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Metabolic engineering of a diazotrophic bacterium improves ammonium release and biofertilization of plants and microalgae

Rafael Ambrosio[a,b,1], Juan Cesar Federico Ortiz-Marquez[a,b,1], Leonardo Curatti[a,b,*]

[a] Instituto de Investigaciones en Biodiversidad y Biotecnología, Consejo Nacional de Investigaciones Científicas y Técnicas, Mar del Plata, Buenos Aires, Argentina
[b] Fundación para Investigaciones Biológicas Aplicadas, Argentina

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ABSTRACT

The biological nitrogen fixation carried out by some Bacteria and Archaea is one of the most attractive alternatives to synthetic nitrogen fertilizers. However, with the exception of the symbiotic rhizobia-legumes system, progress towards a more extensive realization of this goal has been slow. In this study we manipulated the endogenous regulation of both nitrogen fixation and assimilation in the aerobic bacterium *Azotobacter vinelandii*. Substituting an exogenously inducible promoter for the native promoter of glutamine synthetase produced conditional lethal mutant strains unable to grow diazotrophically in the absence of the inducer. This mutant phenotype could be reverted in a double mutant strain bearing a deletion in the *nifL* gene that resulted in constitutive expression of *nif* genes and increased production of ammonium. Under GS non-inducing conditions both the single and the double mutant strains consistently released very high levels of ammonium (> 20 mM) into the growth medium. The double mutant strain grew and excreted high levels of ammonium under a wider range of concentrations of the inducer than the single mutant strain. Induced mutant cells could be loaded with glutamine synthetase at different levels, which resulted in different patterns of extracellular ammonium accumulation afterwards. Inoculation of the engineered bacteria into a microalgal culture in the absence of sources of C and N other than N_2 and CO_2 from the air, resulted in a strong proliferation of microalgae that was suppressed upon addition of the inducer. Both single and double mutant strains also promoted growth of cucumber plants in the absence of added N-fertilizer, while this property was only marginal in the parental strain. This study provides a simple synthetic genetic circuit that might inspire engineering of optimized inoculants that efficiently channel N_2 from the air into crops.

1. Introduction

It is anticipated that the increase in the human population and greater per capita incomes of 2.3 billion people towards 2050 would demand a nearly 100% increase in agricultural crop production (Tilman et al., 2011). The current trends for increasing agricultural production imply intensification with increased productivities in the same land area or extensification with greater land clearing in developed or developing regions of the world, respectively (Tilman et al., 2011).

The use of fertilizers, especially nitrogen, will be crucial to increase productivity since up to 90% (typically 40–60%) of crop yield can be attributed to commercial fertilizer inputs (Stewart and Roberts, 2012). Despite the welfare that the industrial production of N fertilizers by the Haber–Bosch process has signified, and still represents, to food security, both its production and use bring an injurious footprint to the environment (Erisman et al., 2008). Paradoxically, while crop productivity is chronically low due to limited access to N-fertilizers in some regions of the undeveloped world, in other more developed regions, mainly due to wrong application time and dose, the N use efficiency by cereal crops has dropped to nearly 30%. This results in a major loss of the N-fertilizer into the atmospheric, terrestrial and aquatic environments producing a variety of adverse side effects (Tilman et al., 2002; Canfield et al., 2010).

Additionally, concerns on depletion of fossil fuels and environmental decay have precipitated the need to develop alternative and sustainable sources of energy, such as biofuels from crops. Biofuels have been classified as of first, second and third generation according to the nature of its feedstocks as mainly intended for food, agricultural waste (mostly lignocellulose) and microalgae, respectively (Wijffels

* Corresponding author at: Instituto de Investigaciones en Biodiversidad y Biotecnología (INBIOTEC), Vieytes 3103, Mar del Plata 7600, Argentina.
E-mail address: lcuratti@fiba.org.ar (L. Curatti).
[1] These authors contributed equally.

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et al., 2013). Thus biofuels technology would pose an additional constraint towards food security, more prominently in the case of first generation biofuels but also indirectly in those cases that may dispute the use of arable land and agrochemicals, especially N-fertilizers (Ortiz-Marquez et al., 2014).

