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Published version:

DOI:10.1038/ismej.2017.84

Salvioli di Fossalunga, Alessandra; Lipuma, Justine; Venice, Francesco; Dupont, Laurence; Bonfante, Paola. The endobacterium of an arbuscular mycorrhizal fungus modulates the expression of its toxin-antitoxin systems during the life cycle of its host. THE ISME JOURNAL. None pp: 1-5.
DOI: 10.1038/ismej.2017.84

The Endobacterium of an Arbuscular Mycorrhizal Fungus Modulates the Expression of its Toxin-Antitoxin Systems During the Life Cycle of its Host

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Running title: TA systems in the genome of a fungal endobacterium

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Conflict of interest statement: The authors declare no conflict of interest.

Subject Categories: Microbe-microbe and microbe-host interactions

Abstract

Arbuscular mycorrhizal fungi (AMF) are widespread root symbionts that perform important ecological services, such as improving plant nutrient and water acquisition. Some AMF from the *Gigasporaceae* family host a population of endobacteria, *Candidatus* Glomeribacter gigasporarum (*Ca*gg). The analysis of the *Ca*gg genome identified six putative toxin-antitoxin modules (TAs), consisting of pairs of stable toxins and unstable antitoxins that affect diverse physiological functions. Sequence analysis suggested that these TA modules were acquired by horizontal transfer. Gene expression patterns of two TAs (yoeB/yefM and chpB/chpS) changed during the fungal life cycle, with the expression during the presymbiotic phase higher than during the symbiosis with the plant host. The heterologous expression in *Escherichia coli* demonstrated the functionality only for the YoeB-YefM pair. Based on these observations, we speculate that TA modules might help *Ca*gg adapt to its intracellular habitat, coordinating its proliferation with the physiological state of the AMF host.

Arbuscular mycorrhizal fungi (AMF) perform key ecological services, improving nutrient acquisition and water uptake by their plant hosts, while receiving fixed carbon from the host (Smith and Read, 2010). Many fungi, particularly from basal clades, harbor bacterial endosymbionts (Bonfante and Desiro, 2017) and AMF from the *Gigasporaceae* family host a population of *Burkholderia*-related microbes (Bianciotto *et al*., 1996) named *Candidatus* Glomeribacter gigasporarum (*Ca*gg). *Ca*gg is vertically transmitted and currently uncultivable; although not essential for *Gigaspora* survival, *Ca*gg can enhance fungal fitness (Bianciotto *et al*., 2003; Lumini *et al*., 2007; Salvioli *et al*., 2016). Analysis of the 1.72-Mb *Ca*gg genome revealed strong nutritional dependence on the fungal host (Ghignone *et al*., 2012). We wondered whether the *Ca*gg genome possesses genetic determinants involved in its endocellular lifestyle and environmental sensing. Our previous study identified potentially secreted proteins, which could act as effectors (Ghignone *et al*., 2012); the present study identified genes encoding toxin-antitoxin (TA) systems.

Bacterial TA systems (TAs) are typically encoded in operons located on plasmids or on the bacterial chromosome. They can be classified into five types (I to V) with the antitoxin acting as a protein (types II, IV and V) or as a noncoding RNA (types I and III) (Schuster and Bertram, 2013). The well-studied type II TAs include a stable toxin protein and an unstable antitoxin that counteracts the effect of the toxin (Leplae *et al.*, 2011). Depending on the TA superfamily, the type II toxins either interfere with DNA replication (*e.g.* CcdB) or with the translation of mRNA (*e.g.* MazE and RelE) (Mruk and Kobayashi, 2014). It was originally proposed that obligate host-associated bacteria lost their TAs as a consequence of the reduction in genome size experienced during evolution (Pandey and Gerdes, 2005). For example, *Mycobacterium tuberculosis* possesses 88 TAs, but the genome of the obligate intracellular pathogen *Mycobacterium leprae* has no TAs, supporting the idea that TAs help free-living bacteria cope with the changing environment. However, data from high-throughput sequencing have challenged this view by revealing TAs in endocellular bacteria, including pathogens and beneficial symbionts (Sevin and Barloy-Hubler, 2007). By contrast, the genomes of insect endosymbionts do not seem to carry TA operons, except *Wolbachia*, the secondary symbiont of *Drosophila melanogaster*, which encodes seven relBE-like TAs (Sevin and Barloy-Hubler, 2007).

