DNA library. This analysis was carried out using with the Geneious assembler platform (www.geneious.com). The nucleotide sequences of synthetic replicons (*psyn* plasmids) were deposited in the EMBL Nucleotide Sequence Database, accession numbers: *psyn1* (SAMN10699279), *psyn2* (SAMN10699280), *psyn3* (SAMN10699281), *psyn4* (SAMN10699282) and *psyn5* (SAMN10699283).

Phylogenetic and genomic synteny comparisons among *aqpZ* genes and *psyn* plasmids were performed by using Geneious Tree Builder and LASTZ plugins into the Geneious software by using *aqpZ* from the model strain *Ensifer meliloti* 1021 (SMa0627) and the RP4 plasmid (Accession Number: BN000925.1) from *Pseudomonas aeruginosa* as outgroups, respectively. Conjugations of *Pseudomonas fluorescens* Pf-5 with *Ensifer meliloti* K1-K5 harboring *psyn1-5* plasmids were performed on mineral salts medium plates supplemented with octanoate (0.25% w/v) and containing kanamycin (10 μg/ml) as previously described [13]. For antibiotic tolerance assays, overnight cultures were diluted 10^6-fold into fresh LB medium and 100 μl of these dilutions was plated in LB agar (control) or LB agar supplemented with 50-100 μg/ml kanamycin, 5-10 μg/ml tetracycline or 150 μg/ml ampicillin.

3. Results and discussion

We searched for synthetic DNA sequences within the sequenced genomes of five kanamycin-resistant *Ensifer meliloti* strains (K strains) isolated from nodules of uninoculated alfalfa plants. Contrary to the wild-type control strain *Ensifer meliloti* B399, originally called *Rhizobium meliloti* 102F3 [14] and isolated in the 1960s before the birth of genetic engineering [1], kanamycin-resistant strains (here called *Ensifer meliloti* K1, K2, K3, K4 and K5) contain several reads (<4,000) mapping to a high-diversity synthetic DNA library (Table 1), which points to the occurrence of GMMs in agroecosystems. *De novo* assemblies of synthetic constructs using positive reads revealed the presence of <58-kb replicons (here called *psyn1*, *psyn2*, *psyn3*, *psyn4* and *psyn5* plasmids) in K strains (Fig. 1a), which were found to contain ampicillin (ampR), kanamycin (kmR), and tetracycline (tetR and tetA) resistance genes, as well as all essential replication and transfer functions in self-transmissible IncP plasmids (Fig. 1b). We also found that *psyn* plasmids (58,341-59,306 bp) are very similar to the RP4 plasmid (60,096 bp) from *Pseudomonas aeruginosa* (<96.6% nucleotide identity), which suggests that the *psyn* plasmids found in K strains have a *Pseudomonadales* origin (Fig. 1a). In fact, with the exception of two variable regions (here named VR1 and VR2), we found no large gaps in the alignment of *psyn* and RP4 plasmids (Fig. 1a). In VR1, ampR exhibited large deletions in *psyn1*, *psyn2*, *psyn3* and *psyn4* but not in *psyn5* (Fig. 1a and Fig. 1c), whereas in VR2, the non-essential hypothetical gene upf16.5 [15] from the RP4 plasmid had been removed and replaced by the *lacZ* reporter gene in *psyn* plasmids (Fig. 1a and Fig. 1c). However, the VR2 of *psyn* plasmids presented traces of the upf16.5 gene (Fig. 1a and Fig. 1c). Thus, the analysis of the variable regions supports that *psyn* plasmids are derived from the RP4 plasmid itself or other extremely similar replicons.

In agreement with the synthetic origin of *psyn* plasmids, *lacZ* genes were found to contain multiple cloning sites (Fig. 1d), which were identical in all *psyn* plasmids (<100% nucleotide identity). In addition, *lacZ* genes from *psyn* plasmids and their multiple cloning

Table 1
Rapid detection of the occurrence of synthetic DNA sequences in alfalfa rhizobia.

Strain	total reads	reads mapping to gryB	reads mapping to gryB/total reads	reads mapping to synthetic DNA
B399	2,440,468	702	287 10^{-6}	0
K1	2,007,344	625	311 10^{-6}	4,463
K2	2,284,902	667	292 10^{-6}	14,492
K3	2,286,658	715	312 10^{-6}	6,716
K4	2,184,504	617	282 10^{-6}	8,165
K5	1,669,558	490	293 10^{-6}	12,825
1021	2,173,230	619	284 10^{-6}	0

Synthetic DNA sequences within the genomes of five kanamycin-tolerant alfalfa-rhizobia (K strains) isolated from alfalfa nodules in agricultural soils were detected by mapping sequencing reads to a high-diversity synthetic DNA library. The kanamycin-sensitive wild-type strain B399 and the single-copy housekeeping gene gryB were used as GMO-free and post-sequencing quality controls, respectively. As an additional GMO-free control, we included the genomic analysis of wild-type model strain 1021.

