Rev Argent Microbiol. 2018;xxx(xx):xxx–xxx

REVISTA ARGENTINA DE MICROBIOLOGÍA

www.elsevier.es/ram

BRIEF REPORT

Molecular evidence that cellulolytic bacterial genus *Cohnella* is widespread among Neotropical Nasutitermitinae from NE Argentina

Joel Demián Arneodo[a,b,*], Clara Etcheverry[c], Tumelo Thebe[d], Olubukola Oluranti Babalola[d], María Celina Godoy[c], Paola Talia[a,e]

[a] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina*
[b] *Instituto de Microbiología y Zoología Agrícola (IMyZA) – INTA, Hurlingham, Argentina*
[c] *Facultad de Ciencias Exactas y Naturales y Agrimensura, Universidad Nacional de Corrientes, Corrientes, Argentina*
[d] *Faculty of Agriculture, Science and Technology, North-West University, Mmabatho, South Africa*
[e] *Instituto de Biotecnología (IB) – INTA, Hurlingham, Argentina*

Received 22 May 2017; accepted 19 November 2017

KEYWORDS
Termite;
Gut symbiont;
Cellulolytic bacteria;
Cohnella;
Paenibacillaceae

Abstract *Cohnella* is a highly cellulolytic bacterial genus, which can be found in a variety of habitats. The aim of this study was to assess its presence in the digestive tract of termite species collected in North-eastern Argentina: *Nasutitermes aquilinus*, *N. corniger* and *Cortaritermes fulviceps*. Gut homogenates were incubated with cellulosic substrate for bacterial growth. Bacterial 16S rDNA was partially amplified using new primers for *Cohnella* spp. and cloned. Sequences obtained showed highest similarity (97.2–99.9%) with those of *Cohnella* spp. previously reported from diverse environments. Phylogenetic analysis tended to group the clones according to their host species and sampling sites. These results indicate the association of *Cohnella*-related intestinal symbionts with three common Neotropical termites. Their potential industrial application encourages further research.

PALABRAS CLAVE
Termita;
Simbionte intestinal;
Bacteria celulolítica;
Cohnella;
Paenibacillaceae

Evidencia molecular de la ocurrencia de bacterias celulolíticas del género *Cohnella* en especies neotropicales de Nasutitermitinae colectadas en el NEA

Resumen *Cohnella* es un género de bacterias celulolíticas que puede ser encontrado en una variedad de hábitats. El propósito de este estudio fue registrar su presencia en el tracto digestivo de termitas (*Nasutitermes aquilinus*, *N. corniger* y *Cortaritermes fulviceps*) colectadas en el noreste argentino (NEA). Se incubaron homogenados de intestinos en sustrato celulósico

* Corresponding author.
E-mail address: arneodo.joel@inta.gob.ar (J.D. Arneodo).

https://doi.org/10.1016/j.ram.2017.11.008

para multiplicar las bacterias. Utilizando nuevos cebadores para *Cohnella* spp., se amplificó una porción del ADN ribosomal 16S bacteriano, el cual fue posteriormente clonado. Las secuencias obtenidas mostraron su mayor porcentaje de similitud (97,2-99,9%) con *Cohnella* spp., previamente reportadas en diversos ambientes. El análisis filogenético tendió a agrupar a los clones de acuerdo a la especie hospedante y al sitio de muestreo. Estos resultados indican que especies de termitas frecuentes en el NEA albergan simbiontes intestinales relacionados con el género *Cohnella*. Las potenciales aplicaciones industriales de estos microorganismos animan a profundizar los estudios.