TAs have multiple roles, including in programmed cell death (Bayles, 2014), stress adaptation (Ramage *et al*., 2009), and survival in host cells (Helaine *et al*., 2014). For example, persistence of *Salmonella* within macrophages requires the presence of TAs (Helaine *et al*., 2014). In the symbiotic nitrogen-fixing *Sinorhizobium meliloti*, a mutation of the toxin from the *ntrPR* system improved symbiotic efficiency and mutations of the *vapBC-5* pair influenced nitrogen fixation capacity and bacteroid senescence (Olah *et al*., 2001; Lipuma *et al*., 2014). These data suggest that intracellular endobacteria may exploit TAs to modulate their interactions with host cells.

To date, characterization of TAs from fungal endobacteria has been limited to descriptions of their presence in the genome. However, due to their involvement in modulation of growth under stress conditions and survival in host cells, TAs have been hypothesized to play important roles in regulating the life of fungal endobacteria (Lackner *et al*., 2011). Here, we characterize TA systems present in the *Ca*gg genome and test the functionality of two of them.

Analysis of the *Ca*gg genome revealed six complete TAs and three orphan coding sequences (Supplementary Table 1). We also conducted a similar analysis of the genomes of *Ca*gg relatives with different lifestyles, considering two endofungal bacteria, namely *Burkholderia rhizoxinica*, living inside *Rhizopus microsporus* (Partida Martinez *et al*., 2005), and *Mycoavidus cysteinexigens* living inside *Mortierella* (Ohshima *et al*., 2016; Uehling *et al*., 2017), as well as the obligate endosymbiont of the citrus mealybug, *Candidatus* Tremblaya princeps (López-Madrigal *et al*., 2011). The genomes of *B. rhizoxinica*, *M. cysteinexigens*, and *Ca*gg encode TA systems, albeit fewer than in their free-living relatives (Figure 1). By contrast, no TA could be confidently predicted for *Ca.* Tremblaya princeps, probably due to its extremely reduced genome (only 139 kb). Comparison of *Ca*gg TAs with those from its close relative *B. rhizoxinica* revealed a low sequence identity and a lack of collinearity between the two species (Supplementary Table 2). In most cases, the sequences showed more similarity to phylogenetically distant bacteria than to each other (Supplementary Table 1), as also supported by phylogenetic analyses (Supplementary Figure 1). These results are consistent with the acquisition of TAs through lateral gene transfer, as extensively demonstrated for other bacteria (Makarova *et al*., 2009), including endocellular ones as *Rickettsia* (Audoly *et al*., 2011).

To test whether the *Ca*gg TAs play a functional role, we selected two of them, a *yoeB*-*yefM*-like TA pair (CAGGBEG34_v5_20154- CAGGBEG34_v5_20012) and a *chpB*-*chpS*-like pair (CAGGBEG34_v5_60038- CAGGBEG34_v5_60039) from the complete TA systems. These modules are likely to have a chromosomal and plasmidic localization, respectively (see Supplementary Information) and they both belong to the type II TAs (RelE and MazF superfamilies, respectively). Measurement of the expression of these TAs at different stages in the life cycle of *G. margarita*, from the pre-symbiotic stages (non-germinating, germinating, strigolactone-treated spores) to the formation of the symbiotic mycelium (see Supplementary Figure 2 for a depiction of the fungal life cycle), showed that TA gene expression changed throughout the fungal life cycle (Figure 2a and b), with more expression during the fungal pre-symbiotic stages. *G. margarita* is a biotroph, requiring the plant environment to complete its life cycle. The pre-symbiotic stage, when fungal growth mostly depends on its endogenous nutrient supplies, represents a critical step for the fungus as it explores the surrounding soil to find and associate with a plant root. Under these conditions, excessive proliferation of the endobacterium, which acts as an energy sink, might be deleterious for the survival of the fungal/bacterial system. Such stressful conditions might trigger the activation of TA transcription, in contrast to the symbiotic stage, when there is a balanced plant- fungal nutrient exchange. Thus, it is possible that *Ca*gg TAs are involved in the bacterial response to the stress experienced during the fungal pre-symbiotic stages and/or in the survival inside spores, by the induction of a state comparable to the TA-induced persistence state described in *Salmonella* (Helaine *et al*., 2014). Interestingly, the expression of the *Ca*gg cell division gene *ftsZ* inversely mirrors the expression of the tested TAs, suggesting that TA activity negatively correlates with bacterial growth (Anca *et al*., 2009).

