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NOTE

Absence of the Nitrous Oxide Reductase Gene Cluster in Commercial Alfalfa Inoculants Is Probably Due to the Extensive Loss of Genes During Rhizobial Domestication

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Abstract

As other legume crops, alfalfa cultivation increases the emission of the greenhouse gas nitrous oxide (N_2O). Since legume-symbiotic nitrogen-fixing bacteria play a crucial role in this emission, it is important to understand the possible impacts of rhizobial domestication on the evolution of denitrification genes. In comparison with the genomes of non-commercial strains, those of commercial alfalfa inoculants exhibit low total genome size, low number of ORFs and high numbers of both frameshifted genes and pseudogenes, suggesting a dramatic loss of genes during bacterial domestication. Genomic analysis focused on denitrification genes revealed that commercial strains have perfectly conserved the nitrate (NAP), nitrite (NIR) and nitric (NOR) reductase clusters related to the production of N_2O from nitrate but completely lost the nitrous oxide (NOS) reductase cluster (*nosRZDFYLX* genes) associated with the reduction of N_2O to gas nitrogen. Based on these results, we propose future screenings for alfalfa-nodulating isolates containing both nitrogen fixation and N_2O reductase genes for environmental sustainability of alfalfa production.

Keywords Nitrous oxide · NOS cluster · Commercial inoculants · Evolution

Alfalfa, usually known as the "Queen of Forages", is the main source of vegetable protein for meat and milk production systems. In addition to its indirect impact on human nutrition, alfalfa is also the most important legume crop in cultivated area worldwide (30 million ha) after soybean (110 million ha). Due to the fact that the cultivation of this perennial legume forage constitutes an extensive soil-air interface, as well as to its significant emission of the greenhouse gas nitrous oxide (N_2O) [1, 2] and the critical role of legume-symbiotic nitrogen-fixing bacteria in N_2O production via incomplete denitrification [3, 4], it is ecologically relevant to understand the possible impacts of rhizobial domestication on the evolution of denitrification gene clusters. Recently, we have shown that the genomes of the commercial alfalfa inoculants *Ensifer meliloti* B399 (originally named *Rhizobium meliloti* 102F34) and *Ensifer meliloti* B401 (originally named *Sinorhizobium meliloti* B401) are similar to those of other non-commercial strains, including the model bacterium *Ensifer meliloti* 1021 [5, 6]. In concordance with the genomic alignments showing extremely conserved nucleotide sequence without any large-scale DNA rearrangements between the commercial strains B399 and B401 and the model strain 1021 [5, 6], in this study, phylogenetic analysis of *E. meliloti* genomes supported that commercial and non-commercial strains are not divergent lineages and that both B399 and B401 are particularly closely related to strain 1021 (Fig. 1a). Interestingly, the genomes of both B399 and B401 showed significantly lower total size and lower number of ORFs than non-commercial strains (Fig. 1b). Specifically, the genomes of these commercial strains had increases of 148 and 49% in terms of pseudogenes and frameshifted genes with respect to non-commercial strains, respectively (Fig. 1b). These results further support a high selection pressure over genes