Termites depend on both endogenous (host) and exogenous (symbiont) enzymes to accomplish lignocellulose digestion. In higher termites, exogenous cellulolytic activity is mainly due to a variety of bacterial endosymbionts hosted in the guts[3,14]. Such diversity is thought to be influenced in part by the taxonomic position of the termite species, its diet and surrounding environment[3]. Throughout the last decades, there has been an increasing interest in this insect group as a valuable source of novel cellulolytic enzymes for the biofuel industry. South America produces large amounts of biomass from agricultural waste, and Neotropical termites could presumably provide useful glycosyl hydrolases for bioethanol production[1,11]. Much work is still to be done in this field. A previous research dealing with termite-symbiotic bacteria grown on cellulose-rich media indicated the presence of the highly cellulolytic genus *Cohnella*[6] in the digestive tract of *Nasutitermes aquilinus* (Holmgren, 1910) specimens collected in North-eastern (NE) Argentina[2]. The ability of different *Cohnella* strains to degrade the plant cell wall[2,7,10], makes these ubiquitous bacteria particularly interesting in view of their biotechnological potential. Thus, the aim of this study was to further investigate the occurrence of intestinal putative *Cohnella* spp. in different populations of *N. aquilinus* and two additional Nasutitermitinae species: *N. corniger* (Motschulsky, 1855) and *Cortaritermes fulviceps* (Silvestri, 1901). These species are often found in anthropized environments of NE Argentina and neighboring countries, and exploit dissimilar nutritional resources: wood for *Nasutitermes* spp. and various substrates (comprising gramineous plants and wood) for *C. fulviceps*.

Collection sites were located in urban landscapes (Corrientes city, Corrientes, Argentina) separated by 1.4 (min)–6.5 (max) km. Samples of arboreal- (*N. aquilinus* and *N. corniger*) and epigeal- (*C. fulviceps*) termite nests were taken to the laboratory in November 2015, and worker specimens were selected for processing. Three populations of *N. aquilinus* (Boca Unidos, Jardín and Laprida districts), two of *C. fulviceps* (Jardín and Campus) and one of *N. corniger* (Regional) were surveyed for the presence of *Cohnella*-related symbionts. Insects were surface-sterilized with 70% alcohol, entire guts were dissected under binocular microscope and grounded in distillate water. Homogenates corresponding to every species and collection site (10 guts/sample for the two *Nasutitermes* spp. and 15 guts/sample in the case of the smaller *C. fulviceps* individuals) were added to 40 ml of minimal medium (MM) containing pretreated sugarcane bagasse (SCB)[2] as cellulosic substrate. Cultures were incubated on a rotary shaker (37 °C, 250 rpm). Six days after, bacterial growth was clearly visible. No bacterial proliferation was observed in the negative controls included in the experiment (MM + SCB, no gut homogenate added). After substrate sedimentation at 200 × *g* for 5 min, bacterial cultures were transferred to polypropylene microtubes and stored at −20 °C for posterior processing. Bacterial pellets were obtained by centrifugation at 10 000 × *g* for 10 min and resuspended in CTAB extraction buffer. DNA was extracted as mentioned elsewhere[2]. Both the quantity and quality of the purified bacterial DNA were estimated by spectrophotometry and agarose-gel electrophoresis.

Based on sequences available in GenBank, the following primers were designed to amplify an 846-bp-long fragment of 16S rDNA from *Cohnella* spp.: Cohnella Fw (5′-TAAGTCTGGTGTHTAAGTGCGG-3′) and Cohnella Rv (5′-CGRCTTCGGGTGTTGTAAACTC-3′). PCR conditions were set at 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 57 °C for 30 s, 72 °C for 1 min; and 72 °C for 2 min. A highly purified Taq polymerase free of bacterial DNA (INBIO, Tandil, Argentina) was used. Under this restrictive annealing temperature, PCR amplifications yielded a single band of the expected size in every sample tested. Amplicons from each bacterial culture were cloned using the pGEM-T vector (Promega Corp., Madison, USA). Thirty-nine clones corresponding to *N. aquilinus* (13 from Boca Unidos, 14 from Jardín and 12 from Laprida), 34 to *C. fulviceps* (16 from Jardín and 18 from Campus) and 15 to *N. corniger* (from Regional) were sequenced in an ABI PRISM 3500 XL genetic analyzer (Applied Biosystems, USA) at the Instituto de Biotecnología – INTA (Hurlingham, Argentina). Obtained sequences were screened for chimeras using DECIPHER[15] and compared to those in public databases. All sequences were deposited at GenBank with accession numbers KY230519 to KY230606. As presumed, BLAST search revealed highest sequence similarity with previously reported *Cohnella* spp., with identity values ranging from 97.2 to 99.9%. These data proved the efficacy of the primer set and evidenced the occurrence of *Cohnella*-related bacteria in all three termite species studied. A phylogenetic tree was constructed with the

