Metabolic Engineering 23 (2014) 154–164

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

Metabolic engineering of ammonium release for nitrogen-fixing multispecies microbial cell-factories

Juan Cesar Federico Ortiz-Marquez [a,b], Mauro Do Nascimento [a,b], 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

ARTICLE INFO

Article history:
Received 15 November 2013
Received in revised form
26 February 2014
Accepted 19 March 2014
Available online 26 March 2014

Keywords:
Nitrogen fixation
Nitrogen assimilation
Ammonium excretion
Fertilizer
Microalgae biomass
Synthetic consortia

ABSTRACT

The biological nitrogen fixation carried out by some Bacteria and Archaea is one of the most attractive alternatives to synthetic nitrogen fertilizers. In this study we compared the effect of controlling the maximum activation state of the *Azotobacter vinelandii* glutamine synthase by a point mutation at the active site (D49S mutation) and impairing the ammonium-dependent homeostatic control of nitrogen-fixation genes expression by the *ΔnifL* mutation on ammonium release by the cells. Strains bearing the single D49S mutation were more efficient ammonium producers under carbon/energy limiting conditions and sustained microalgae growth at the expense of atmospheric N_2 in synthetic microalgae–bacteria consortia. Ammonium delivery by the different strains had implications for the microalga's cell-size distribution. It was uncovered an extensive cross regulation between nitrogen fixation and assimilation that extends current knowledge on this key metabolic pathway and might represent valuable hints for further improvements of versatile N_2-fixing microbial-cell factories.

1. Introduction

Concerns on energy depletion and environmental decay highlight a need for the development of alternative and sustainable sources of energy, food, feed and biomaterials. Since agriculture has a limited capacity to satisfy the current demand of all these services, over-exploitation according to available technology may incur severe environment deterioration (Sayer and Cassman, 2013). Microalgae-based bioprocesses represent a very promising set of alternatives to plant-based biofuels, feed and bulk chemicals (Wijffels et al., 2013). These bioprocesses might alleviate pressure on agriculture for food supply and extensive land-use change (Brennan and Owende, 2010). Currently, microalgal biomass is produced commercially mostly as high value products such as human food supplements, animal feed, cosmetics and pharmaceuticals (Gong et al., 2011). However, biofuels from microalgae are not commercial yet mostly because production of algae biomass is currently too expensive and too energy intensive for low commercial-value products (Chisti, 2013).

Similar to intensive agriculture, one of the drawbacks of implementing massive cultivation of microalgae is the unsustainable requirements of fertilizers, especially N. Microalgae have an average composition of $CH_{1.7}O_{0.4}N_{0.15}P_{0.0094}$, with N accounting for 4–8% on a dry biomass basis making the bioprocess considerably more N-intensive than traditional agriculture. For example, it has been estimated that for the production of 1 kg of triacylglycerol (the principal feedstock of biodiesel) from microalgae biomass it would be needed 0.36 kg of N (0.46 kg NH_4^+ or 1.6 kg NO_3^-) (Peccia et al., 2013). This situation may not only negatively impact on production costs, but also represents a significant share of total energy inputs, may promote competence with agriculture and also direct and/or indirect detrimental effects on the environment (Crutzen et al., 2007; Miller, 2010).

Thus several alternatives have been proposed such as the use of waste water as a source of inexpensive nutrients (Olguín, 2012), N-recycling from biomass after separation of the energy carrier (Peccia et al., 2013; Wernick and Liao, 2013) and the use of N from the air by biological N_2 fixation (BNF) (Ortiz-Marquez et al., 2013, 2012).

All known N_2-fixing organisms (diazotrophs) correspond to the bacterial or archaeal domains of life (Raymond et al., 2004). Nonetheless, some eukaryotes (including plants and algae) engage in N_2-fixing symbioses with bacteria that allow them to take N, although indirectly, from the air (Zehr, 2013).

BNF is catalyzed by nitrogenases in a high energy-demanding reaction requiring at least 16 ATP to fix 1 N_2. The molybdenum nitrogenase is an oxygen sensitive complex of dinitrogenase (NifDK heterotetramer) and dinitrogenase reductase (NifH homodimer).

* Correspondence to: Instituto de Investigaciones en Biodiversidad y Biotecnología (INBIOTEC), Vieytes 3103, Mar del Plata 7600, Argentina. Fax: +54 223 475 7120.
E-mail addresses: lcuratti@fiba.org.ar, leocuratti@gmail.com (L. Curatti).

http://dx.doi.org/10.1016/j.ymben.2014.03.002
1096-7176/

The dinitrogenase contains at its active site for N_2 reduction, the iron and molybdenum cofactor (FeMo-co) which biosynthesis comprises dozens of gene products, among which NifB catalyzes the first committed step in the pathway (Rubio and Ludden, 2008). 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 works as a regulatory switch that 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). NifL senses autonomously the oxygen status by its FAD-containing PAS domain and the N status by interacting and receiving signals from GlnK (Dixon and Kahn, 2004). In *A. vinelandii* ammonium is incorporated into amino acids by a "low ammonia pathway" catalized by 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 protein 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).

Symbiotic bacteria normally execute a sophisticated interplay of signals with their partners to assure the specificity of the interaction before starting to cross-feed N-fixation products to their hosts (Charpentier and Oldroyd, 2010). On the other hand, free-living diazotrophs fix sufficient N_2 for their own needs and typically do not excrete significant amounts of N_2-fixation products into their environment (Colnaghi et al., 1997). However, two different kinds of mutations have been associated with an enhanced capacity of ammonium excretion in diverse diazotrophic bacteria: disruption of the NifA/NifL-dependent ammonium control of *nif* genes expression or partial inhibition of GS or GOGAT for deficient ammonium assimilation (Colnaghi et al., 1997). The first aspect has been addressed quite successfully in *A. vinelandii* by either overexpression of NifA or deletion of NifL (Bali et al., 1992; Brewin et al., 1999; Colnaghi et al., 1997; Ortiz-Marquez et al., 2012).