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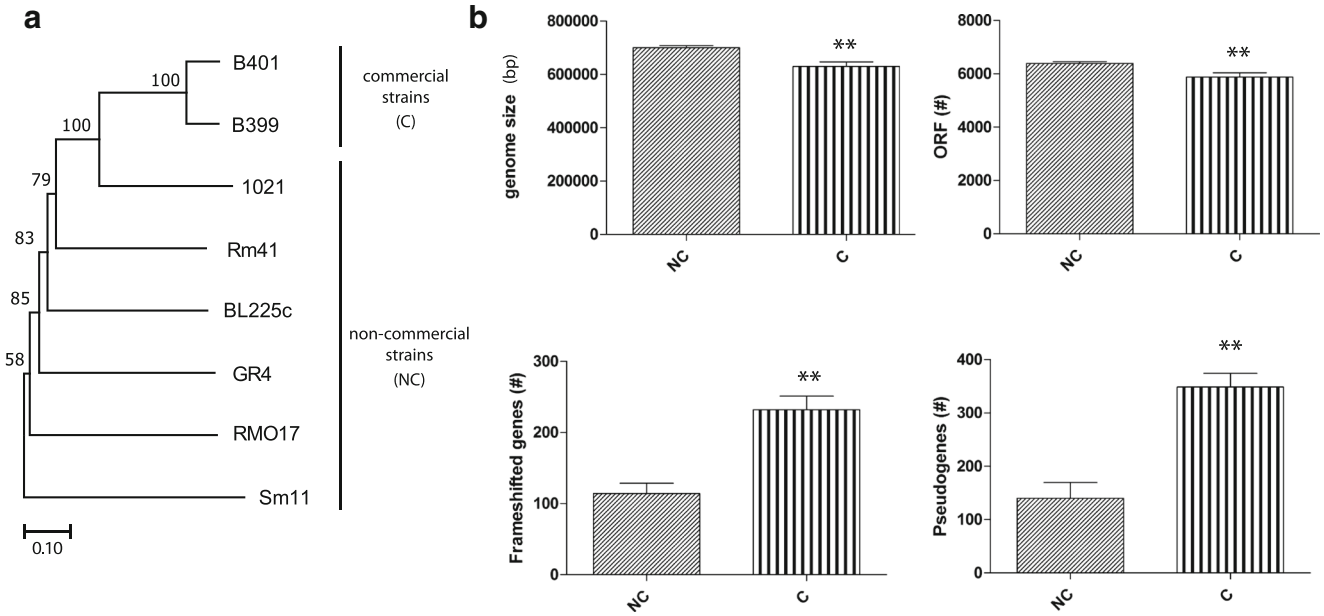
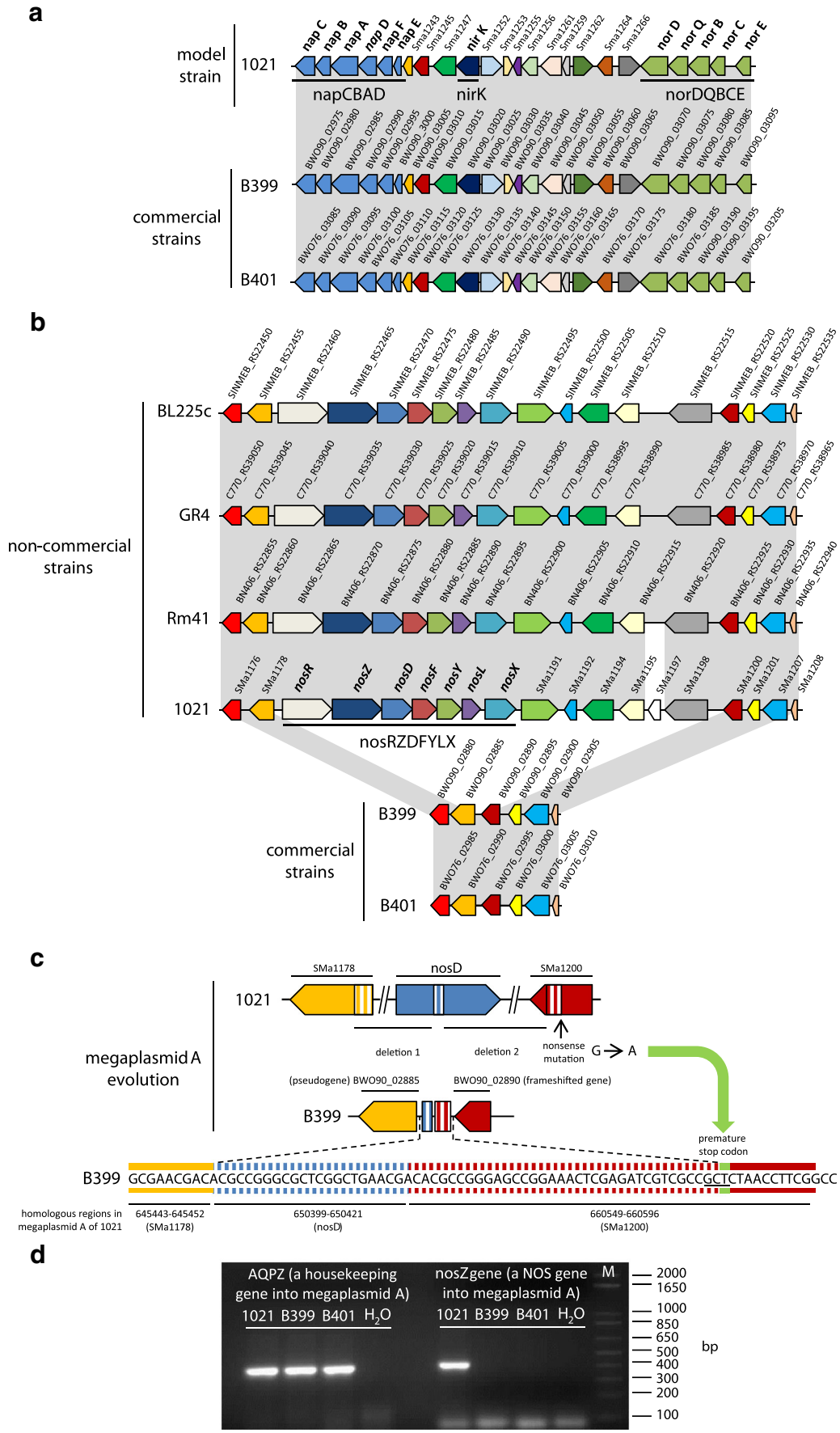


