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Short communication

Stable symbiotic nitrogen fixation under water-deficit field conditions by a stress-tolerant alfalfa microsymbiont and its complete genome sequence

Cintia Jozefkowicz[a,b], Silvina Brambilla[b], Romina Frare[a,b], Margarita Stritzler[a,b], Carlos Piccinetti[c], Mariana Puente[c], Carolina Andrea Berini[d], Pedro Reyes Pérez[b], Gabriela Soto[a,b], Nicolás Ayub[a,b,*]

[a] Consejo Nacional de Investigaciones Científicas y Técnicas, CABA, Argentina
[b] Instituto de Genética Ewald A. Favret (INTA), Buenos Aires, Argentina
[c] Instituto de Microbiología y Zoología Agrícola (INTA), Buenos Aires, Argentina
[d] Instituto de Investigaciones Biomédicas en Retrovirus y SIDA (INBIRS), CABA, Argentina

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ABSTRACT

We here characterized the stress-tolerant alfalfa microsymbiont *Sinorhizobium meliloti* B401. B401-treated plants showed high nitrogen fixation rates under humid and semiarid environments. The production of glycine betaine in isolated bacteroids positively correlated with low precipitation levels, suggesting that this compound acts as a critical osmoprotectant under field conditions. Genome analysis revealed that strain B401 contains alternative pathways for the biosynthesis and uptake of glycine betaine and its precursors. Such genomic information will offer substantial insight into the environmental physiology of this biotechnologically valuable nitrogen-fixing bacterium.

Alfalfa (*Medicago sativa* L.), commonly known as the "Queen of Forages", is the most important forage legume, and thus, the basis of meat and milk productions worldwide. However, cultivation of this legume crop has been displaced to marginal areas, e.g. arid regions and saline soils, by other crops of greater economic importance for direct human consumption such as wheat, maize, rice and soybean (Cornacchione and Suarez, 2017; Sandhu et al., 2017). Thus, to maintain high levels of alfalfa productivity in the next years, it is necessary to understand the mechanisms of stress tolerance of alfalfa microsymbionts. Since the complete genome sequence of the model strain *Sinorhizobium meliloti* 1021 became available, genomic data and genetic studies of this species have increased rapidly, providing useful information about the genetic features involved in root colonization, nodule formation and nitrogen fixation (diCenzo et al., 2016; diCenzo and Finan, 2015; Galardini et al., 2013; Schneiker-Bekel et al., 2011). In contrast, the genetic factors associated with the adaptation of symbiotic bacteria to different environments are poorly understood. In this context, we decided to study the stress-tolerant nitrogen-fixing bacterium *Sinorhizobium meliloti* B401. This strain, isolated from the agronomic soil in alfalfa monoculture under water-deficit field conditions in the Pampean region (32° 14′S, 65° 14′W) by the National Institute of Agricultural Technology of Argentina (https://inta.gob.ar/).

Contrary to the model strain *Sinorhizobium meliloti* 1021, which has a high performance in some european soils, high levels of biological nitrogen fixation were observed in alfalfa plants inoculated with strain B401 in both humid and semiarid environments of the Pampean region of South America (Fig. 1a). Particularly, eight months after inoculation, and relative to uninoculated plants, the nitrogen derived from gaseous nitrogen (%Ndfa) in B401-treated alfalfa plants was high and similar in both stress and optimal field conditions, with an average of 86.9% and 85.3%, respectively. This result shows that these B401-inoculated alfalfa plants are incorporating vast amounts of nitrogen via symbiotic nitrogen fixation regardless of the environmental conditions. Interestingly, alfalfa plants inoculated with strain 1021 were unable to produce nodules under semiarid environments (not data shown), probably due its low adaptation to the Pampean region. In addition, the accumulation of glycine betaine positively correlated with low precipitation levels, suggesting that this compound acts as a compatible solute under field conditions (Fig. 1b). Genome sequencing analysis showed that the complete genome sequence of the bacterium B401 (6,140,999 bp) consists of a circular chromosome (3,459,457 bp) and two large extrachromosomal replicons named megaplasmid A (1,096,825 bp) and megaplasmid B (1,584,717 bp) (Table 1), and that the G + C content (62.4%) is similar to that of other *S. meliloti* strains (Galardini et al., 2013). Pairwise alignments of genomes showed that *S. meliloti* B401 has high nucleotide identity to other *S. meliloti* strains (Table 2), including

* Corresponding author at: De los Reseros S/N, Castelar C25(1712), Buenos Aires, Argentina.
E-mail addresses: nicoayub@gmail.com, ayub.nicolas@inta.gob.ar (N. Ayub).