It was recently shown that conversely to the *A. vinelandii* wild type strain a *ΔnifL* mutant engaged in an artificial C for N mutualistic relationship with eukaryotic oleaginous microalgae. This genetically-engineered multispecies platform produced oil up to 30% on a dry biomass basis at the expense of C and N from the air (Ortiz-Marquez et al., 2012).

On the other hand, the ammonium assimilation by the GS–GOGAT pathway has remained difficult to modify in *A. vinelandii* probably because this bacterium relies exclusively on this pathway for N-assimilation and/or its inability to transport glutamine has precluded the isolation of conditional mutant strains as in other bacteria (Colnaghi et al., 2001, 1997).

Thus understanding the regulation of N_2 fixation and assimilation and especially ammonium release by aerotolerant non-symbiotic bacteria is of prime interest for the rational design of versatile biofertilizers for sustainable agriculture and/or 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).

In this work we show the ammonium excretion properties of *A. vinelandii* mutant strains impaired in the regulation of N_2 fixation and/or ammonium assimilation. Glutamine synthetase-deficient strains presented a moderate diazotrophic growth defect, excreted significant amounts of ammonium into the medium and show a higher ratio of excreted ammonium to sugar spent than mutant strains impaired in the regulation of N_2 fixation. Accordingly, these strains improved the growth of microalgae at the expense of atmospheric N_2 in a synthetic microbial consortium.

2. Materials and methods

2.1. Bacterial strains and culture conditions

A. vinelandii strain DJ was the *wt* strain used in this study. DJ and DJ33 (*ΔnifDK*) were kindly provided by Dennis Dean (Virginia Tech). *A. vinelandii* strains AV2 (*ΔnifA::sp*), and AV3 (*ΔnifL*) isolation has been described previously (Ortiz-Marquez et al., 2012). *A. vinelandii* strains AV4 (DJ, *glnA* D49), AV5 (*ΔnifL*, *glnA* D49), AV6 (DJ, *glnA* D49S) and AV7 (*ΔnifL*, *glnA* D49S) were isolated in this work (Table 1). All *A. vinelandii* strains were maintained in Burk's modified medium (Strandberg and Wilson, 1968), incubated at 29 ± 1 °C with shaking at 200 rpm. When required the media were supplemented with 20 μg ml^{-1} spectinomycin or 50 μg ml^{-1} ampicillin. Inocula were routinely prepared from cells cultivated in the presence of 29 mM NH_4Cl or ammonium acetate for solid or liquid medium, respectively and represented the ammonium-replete condition. For derepression of BNF and/or diazotrophic growth analysis cells were collected by centrifugation for 3 min at 1136*g* and then transferred to ammonium-free medium.

For ammonium or glucose consumption experiments, cells were transferred to fresh Burk's modified medium containing 5.6 mM ammonium acetate or 55.5 mM glucose substituting for sucrose, respectively.

Chlorella sorokiniana strain RP maintenance and assays were conducted in nitrogen-free BG11 medium (Rippka et al., 1979), supplemented with 2 mM ammonium or inoculated with *A. vinelandii* strains at initial cell-ratio of 1:1 in 500 ml sterile-air bubbled-bottles at 30 °C and 50 μmol photons m^{-2} s^{-1} continuous white light, as reported before (Ortiz-Marquez et al., 2012) with modifications. For ammonium-limiting conditions *C. sorokiniana* cells were cultivated for 24 h at the expense of 0.5 mM ammonium until the nitrogen source was completely exhausted from the medium and then supplemented with ammonium or inoculated with *A. vinelandii* strains. For extreme ammonium-limiting conditions ammonium-cultivated cells were collected, rinsed with ammonium-free medium and incubated under nitrogen-deprivation condition for 24 h until ammonium addition or inoculation with bacterial cells.

Escherichia coli strain DH5α was used for molecular cloning purposes and was cultured in Luria-Bertani medium, supplemented with appropriate antibiotics at 37 °C with shaking at 150 rpm.

2.2. Isolation of A. vinelandii site-directed mutants of glnA

For site-directed mutagenesis of *A. vinelandii* GS encoded by the *glnA* gene, sequence alignments of GSs were carried out to identify a candidate amino acid in *A. vinelandii* GS that might display the critical function of D50 or D51 in ammonium binding and deprotonation as in *E. coli* (Liaw et al., 1995; Liaw and Eisenberg, 1994) or *Anabaena azollae* (Crespo et al., 1999) GSs, respectively. Thus the candidate D49 was identified for *A. vinelandii* GS. To construct a D49S mutant a 831-bp DNA fragment was amplified by PCR, corresponding to the promoter region of *glnA* and the region comprising the codon for amino acid D49, using the primers *glnA*-F (5′-CAG GCA AAA GAG GGG GCG GGA TTA TAG C-3′) and *glnA*-R (5′-TGGCGGTGCGGATTTCGTGGTCGTGGT-3′) and ligated into the pGEM-T Easy vector (Promega). After sequence confirmation, the D49S mutation was introduced by PCR amplification with the mutagenic primers D49S-F (5′-CGG CAA GAT GTT CTC CGG CTC