Fig. 1 Genomic analyses of *Ensifer meliloti* strains including commercial (B399 and B401) and non-commercial (1021, BL225, GR4, RMO17, Rm41 and Sm11) strains. a Evolutionary analysis of *E. meliloti* strains confirming that commercial and non-commercial strains are not divergent lineages. **b** Genomic comparisons between commercial (C) and non-commercial (NC) *E. meliloti* strains showing massive loss of genes in strains B399 and B401. Statistical analysis was carried out with Student's *t* test (**$p < 0.01$). Values are mean ± SEM

during the process of domestication of alfalfa rhizobia. Probably, this massive gene loss in commercial strains was possible due to the exceptionally high genetic redundancy in the three replicons of *Ensifer meliloti* strains [7, 8]. In addition, we observed a complete integrity of the nitrate (NAP), nitrite (NIR) and nitric oxide (NOR) clusters associated with the production of N_2O from nitrate in both B399 and B401 (Fig. 2a), which have a 100% nucleotide identity to that previously described in strain 1021 [9]. In contrast, the genomes of strains B399 and B401 showed a 15,004-bp deletion, which includes the nitrous oxidase (NOS) reductase cluster codifying *nosRZDFYLX* genes (Fig. 2b, c) related to the reduction of N_2O to gaseous nitrogen in strain 1021 and other microbes [3, 4, 10]. Genomic PCR analysis confirmed the absence of the *nosZ* gene (an essential factor for nitrous oxidase complex) in commercial strains B399 and B401 and the presence of this gene in model strain 1021, where AQPZ gene (*Ensifer meliloti* house-keeping gene) was used as an internal control for DNA template quality (Fig. 2d).

The commercial strains B399 and B401 have been the inoculants most used in alfalfa production in the last 50 years. Since the domestication of these strains was prior to the emergence of global environmental change as a critical topic in the research community and society in general, the conservation of their genetic factors related to the mitigation of N_2O emission was not considered. The general pattern of loss of genes and the dissimilar conservation of dinitrification gene clusters in B399 and B401 suggest that the NAP, NIR and NOR clusters but not the NOS cluster make significant fitness contribution to rhizobia and, more importantly, provide evidence of the potential environmental risk of the domestication of alfalfa rhizobia (Fig. 3). In this context, we propose novel screenings for alfalfa-nodulating isolates containing both nitrogen fixation and N_2O reductase genes for a sustainable production of alfalfa at both economic and ecological levels.

Fig. 2 Comparison of codified regions for denitrification reductases (NAP-NIR-NOR-NOS) from commercial strains B399 and B401 and their phylogenetic-related non-commercial strains. **a** The genomic analysis of commercial and non-commercial strains shows a conserved synteny in the megaplasmid A region that codifies for nitrate (NAP), nitrite (NIR) and nitric oxide (NOR) reductases associated with the production of nitrous oxide (N_2O) from nitrate (NO_3^-). **b** Genomic synteny comparison between commercial and non-commercial strains shows that the commercial strains suffered a 15-kb deletion in the megaplasmid A region that contains the NOS cluster (*nosRZDFYLX*) codifying the N_2O reductase related to the production of nitrogen gas (N_2) from N_2O. **c** An exhaustive analysis of this genomic region showed three evolutionary events that caused the loss of NOS cluster and the alteration of flanking genes (BWO90_02885 and BWO90_02890) in commercial strain B399: two large deletions and one nonsense mutation. In addition, traces of *nosD* gene and Sma1200 were found in BWO90_02885–BWO90_02890 intergenic regions. In genomic analysis of NAP, NIR, NOR and NOS clusters, orthologous genes have the same colour, orthologous block of genes are represented with a grey shadow and arrows represent gene orientation (Supplementary material). **d** PCR of *nosZ* (essential gene for nitrous oxidase complex) and AQPZ (*Ensifer meliloti* house-keeping gene) genes of strains 1021, B399 and B401. AQPZ was used as an internal control for DNA template quality. Water (H_2O) was used as negative control. PCR marker: 1 Kb Plus DNA Ladder (INVITROGEN)