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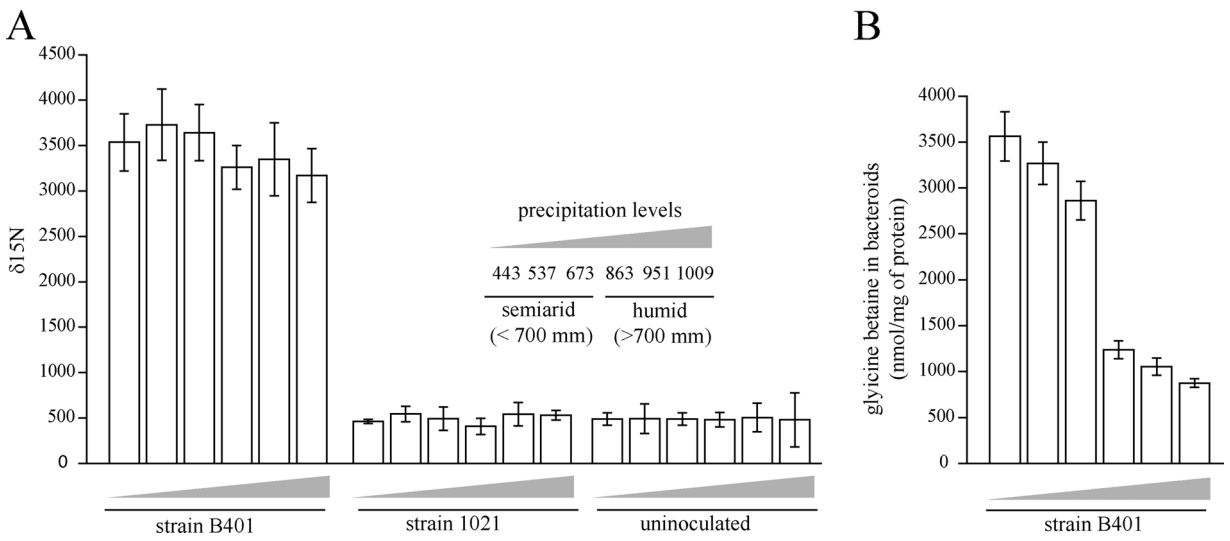

Fig. 1. Biological nitrogen fixation and glycine betaine accumulation in strain B401. (A) The biological nitrogen fixation in plants without inoculation or inoculated with model strain 1021, commercial strain B401 and (B) the intracellular levels of glycine betaine in isolated bacteroids from plants inoculated with commercial strain B401 were analyzed in six experimental sites within the Pampean region of Argentina with different annual precipitations (Additional File 1) All values are means + SEM of ten biological replicates.

Table 1
Genome features of the commercial inoculant *Sinorhizobium meliloti* B401.

Features	Values
Genome size	6,140,999 bp
G + C content	62.4%
Total genes	6116
Coding genes	5727
rRNA	9
tRNA	53

Table 2
Relationship of bacterium B401 to other *Sinorhizobium* strains. Genomic alignments of strain B401 against other *Sinorhizobium* strains were performed by LASTZ (www.geneious.com). The accession numbers used in these alignments were NC_003047, NC_003037 and NC_003078 for strain 1021; NC_015590, NC_015591 and NC_015596 for strain AK83; NC_017322, NC_017324 and NC_017323 for strain BL225; NC_017325, NC_017327 and NC_017326 for strain Sm11; NC_019845, NC_019848 and NC_019849 for strain GR4; NZ_CP009144, NZ_CP009145 and NZ_CP009146 for strain RMO17; and NC_018700, NC_018683 and NC_018701 for strain Rm41.