To test whether the *Ca*gg TAs encode functional toxins and antitoxins, we heterologously expressed the proteins from an inducible promoter, as *Ca*gg currently cannot be cultivated *in vitro*. The expression of the YoeB protein strongly affected the growth of *E. coli* cells, producing a decrease in numbers of living cells as early as 3 h after toxin induction. By contrast, the expression of YoeB toxin together with its cognate antitoxin YefM did not affect *E. coli* growth (Figure 2c and d, Supplementary Figure 3). These results demonstrate that YoeB affects *E. coli* cell viability, while YefM prevents its toxic effect, and that this system acts as a TA module. The activity of the ChpB toxin was also analyzed but ChpB showed no effect on *E. coli* growth and viability in our experimental conditions (data not shown).

TAs from the RelE and MazF superfamilies help bacteria cope with nutritional stresses (Gerdes *et al*., 2005). Recent evidence indicates that *Mycobacterium tuberculosis Rel* loci also react to other stresses, such as oxidative and nitrogen-limiting conditions (Korch *et al*., 2015). For this reason, we next tested whether an oxidative stress can induce the YoeB-YefM TA gene expression. *Ca*gg has been shown to promote antioxidative responses in its fungal host (Vannini *et al*., 2016); therefore, we challenged germinating spores with H_2O_2. This stress induced the up-regulation of TA gene expression, with statistically significant upregulation of the *yefM* antitoxin gene (Supplementary Figure 4).

The observations described above demonstrate the functionality of a TA module from an endobacterium that lives inside a fungus that lives inside a plant. TA gene expression is regulated throughout the fungal life cycle, and we speculate that it can respond to external stimuli by modulating bacterial cell division. In conclusion, our findings suggest that TAs might represent one of the genetic determinants that coordinate the *Ca*gg population dynamics with the life cycle of its fungal host.

Acknowledgements

Research was funded by the local project, 60% to PB. Exchanges between Torino and Nice University were supported by the Italian-French PRES Agreement. The authors wish to express their thanks to Jennifer Mach for the language revision. They also thank Stefano Ghignone, Mara Novero, and Veronica Volpe, as well as the RASTA software developers.

Supplementary information is available at The ISME Journal’s website (http://www.nature.com/ismej).

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Figure legends

Figure 1 Numbers of TA systems found in the genome of the *Ca*gg endosymbiont and its closest relatives. The approximate genome size is given in circles and the number of TAs is given below the circles. Genomes were scanned for TAs using the RASTA and TA finder online tools.

**B. phymatum* is a free-living microbe that can nodulate legume roots (Vandamme *et al*., 2002).

Figure 2 Relative expression of the bacterial gene pairs *yoe*B/*yef*M (a) and *chp*B/*chp*S (b) obtained by RT-qPCR according to the $2^{-\Delta\Delta ct}$ method. Means with different superscripts differ significantly (Kruskal-Wallis, $p<0.05$). The biological material includes the fungal host *G. margarita*, which contains the endobacterium *Ca*gg. The tested conditions where the following: non-germinating spores (control); germinating spores; strigolactone-treated spores; symbiotic intra-radical mycelium colonizing *Lotus japonicus* roots; extra-radical mycelium, developing at the root surface. Strigolactones are phytohormones that stimulate AMF hyphal growth and branching (Akyiama *et al*., 2005). Compared with the pre-symbiotic stages (spores), the expression of the TA operons decreased when the fungus formed the symbiotic mycelium (extra- and intra-radical).

Growth (c) and viability (d) of *E. coli* expressing the YoeB toxin from plasmid pBAD24-YoeB and the YoeB and YefM proteins from plasmid pBAD24-YoeBYefM. The expression was induced by the addition of 1% arabinose to the bacterial cultures. Control cells carried the empty vector pBAD24. Samples taken at 0, 3, and 4 hours after induction were plated and living cell numbers were calculated from three independent experiments. Bars represent standard deviations.