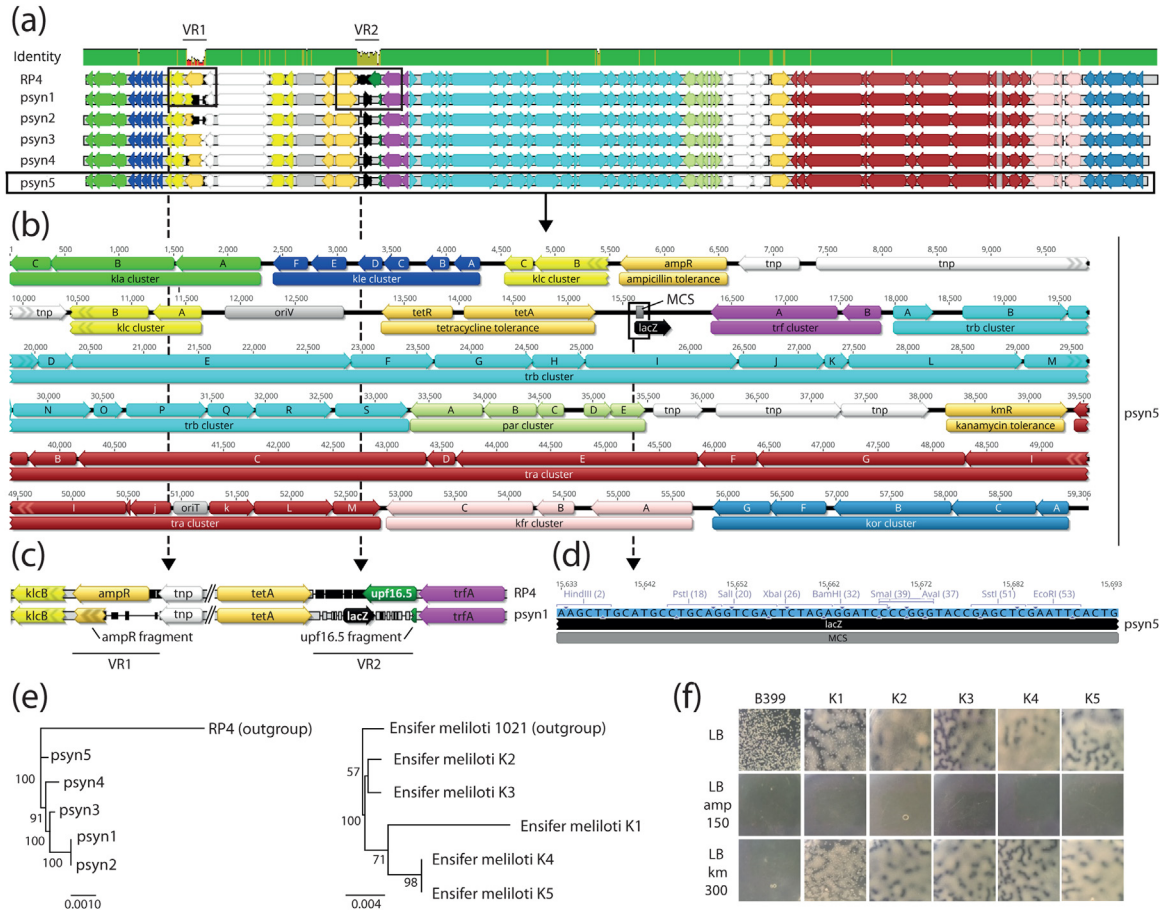

Fig. 1. Kanamycin-tolerant rhizobia (K strains) isolated from alfalfa nodules in agricultural soils contain large synthetic replicons (*psyn* plasmids). (a) *psyn* plasmids from K strains are similar to the self-transmissible, broad host-range plasmid RP4 from *Pseudomonas aeruginosa*, with the exception of two variable regions (VR1 and VR2). (b) *psyn* plasmids contain ampicillin (ampR), kanamycin (kmR) and tetracycline (tetR and tetA) resistance genes, the reporter gene *lacZ*, multiple cloning sites (MCS), and conjugal transfer and replication regions (*kla*, *kle*, *klc*, *trf*, *trb*, *par*, *tra*, *kfr* and *kor* clusters). (c) Detailed description of VR1 and VR2 from *psyn1* and RP4, showing that *psyn1* has the ampicillin resistance (left panel) and the upf16.5 (right panel) genes partially deleted. In the last case, the hypothetical gene upf16.5 has been replaced by the reporter gene *lacZ*. (d) Detailed description of the MCS from *psyn8* showing artificial restriction enzyme recognition sites (HindIII, PstI, SalI, XbaI, BamHI, SmaI, AvaI, SstI and EcoRI). (e) Phylogenetic trees of *psyn* plasmids (left panel) and organism (righ panel) based on neighbor-joining analysis with genetic distances computed using Jukes-Cantor model. Bootstrap percentages are indicated at the branch points. AQPZ, variable housekeeping gene [11], was used to analyse the evolution of K strains. AQPZ from the model strain *Ensifer meliloti* 1021 and RP4 plasmid from *Pseudomonas aeruginosa* were used as outgroups in *psyn* plasmid and organismal trees, respectively. The incongruence (i.e. different topology) between *psyn* plasmid and organismal trees suggests the occurrence of horizontal transfer events of *psyn* plasmids in nature. (f) K strains showed tolerance to kanamycin but not to ampicillin. This partial conservation of the antibiotic resistance traits in K strains could be associated with the lack of strong selective pressures on antibiotic resistance markers in nature.