Replicons of strain B401	Strains – pairwaise% identity –						
	1021	AK83	BL225	Sm11	GR4	RMO17	Rm41
chromosome	99.8	99.6	99.8	99.7	99.7	99.8	99.3
megaplasmid A	98.0	98.6	97.9	98.3	97.7	98.7	98.5
megaplasmid B	98.6	98.4	98.5	98.5	98.1	98.5	98.4

the model strain *S. meliloti* 1021 (Galibert et al., 2001). Similar to strain 1021, strain B401 contains a set of genes for biosynthesis and uptake of glycine betaine (Fig. 2). Strain B401 has two pathways for glycine betaine biosynthesis: the oxidation of choline by choline dehydrogenase (BetA) and betaine aldehyde dehydrogenase (BetB) (Pocard et al., 1997), the oxidation of choline sulfate by choline sulfatase (BetC) (Mandon et al., 2003) and the methylation of sarcosine by betaine-homocysteine methyltransferase (Bmt) (Barra et al., 2006) (Fig. 2). The latter enzyme shows significant amino acid identity (25%) to the Bmt enzyme of humans (Millian and Garrow, 1998) and other eukaryotes but not to other strains belonging to the Eubacteria domain, with the exception of *Sinorhizobium* strains. In addition to the betaine transporter (BetS) (Boscari et al., 2002; Boscari et al., 2006), strain B401 has the ABC gene cluster (Dawson and Locher, 2006) for choline transport (ChoXWV) (Dupont et al., 2004) (Fig. 2). Therefore, BetAB proteins can probably synthesize glycine betaine using both endogenous and exogenous chlorine. Moreover, strain B401 has a complete set for the methionine cycle, including the key enzyme methionine synthase codified by the *metH* gene (Barra et al., 2006) (Fig. 2). In addition to the chromosomal cluster BetS-ChoXWV (BWO76_26325-26340), and contrary to other strains belonging to the *Sinorhizobium* genus, strain B401 has an additional cluster (ChoXWV-BetS) for choline and betaine uptake (BWO76_03765-BWO76_03780) within the megaplasmid A (Fig. 2), the most variable replicon in strains belonging to *S. meliloti* species (Galardini et al., 2013). The localization of cluster ChoXWV-BetS of strain B401 into a highly variable replicon, the different gene order within the chromosomic (BetS-ChoXWV) and plamidic (ChoXWV-BetS) clusters, the low amino acid identities between each individual genes of these clusters (> 50%), and the absence of ChoXWV-BetS cluster in other *Sinorhizobium* strains suggest that it was acquired via horizontal transfer, probably as a mechanism for adaptation to abiotic stress conditions. The availability of the genome presented here provides the source for analysis of regulation of glycine betaine production in strain B401 and in other stress-tolerant related rhizobial species which are able to fix nitrogen in association with legume plants under stressed field conditions.

Genome sequencing and annotations. Genomic DNA of strain B401 was isolated from overnight cultures using the Wizard Genomic DNA Purification Kit (#A1120, Promega, USA). Genome sequencing was performed at INDEAR (http://www.indear.com/). As a result, a total of 22.04 mega reads (2.23 Gp) were obtained from Ilumina Hi-seq1500 technology. De novo assembly and annotations were carried out using the Geneious software (www.geneious.com) and Pipeline (https://www.ncbi.nlm.nih.gov) with a N50 scaffold length of 1.4 Mb. The coding DNA sequences were classified into subsystems by means of RAST (http://rast.nmpdr.org). The nucleotide sequences of the three replicons obtained from strain B401 were deposited at the EMBL Nucleotide Sequence Database, accession numbers: CP019485 for the chromosome, CP019483 for megaplasmid A, and CP019484 for megaplasmid B. Strain B401 is available in two public collections: BNM (http://inba.agro.uba.ar/) and IMyZA (https://inta.gob.ar/imyza).