One of the most promising alternatives for affordable and environmentally benign N-fertilizers is promoting the agricultural use of biological nitrogen fixation (BNF), consisting of the biological production of ammonia from atmospheric N_2 (Canfield et al., 2010). BNF is an essential part of the geochemical cycle of N that accounts for about two-thirds of the total fixed N_2 while most of the remainder is due to the Haber–Bosch process. While the only known strict diazotrophs belong to the Domains Bacteria or Archaea, some eukaryotes (mainly plants and algae) engage in trophic associations with N_2-fixing bacteria that allow them to gain access to the atmospheric N_2. The legume–rhizobium symbiosis has been successfully used in agriculture for decades and also inoculation of crop plants with different N_2-fixing bacteria has been proved satisfactory towards the reduction of synthetic N-fertilizer requirements (Curatti and Rubio, 2014).

More recently, it has been proposed that metabolic engineering/synthetic biology has potential to take agricultural exploitation of BNF for crops production to a next level by means of (1) engineering new symbioses between plants and N_2-fixing bacteria (Oldroyd and Dixon, 2014; Mus et al., 2016); (2) enhancing of N_2-fixing bacterial endophytes or free living diazotrophs (Geddes et al., 2015); and (3) directly transferring of bacterial *nif* genes into crops (Curatti and Rubio, 2014; López-Torrejón et al., 2016; Ivleva et al., 2016).

BNF is catalyzed by oxygen sensitive nitrogenases in a high energy-demanding reaction requiring at least 16 ATP to fix 1 N_2. The molybdenum nitrogenase is a complex of dinitrogenase (NifDK heterotetramer) and dinitrogenase reductase (NifH homodimer) that requires dozens of additional genes to assemble an active enzyme. The γ-proteobacterium *Azotobacter vinelandii* is a free-living diazotroph that is exquisitely adapted to carry out aerobic BNF among other anaerobic metabolic pathways (Setubal et al., 2009). In this bacterium, the N_2 fixation genes for the molybdenum nitrogenase (*nif* genes) are activated by NifA-RpoN, while the antiactivator NifL interacts with and inhibits NifA to prevent *nif* genes expression when ammonium is available and/or in response to elevated concentrations of oxygen (Dixon and Kahn, 2004) (Fig. 1). In *A. vinelandii,* ammonium is incorporated into amino acids by a cyclic pathway comprising glutamine synthetase and glutamate synthase (GS-GOGAT pathway) (Kleinschmidt and Kleiner, 1978). In bacteria, GS is tightly regulated to attain cellular N homeostasis; under N-limiting conditions GlnD uridylylates the signal transduction proteins PII and GlnK increasing their rate of deadenylylation of GS (activation) by stimulating the adenylyl-removing activity of GlnE. Conversely, under N-sufficiency GlnD deuridylylates PII and GlnK to revert the GlnE-dependent activation of GS (Colnaghi et al., 2001) (Fig. 1).

Thus, *A. vinelandii*, as most free-living diazotrophs, makes use of concerted mechanisms for cellular N homeostasis and typically does not fix N_2 in excess nor excretes significant amounts of N_2-fixation products (Ortiz-Marquez et al., 2012). Three different kinds of mutations have been associated with an enhanced capacity of ammonium excretion in *A. vinelandii* and other diazotrophic bacteria: 1) disruption of the NifA/NifL-dependent ammonium control of *nif* genes expression; 2) partial inhibition of GS or GOGAT for deficient ammonium assimilation (Ortiz-Marquez et al., 2014); and 3) disruption of the ammonium/methylammonium transporter AmtB, although to lower relative level (Barney et al., 2015) (Fig. 1). *A. vinelandii* cells bearing a point mutation at the active site of GS (*glnA* D49S) displayed a moderate mutant phenotype under diazotrophyc growth conditions and excreted ammonium into the medium up to 1.7 mM. Conversely, double mutant strains (*ΔnifL*, *glnA* D49S) presented a stronger diazotrophic growth defect and although presented an increased initial rate of ammonium release into the medium, the maximum concentration remained lower than the single mutant cells at 1.0 mM, presumably as a consequence of the severe growth defect imposed by the combination of the mutations. Inoculation of either single or double mutant strains into a microalgal culture sustained microalgae growth at the expense of atmospheric CO_2 and N_2 in a synthetic microalgae-bacteria consortium (Ortiz-Marquez et al., 2014).