Please cite this article in press as: Arneodo JD, et al. Molecular evidence that cellulolytic bacterial genus *Cohnella* is widespread among Neotropical Nasutitermitinae from NE Argentina. Rev Argent Microbiol. 2018. https://doi.org/10.1016/j.ram.2017.11.008

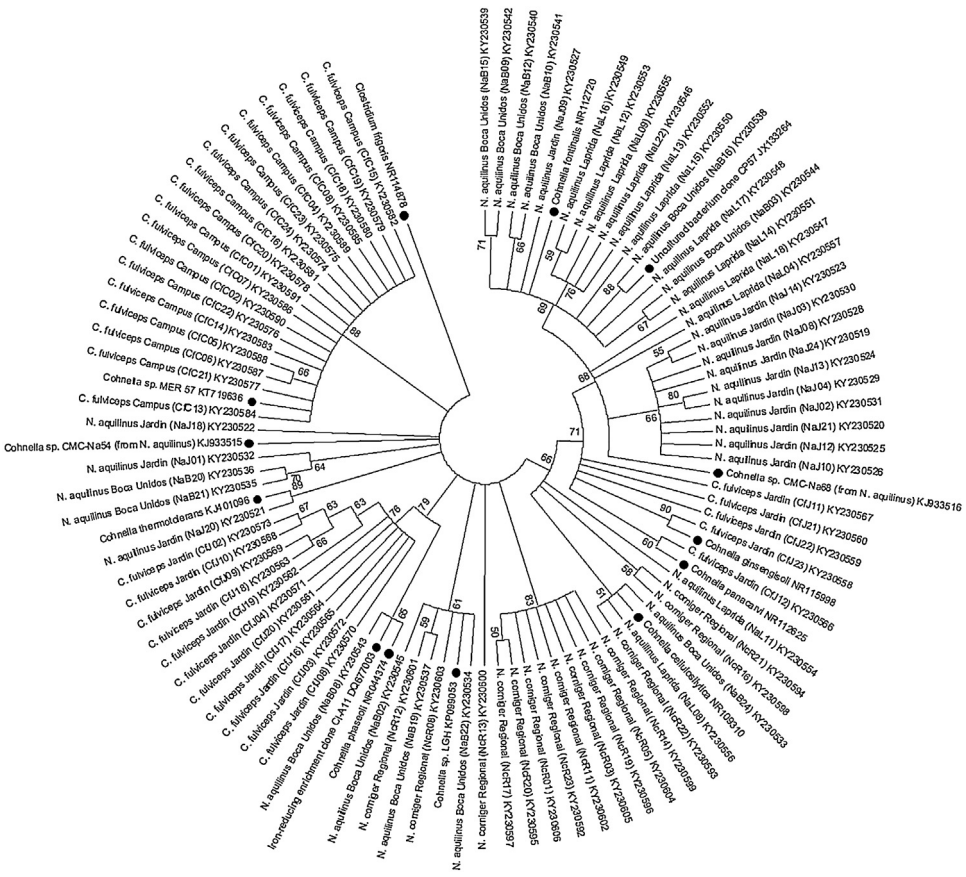

Figure 1 Phylogenetic tree inferred by the maximum likelihood method based on the partial bacterial 16S rDNA sequences obtained in this work. Host species, collection site and clone identification are given at the branch tips. Reference and previously reported sequences sharing highest nucleotide identity are highlighted by a black circle. All sequences are identified by their corresponding accession numbers. Branches with <50% Bootstrap support are collapsed (values indicated at the nodes).

sequenced clones by MEGA5[12] using the maximum likelihood method with 1000 bootstrap replicates. Bacterial sequences from GenBank that showed maximum identity percentages by BLAST were also incorporated in the analysis. The Firmicute *Clostridium frigoris* served as outgroup. In general, the investigated 16S rDNA sequences tended to group together according to the sample, *i.e.* host species and collection site (Fig. 1). A few exceptions were observed within *Nasutitermes* spp.: some clusters were formed by bacterial sequences obtained from distinct samples (different districts and/or host species). Concerning the 16S rDNA clones from *C. fulviceps*, sequences were grouped by district origin in more homogeneous clusters.