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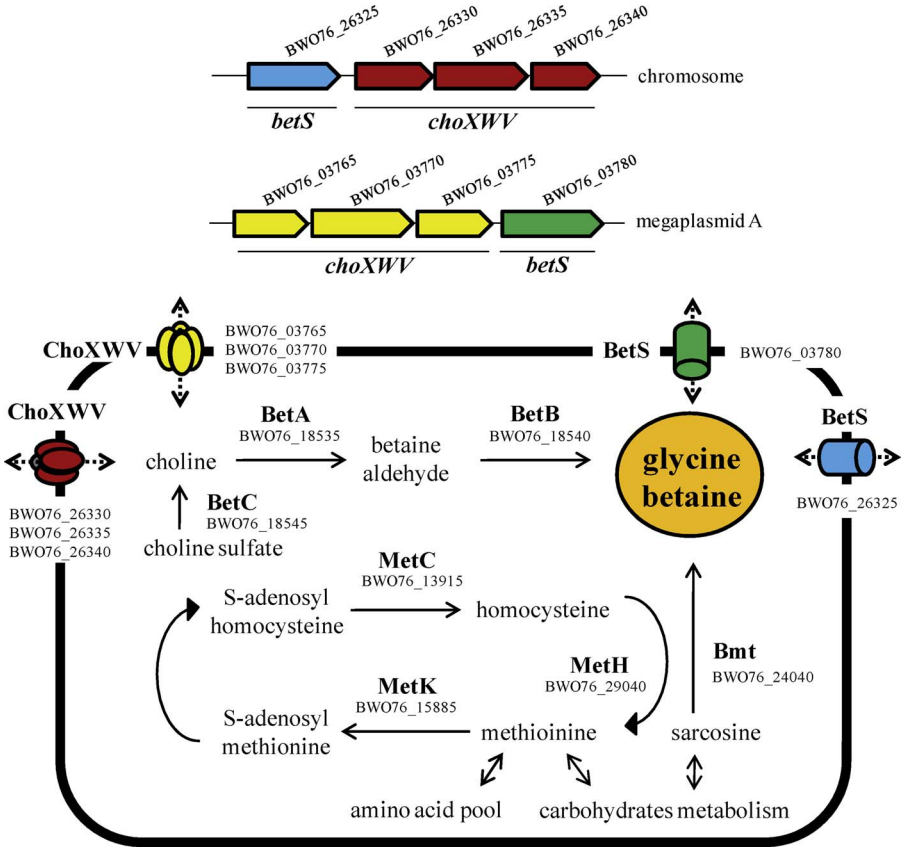

Fig. 2. Identification of genes potentially involved in the biosynthesis, degradation, uptake and transport of the osmoprotectant glycine betaine in strain B401 genome. There are three clusters involved in the complex metabolic network of glycine betaine: BWO76_18535 – BWO76_18540 – BWO76_18545 for the biosynthesis of glycine betaine from choline (*BetABC*), BWO76_26325 – BWO76_26330 – BWO76_26335 – BWO76_26340 (*BetS-ChoXWV*) and BWO76_03765 – BWO76_03770 – BWO76_03775 – BWO76_03780 (*ChoXWV-BetS*) for the transport of choline (*Cho*) and glycine betaine (*Bet*) molecules. The rest of the genes related to glycine betaine biosynthesis and degradation are in single locus: *metC* (BWO76_13915), *metK* (BWO76_15885), *metH* (BWO76_29040) and *bmt* (BWO76_24040).

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.jbiotec.2017.10.007.

References

Barra, L., Fontenelle, C., Ermel, G., Trautwetter, A., Walker, G.C., Blanco, C., 2006. Interrelations between glycine betaine catabolism and methionine biosynthesis in Sinorhizobium meliloti strain 102F34. J. Bacteriol. 188, 7195–7204.

Boscari, A., Mandon, K., Dupont, L., Poggi, M.C., Le Rudulier, D., 2002. BetS is a major glycine betaine/proline betaine transporter required for early osmotic adjustment in Sinorhizobium meliloti. J. Bacteriol. 184, 2654–2663.

Boscari, A., Van de Sype, G., Le Rudulier, D., Mandon, K., 2006. Overexpression of BetS, a Sinorhizobium meliloti high-affinity betaine transporter, in bacteroids from Medicago sativa nodules sustains nitrogen fixation during early salt stress adaptation. Mol. Plant Microbe Interact. 19, 896–903.