sites are very similar (but not identical) to pUC19 [16]. The average percent GC content in *psyn* plasmids (61.7%) was similar to that in genomes of *E. meliloti* strains (61.8%) [17], which suggests an optimal and long-term adaptation of *psyn* plasmids to the genomic background of *E. meliloti*. Phylogenetic analysis showed incongruence between the *psyn* plasmid and organismal trees (Fig. 1e), suggesting that the ancestors of K strains may have obtained the *psyn* plasmids by different horizontal transfer events. In concordance with the low selective pressures on antibiotic resistance markers in nature, K strains exhibited resistance to kanamycin but not to ampicillin (Fig. 1f). Complementation analyses were performed to determine the ability of *psyn* plasmids to transfer the antibiotic resistance to other unrelated bacteria. Thus, *psyn1-5* plasmid from *Ensifer meliloti* K1-K5 was introduced by conjugation

Table 2
Antibiotic tolerance of wild-type strain *Pseudomonas fluorescens* Pf-5 and recombinant *Pseudomonas* strains containing *psyn1*-5 plasmid from Ensifer meliloti K1-K5. *P. fluorescens* Pf-5 and its derived recombinant strains were growth in LB or in LB supplemented with 50-100 μg/ml kanamycin (km) or 5-10 μg/ml tetracycline (tc).

Strain	Lysogeny broth	Lysogeny broth km50	Lysogeny broth km75	Lysogeny broth km100	Lysogeny broth tc5	Lysogeny broth tc10
***P. fluorescens* Pf-5**	+	-	-	-	-	-
P. fluorescens* Pf-5 *psyn1	+	+	+	-	+	-
P. fluorescens* Pf-5 *psyn2	+	+	-	-	+	+
P. fluorescens* Pf-5 *psyn3	+	+	+	+	+	+
P. fluorescens* Pf-5 *psyn4	+	+	-	-	+	-
P. fluorescens* Pf-5 *psyn5	+	+	+	+	+	-

in *Pseudomonas fluorescens* Pf-5. All *psyn* plasmids conferred significant kanamycin (>50 μg/ml) and tetracycline (>5 μg/ml) tolerance to the host (Table 2). This result strongly suggested that kanamycin and tetracycline resistance genes within *psyn* plasmids were functionally active in *Pseudomonas* background.

4. Conclusions

Based on our results and the potential risk of GMMs, we propose to add a basic molecular study (sequencing and automatic analysis of bacterial genomes) within the necessary prerequisites for the massive release of putative natural microbes to the environment. In the long term, and with the help of low-cost sequencing technologies, crop inoculation using natural microbes can offer improved safety.

Declarations

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Competing Interests: None declared.

Ethical approval: Not required.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jgar.2020.01.015.