Since its separation from the genus *Paenibacillus*[6], several species have been characterized within the genus *Cohnella*, most of them with cellulolytic or xylanolytic activities[2,7,10]. Members of *Cohnella* were isolated from a variety of environments, including industrial samples, plant root nodules, soil, water and animal feces[6,7,10]. With respect to Nasutitermitinae, metagenomic analyses regarding the microbial diversity of *N. corniger* revealed the regular presence of Firmicutes, though at comparatively low abundance compared to other bacterial phyla[8,9,14]. None of the latter studies referred to *Paenibacillus* or *Cohnella* spp. More recently, however, a *Paenibacillus* sp. has been reported in *Nasutitermes arborum* by 16S rRNA gene pyrosequencing[5], and *Paenibacillus nasutitermitis* was described as an intestinal symbiont of *Nasutitermes* sp[13]. In a preceding study restricted to a single *N. aquilinus* nest, *Cohnella* was shown to form part of the cellulolytic microbial community associated to this wood-eating termite[2]. The present paper supports this finding and demonstrates that such situation is a common feature in different *N. aquilinus* colonies. Moreover, 16S rDNA sequences affiliated with *Cohnella* spp. were consistently determined in the taxonomically close *N. corniger* and *C. fulviceps*. In all cases, DNA amplification and sequencing was made after bacterial multiplication in cellulose medium. When PCR was directly performed on total gut DNA extractions, only a few samples gave positive reactions (not shown). This could be due to their relatively low abundance in the termite guts, counteracted by elevated growth rates in enrichment media. In this sense, a recent survey carried out on a wood-feeding lower termite showed that the largest amount of culturable cellulolytic bacteria belonged to the genera *Bacillus* and *Paenibacillus*[4]. In digestive symbioses, different symbiont transmission routes have been postulated to play a concomitant role in shaping the intestinal microbiota[3]. Social exchanges (including stomodeal trophallaxis) have been proposed as a chief way of gut-specific bacterial colonization in higher termites. In

Please cite this article in press as: Arneodo JD, et al. Molecular evidence that cellulolytic bacterial genus *Cohnella* is widespread among Neotropical Nasutitermitinae from NE Argentina. Rev Argent Microbiol. 2018. https://doi.org/10.1016/j.ram.2017.11.008

addition, the possibility of direct acquisition of facultative symbionts from the environment cannot be excluded[3,5]. Whether culturable *Cohnella*-related bacteria associated to Nasutitermitinae also exist as free-living organisms in nature is to be elucidated.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This work was funded by Agencia Nacional de Promoción Científica y Tecnológica through Grant PICT-2013-1454, Instituto Nacional de Tecnología Agropecuaria, Secretaría General de Ciencia y Técnica – Universidad Nacional del Nordeste (Argentina), Bilateral Project MinCyT-DST SA/13/10 (Argentina/South Africa), the National Research Foundation UID 91990 and North-West University (South Africa). Authors are indebted to Facundo Felice (IB-INTA) for Technical Assistance.