Cornacchione, M.V., Suarez, D.L., 2017. Evaluation of alfalfa (Medicago sativa L.) populations' response to salinity stress. Crop Sci. 57, 137–150.

Dawson, R.J., Locher, K.P., 2006. Structure of a bacterial multidrug ABC transporter. Nature 443, 180–185.

Dupont, L., Garcia, I., Poggi, M.C., Alloing, G., Mandon, K., Le Rudulier, D., 2004. The Sinorhizobium meliloti ABC transporter Cho is highly specific for choline and expressed in bacteroids from Medicago sativa nodules. J. Bacteriol. 186, 5988–5996.

Galardini, M., Pini, F., Bazzicalupo, M., Biondi, E.G., Mengoni, A., 2013. Replicon-dependent bacterial genome evolution: the case of Sinorhizobium meliloti. Genome. Biol. Evol. 5, 542–558.

Galibert, F., Finan, T.M., Long, S.R., Puhler, A., Abola, P., Ampe, F., Barloy-Hubler, F., Barnett, M.J., Becker, A., Boistard, P., Bothe, G., Boutry, M., Bowser, L., Buhrmester, J., Cadieu, E., Capela, D., Chain, P., Cowie, A., Davis, R.W., Dreano, S., Federspiel, N.A., Fisher, R.F., Gloux, S., Godrie, T., Goffeau, A., Golding, B., Gouzy, J., Gurjal, M., Hernandez-Lucas, I., Hong, A., Huizar, L., Hyman, R.W., Jones, T., Kahn, D., Kahn, M.L., Kalman, S., Keating, D.H., Kiss, E., Komp, C., Lelaure, V., Masuy, D., Palm, C., Peck, M.C., Pohl, T.M., Portetelle, D., Purnelle, B., Ramsperger, U., Surzycki, R., Thebault, P., Vandenbol, M., Vorholter, F.J., Weidner, S., Wells, D.H., Wong, K., Yeh, K.C., Batut, J., 2001. The composite genome of the legume symbiont Sinorhizobium meliloti. Science 293, 668–672.

Mandon, K., Osteras, M., Boncompagni, E., Trinchant, J.C., Spennato, G., Poggi, M.C., Le Rudulier, D., 2003. The Sinorhizobium meliloti glycine betaine biosynthetic genes (betlCBA) are induced by choline and highly expressed in bacteroids. Mol. Plant Microbe Interact. 16, 709–719.

Millian, N.S., Garrow, T.A., 1998. Human betaine-homocysteine methyltransferase is a zinc metalloenzyme. Arch. Biochem. Biophys. 356, 93–98.

Pocard, J.A., Vincent, N., Boncompagni, E., Smith, L.T., Poggi, M.C., Le Rudulier, D., 1997. Molecular characterization of the bet genes encoding glycine betaine synthesis in Sinorhizobium meliloti 102F34. Microbiology 143 (Pt. 4), 1369–1379.

Sandhu, D., Cornacchione, M.V., Ferreira, J.F.S., Suarez, D.L., 2017. Variable salinity responses of 12 alfalfa genotypes and comparative expression analyses of salt-response genes. Sci. Report. 7, 42958.

Schneiker-Bekel, S., Wibberg, D., Bekel, T., Blom, J., Linke, B., Neuweger, H., Stiens, M., Vorhölter, F.-J., Weidner, S., Goesmann, A., Pühler, A., Schlüter, A., 2011. The complete genome sequence of the dominant Sinorhizobium meliloti field isolate SM11 extends the S. meliloti pan-genome. J. Biotechnol. 155, 20–33.

diCenzo, G.C., Finan, T.M., 2015. Genetic redundancy is prevalent within the 6.7 Mb Sinorhizobium meliloti genome. Mol. Genet. Genomics 290, 1345–1356.

diCenzo, G.C., Checcucci, A., Bazzicalupo, M., Mengoni, A., Viti, C., Dziewit, L., Finan, T.M., Galardini, M., Fondi, M., 2016. Metabolic modelling reveals the specialization of secondary replicons for niche adaptation in Sinorhizobium meliloti. Nat. Commun. 7, 12219.