a
model strain
1021
napCBAD
nirK
norDQBCE
commercial strains
B399
B401
b
non-commercial strains
BL225c
GR4
Rm41
1021
nosRZDFYLX
commercial strains
B399
B401
c
megaplasmid A evolution
1021
SMa1178
nosD
SMa1200
deletion 1
deletion 2
nonsense mutation G → A
(pseudogene) BWO90_02885
BWO90_02890 (frameshifted gene)
B399
premature stop codon
B399 GCGAACGACACGCCGGGCGCTCGGCTGAACGACACGCCGGGAGCCGGAAACTCGAGATCGTCGCCGCTCTAACCTTCGGCC
homologous regions in megaplasmid A of 1021
645443-645452 (SMa1178)
650399-650421 (nosD)
660549-660596 (SMa1200)
d
AQPZ (a housekeeping gene into megaplasmid A)
1021 B399 B401 H2O
nosZgene (a NOS gene into megaplasmid A)
1021 B399 B401 H2O
M
2000
1650
1000
850
650
500
400
300
200
100
bp

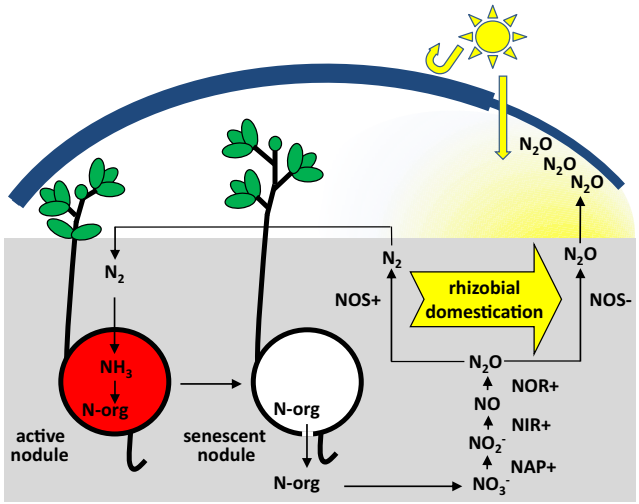


Fig. 3 Environmental risk of the rhizobial domestication. After nodule senescence, increased amount of nitrate (NO_3^-) derived from organic nitrogen (N-org) becomes available for rhizosphere microorganisms including symbiotic nitrogen-fixing commercial inoculants. These symbiotic bacteria can reduce NO_3^- to gas nitrogen (N_2) via the activity of nitrate reductase (NAP+), nitrite reductase (NIR+), nitric oxide reductase (NOR+) and nitrous oxide reductase (NOS+) [3, 12]. However, stability of NAP, NIP and NOR genes combined with loss of NOS cluster (NOS-) during rhizobial domestication can conduce to incomplete denitrification with the consequently increase of N_2O production

Methods Genomic DNA of strains 1021, B399 and B401 was isolated from overnight cultures using the Wizard Genomic DNA Purification Kit (#A1120, Promega, USA). For the PCR assays, the 408- and 398-bp fragments of the *nosZ* and AQPZ genes were obtained by PCR amplification using NosZ-U = TTACAGACGGCACCTATGACGGGCGCTATCTC and NosZ-L = GCTCGCTCGCCATCATGTCCGCCAGG GTAAAG and AQPZ-U = AGGTCGTGATGACATTCTTC TT and AQPZ-L = GTAATCCAGAAGAGCCAGAGTT primers designed in this work, respectively, and a program of 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 60 °C for 45 s and 72 °C for 30s and a final cycle of 72 °C for 10 min. Phylogenetic and genomic synteny comparisons among *Ensifer meliloti* strains were performed by using FastTree and LASTZ plugins into Geneious v10.1 software [11] using strain Sm11 as outgroup. Total genome size and numbers of ORFs, pseudogenes and frameshifted genes from *Ensifer meliloti* genome were determined by Pipeline (https://www.ncbi.nlm.nih.gov). The accession numbers used in these analyses were NZ_CP019486, NZ_CP019487 and NZ_CP019488 for strain B399; NZ_CP019485, NZ_CP019485 and NZ_CP019485 for strain B401; NC_003047, NC_003037 and NC_003078 for strain 1021; NC_017322, NC_017324 and NC_017323 for strain BL225; NC_019845, NC_019848 and NC_019849 for strain GR4; NZ_CP009144, NZ_CP009145 and NZ_CP009146 for strain RMO17; NC_018700, NC_018683 and NC_018701 for strain Rm41; and NC_017325, NC_017327 and NC_017326 for Sm11.

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