References

1. Batista-García RA, del Rayo Sánchez-Carbente M, Talia P, Jackson SA, O'Leary ND, Dobson ADW, Folch-Mallol JL. From lignocellulosic metagenomes to lignocellulolytic genes: trends, challenges and future prospects. Biofuel Bioprod Biorefin. 2016;10:864–82.
2. Ben Guerrero E, Arneodo J, Bombarda Campanha R, Abrão de Oliveira P, Veneziano Labate MT, Regiani Cataldi T, Campos E, Cataldi A, Labate CA, Martins Rodrigues C, Talia P. Prospection and evaluation of (Hemi) cellulolytic enzymes using untreated and pretreated biomasses in two Argentinean native termites. PLoS ONE. 2015;10:e0136573, http://dx.doi.org/10.1371/journal.pone.0136573.
3. Brune A, Dietrich C. The gut microbiota of termites: digesting the diversity in the light of ecology and evolution. Annu Rev Microbiol. 2015;69:145–66.
4. Butera G, Ferraro C, Alonzo G, Colazza S, Quatrini P. The gut microbiota of the wood-feeding termite *Reticulitermes lucifugus* (Isoptera: Rhinotermitidae). Ann Microbiol. 2016;66:253–60.
5. Diouf M, Roy V, Mora P, Frechault S, Lefebvre T, Hervé V, Rouland-Lefèvre C, Miambi E. Profiling the succession of bacterial communities throughout the life stages of a higher termite *Nasutitermes arborum* (Termitidae Nasutitermitinae) using 16S rRNA gene pyrosequencing. PLOS ONE. 2015;10.
6. Kämpfer P, Rosselló-Mora R, Falsen E, Busse H, Tindall BJ. *Cohnella thermotolerans* gen. nov., sp. nov., and classification of '*Paenibacillus hongkongensis*' as *Cohnella hongkongensis* sp. nov. Int J Syst Evol Microbiol. 2006;56:781–6.
7. Khianngam S, Tanasupawat S, Akaracharanya A, Kim KK, Lee KC, Lee J. *Cohnella cellulosilytica* sp. nov., isolated from buffalo faeces. Int J Syst Evol Microbiol. 2012;62:1921–5.
8. Köhler T, Dietrich C, Scheffrahn RH, Brune A. High-resolution analysis of gut environment and bacterial microbiota reveals functional compartmentation of the gut in wood-feeding higher termites (*Nasutitermes* spp.). Appl Environ Microbiol. 2012;78:4691–701.
9. Mikaelyan A, Strassert JFH, Tokuda G, Brune A. The fibre-associated cellulolytic bacterial community in the hindgut of wood-feeding higher termites (*Nasutitermes* spp.). Environ Microbiol. 2014;16:2711–22.
10. Shiratori H, Tagami Y, Beppu T, Ueda K. *Cohnella fontinalis* sp. nov., a xylanolytic bacterium isolated from fresh water. Int J Syst Evol Microbiol. 2010;60:1344–8.
11. Talia P, Arneodo J. Lignocellulose degradation by termites. In: Sustainable termite management. Vol. 1: Biology, social behaviour and economic importance. Springer series: sustainability in plant and crop protection; 2018; p. 101-17.
12. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28:2731–9.
13. Wang XM, Ma S, Yang SY, Peng R, Zheng Y, Yang H. *Paenibacillus nasutitermitis* sp. nov., isolated from a termite gut. Int J Syst Evol Microbiol. 2016;66:901–5.
14. Warnecke F, Luginbühl P, Ivanova N, Ghassemian M, Richardson TH, Stege JT, Cayouette M, McHardy AC, Djordjevic G, Aboushadi N, Sorek R, Tringe SG, Podar M, Garcia-Martin H, Kunin V, Dalevi D, Madejska J, Kirton E, Platt D, Szeto E, Salamov A, Barry K, Mikhailova N, Kyrpides NC, Matson EG, Ottesen EA, Zhang X, Hernández M, Murillo C, Acosta LG, Rigoutsos I, Tamayo G, Green BD, Chang C, Rubin EM, Mathur EJ, Robertson DE, Hugenholtz P, Leadbetter JR. Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. Nature. 2007;450:560–5.
15. Wright ES, Yilmaz LS, Noguera DR. DECIPHER, a search-based approach to chimera identification for 16S rRNA sequences. Appl Environ Microb. 2012;78:717–25.

Please cite this article in press as: Arneodo JD, et al. Molecular evidence that cellulolytic bacterial genus *Cohnella* is widespread among Neotropical Nasutitermitinae from NE Argentina. Rev Argent Microbiol. 2018. https://doi.org/10.1016/j.ram.2017.11.008