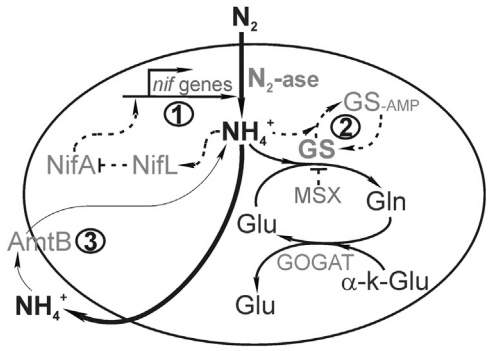

Fig. 1. Simplified schematic of N_2 fixation, ammonium assimilation and control of nitrogen homeostasis in *A. vinelandii*. Nitrogenase catalyses the conversion of atmospheric N_2 into ammonium, which is further assimilated into glutamate by the GS-GOGAT cycle. When cellular ammonium sufficiency is attained by supplementation of a suitable source of nitrogen or as a result of N_2 fixation, increased cellular level of ammonium down-regulates *nif* genes expression by a NifLA-dependent pathway (1) and promotes adenylylation of GS (inhibition of the Gln biosynthetic reaction and activation of the non-physiological γ-glutamyl transferase activity) (2). While ammonium diffuses in and out of the cell, the high-affinity ammonium-methylammonium transporter AmtB scavenges and uptakes ammonium at μM concentrations (3). It has been shown that modification of either of these three pathways promotes ammonium accumulation in the growth medium of *A. vinelandii* cells (see main text for details). N_2-ase, Fe-Mo nitrogenase; GS, glutamine synthetase; GS-AMP, adenylylated GS; GOGAT, glutamine-2-oxoglutarate aminotransferase; Glu, glutamate; Gln, glutamine; α-k-Glu, α-2-oxoglutarate; MSX, methionine sulfoximine. Solid lines indicate metabolic flux and dash lines regulatory circuits.

Thus, genetic engineering of ammonium release by aerotolerant non-symbiotic bacteria would be of prime interest for: 1) the development of versatile N-biofertilizers for sustainable agriculture (Geddes et al., 2015; Mus et al., 2016) and/or 2) the construction of exchangeable N_2-fixing parts for synthetic biology approaches for the development of multispecies microbial cell-factories comprising CO_2-fixing and O_2 evolving microalgae or cyanobacteria (Ortiz-Marquez et al., 2013; Smith and Francis, 2016).

This report shows high levels of ammonium accumulation (up to 20 mM) in the growth medium of *A. vinelandii* conditionally-lethal mutant strains that express *glnA* under an exogenously inducible promoter (trc_P-*glnA*). Single mutant strains (trc_P-*glnA*) excreted ammonium at very low concentrations of the inducer, which corresponds with extremely slow growth. On the other hand double mutant strains (trc_P-*glnA; ΔnifL)* grew and excreted high levels of ammonium under a wider range of concentrations of the inducer. Mutant cells could be loaded with GS activity at different levels allowing an additional level of control of cells population and ammonium release. Inoculation of the engineered bacteria proved to be a good replacement of N-fertilizers for growth of both microalgae and cucumber plants.

2. Materials and methods

2.1. Organisms and culture conditions

A. vinelandii strain DJ was the *wt* strain used in this study and was kindly provided by Dennis Dean (Virginia Tech). *A. vinelandii* strain AV3 (*ΔnifL*) isolation has been described previously (Ortiz-Marquez et al., 2012). *A. vinelandii* strains DJ, AV3, AV11 (trc_P-*glnA*), AV12 (*ΔnifL*, trc_P-*glnA*), and AV13 (trc_P-*glnA; ΔnifA::sp*) were maintained in Burk's modified medium (Strandberg and Wilson, 1968), incubated at 29 ± 1 °C with shaking at 200 rpm. When required media were