References

[1] Cohen SN, Chang AC, Boyer HW, Helling RB. Construction of biologically functional bacterial plasmids in vitro. Proc Natl Acad Sci U S A 1973;70:3240–4.
[2] Jozefkowicz C, Frare R, Fox R, Odorizzi A, Arolfo V, Pagano E, Basigalup D, Ayub N, Soto G. Maximizing the expression of transgenic traits into elite alfalfa germplasm using a supertransgene configuration in heterozygous conditions. Theor Appl Genet 2018;131:1111–23, doi:http://dx.doi.org/10.1007/s00122-018-3062-1.
[3] ISAAA. ISAAA Global Status of Commercialized Biotech/GM Crops. 2016.
[4] Wilkinson DM, Koumoutsaris S, Mitchell EAD, Bey I. Modelling the effect of size on the aerial dispersal of microorganisms. Journal of Biogeography 2012;39:89–97, doi:http://dx.doi.org/10.1111/j.1365-2699.2011.02569.x.
[5] Glandorf DCM. Re-evaluation of biosafety questions on genetically modified biocontrol bacteria. European Journal of Plant Pathology. 2018, doi:http://dx.doi.org/10.1007/s10658-018-1598-1.
[6] Mazel D, Davies J. Antibiotic resistance in microbes. Cell Mol Life Sci 1999;56:742–54.
[7] Read AF, Woods RJ. Antibiotic resistance management. Evol Med Public Health 2014;147:, doi:http://dx.doi.org/10.1093/emph/eou024.
[8] Michael CA, Dominey-Howes D, Labbate M. The antimicrobial resistance crisis: causes, consequences, and management. Front Public Health 2014;2:145, doi:http://dx.doi.org/10.3389/fpubh.2014.00145.
[9] Robinson TP, Bu DP, Carrique-Mas J, Fevre EM, Gilbert M, Grace D, Hay SI, Jiwakanon J, Kakkar M, Kariuki S, Laxminarayan R, Lubroth J, Magnusson U, Thi Ngoc P, Van Boeckel TP, Woolhouse ME. Antibiotic resistance is the quintessential One Health issue. Trans R Soc Trop Med Hyg 2016;110:377–80, doi:http://dx.doi.org/10.1093/trstmh/trw048.
[10] Stritzler M, Soto G, Ayub N. Plant Growth-Promoting Genes can Switch to be Virulence Factors via Horizontal Gene Transfer. Microb Ecol 2018;76:579–83, doi:http://dx.doi.org/10.1007/s00248-018-1163-7.
[11] Brambilla S, Frare R, Soto G, Jozefkowicz C, Ayub N. Absence of the Nitrous Oxide Reductase Gene Cluster in Commercial Alfalfa Inoculants Is Probably Due to the Extensive Loss of Genes During Rhizobial Domestication. Microb Ecol 2018;76:299–302, doi:http://dx.doi.org/10.1007/s00248-018-1145-9.
[12] Brambilla S, Soto G, Odorizzi A, Arolfo V, McCormick W, Primo E, Giordano W, Jozefkowicz C, Ayub N. Spontaneous Mutations in the Nitrate Reductase Gene napC Drive the Emergence of Eco-friendly Low-N2O-Emitting Alfalfa Rhizobia in Regions with Different Climates. Microb Ecol. 2019, doi:http://dx.doi.org/10.1007/s00248-019-01473-w.
[13] Ayub ND, Julia Pettinari M, Mendez BS, Lopez NI. Impaired polyhydroxybutyrate biosynthesis from glucose in Pseudomonas sp. 14-3 is due to a defective beta-ketothiolase gene. FEMS Microbiol Lett 2006;264:125–31, doi:http://dx.doi.org/10.1111/j.1574-6968.2006.00446.x.
[14] Burton J. Alfalfa Science and Technology. 1972 Edited by Clarence, HH Am Soc Agron.
[15] Bartosik AA, Glabski K, Kulinska A, Lewicka E, Godziszewska J, Markowska A, Jagura-Burdzy G. Convenient broad-host-range unstable vectors for studying stabilization cassettes in diverse bacteria. BMC Microbiol 2016;16:59, doi:http://dx.doi.org/10.1186/s12866-016-0674-y.
[16] Yanisch-Perron C, Vieira J, Messing J. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 1985;33:103–19, doi:http://dx.doi.org/10.1016/0378-1119(85)90120-9.
[17] Galardini M, Pini F, Bazzicalupo M, Biondi EG, Mengoni A. Replicon-dependent bacterial genome evolution: the case of Sinorhizobium meliloti. Genome Biol Evol 2013;5:542–58, doi:http://dx.doi.org/10.1093/gbe/evt027.