SYMBIOGENETICS

Supply of acetyl-CoA to N_2-fixing bacteroids: insights from the mutational and proteomic analyses of *Sinorhizobium meliloti*

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Abstract

Rhizobia represent a diverse group of gram-negative bacteria capable of fixing atmospheric nitrogen in symbiosis with leguminous plants. Mechanisms of symbiotic efficiency are important to study not only to reveal the "fine tuning" of the host–symbiont supra-organismal genetic system emergence, but also to develop agriculture with minimal environmental risks. In this paper we demonstrate that among seven genes whose inactivation by Tn5 insertions results in an increased efficiency of rhizobia (*Sinorhizobium meliloti*) symbiosis with alfalfa (Eff^{++} phenotype), six genes are involved in the metabolism of small molecules. One of them (SMc04399) encodes for acetate-CoA transferase catalyzing the formation of acetyl-CoA from acyl-CoA. Since acetyl-CoA is required for operation of the Krebs cycle, providing ATP for symbiotic N_2 fixation, we suggest that a significant portion of this coenzyme utilized by bacteroids is provided by the plant cell supporting the energy-consuming nitrogenase reaction. Proteomic data analysis allow us to reveal the lability of enzymatic pathways which are involved in bacteroids in the production and catabolism of acetyl-CoA and which should be modified to obtain the Eff^{++} phenotype. This phenotype was developed also after inactivation of NoeB protein which is involved in the host-specific nodulation and is characterized by an elevated production in wild type *S. meliloti* bacteroids, suggesting a multifunctional role of *noe*B in the symbiosis operation.

Keywords: *Sinorhizobium meliloti*, symbiotic efficiency, metabolism of small molecules, acetyl CoA, acetate-CoA transferase, transposon (Tn5) mutagenesis, proteomic analysis

Introduction

Fixation of atmospheric N_2 by nodule bacteria (rhizobia) in symbiosis with leguminous plants is associated with differentiation of intracellular bacteroids, which differ from non-differentiated cells in the activity of many metabolic genes (Green et al., 2019). Specifically, bacteroids exhibit high activity of genes encoding for nitrogenase as well as for the Krebs cycle enzymes, which provide energy for N_2 fixation due to catabolism of acetyl-CoA. However, glycolysis, representing the major source of pyruvate from which acetyl-CoA is derived, is suppressed in bacteroids (Dunn, 1988). Although activities of pyruvate dehydrogenase and pyruvate decarboxylase were detected in bacteroids (Cabanes, Boistard and Batut, 2000), rhizobia mutants with impaired activities of these enzymes retain the ability to fix N_2 (Dunn, 1988), indicating the necessity to clarify the sources of acetyl-CoA used by bacteroids.

Leguminous plants are known to supply bacteroids with dicarboxylic acids, mostly malate, which is catabolized via the Krebs cycle, but can be also involved in pyruvate formation via malic enzymes (Liu, Contador, Fan and Lam, 2018). It

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was demonstrated that in pea and vetch rhizobia (*Rhizobium leguminosarum* bv. *viciae*) the simultaneous inactivation of two malic enzymes, as well as of phosphoenolpyruvate carboxykinase and pyruvate kinase, leads to the loss of nitrogenase activity. However, in alfalfa rhizobia, *Sinorhizobium meliloti*, deletion of the phosphotransacetylase domain from the malic enzyme leads to a significant (40–50 %) increase of nitrogenase activity and of the root nodule mass. Moreover, activation of phosphoenolpyruvate carboxykinase in bacteroids leads to a 70 % reduction of N_2-fixing activity compared to wild-type bacteroids (Mulley et al., 2010), suggesting that operation of this enzyme is disadvantageous for the symbiosis operation.

Earlier, we revealed in alfalfa rhizobia (*Sinorhizobium meliloti*) a number of *eff* genes whose inactivation by Tn5 insertions leads to an increased symbiotic efficiency (SE) — the impact of bacteria inoculation on host plant productivity (Sharypova et al., 1994; Onishchuk, Sharypova and Simarov, 1994). Among these genes, addressed as the negative regulators of symbiosis (Provorov et al., 2014), several groups were identified as being involved in: (i) accumulation of storage nutrients (glycogen, GABA), which reduces the catabolic activity of bacteroids; (ii) negative control of respiration, which restricts the energy flow to nitrogenase; (iii) synthesis of surface polysaccharides, which elicit host defense reactions, thereby reducing the persistence of the endo-symbiotic bacterial population.

Our paper demonstrates that among seven newly obtained *S. meliloti* Tn5 mutants selected for an increased SE (Eff^{++} phenotype), six mutants are disturbed in the metabolism of small molecules (Werner, Semsey, Sneppen and Krishna, 2009). In one of these mutants, the inactivated gene encodes for acetate-CoA transferase, responsible for production of acetyl-CoA from acyl-CoA. The proteomic analysis enabled us to identify a range of enzymes for pyruvate formation and catabolism which are induced in *S. meliloti* bacteroids with respect to free-living cells (Dme, PpdK, Tme, PdhA). The presented data suggest the lability of pyruvate metabolism in bacteroids, which is correlated to the availability of acetyl-CoA. Specifically, limitations in cellular pyruvate production by bacteroids may activate the acetyl-CoA import from the host cell, which is required to develop the Eff^{++} phenotype in agronomically promising rhizobia strains.

Materials and methods

Bacterial strains and plasmids. The objects of our study include the laboratory strain CXM1-188 of alfalfa rhizobia, *Sinorhizobium meliloti*, UV-induced Sm^r mutant of symbiotically effective (Eff$^+$) strain 425a (Fedorov and Simarov, 1987) and its Tn5 mutants selected for an increased SE (Eff^{++}). For non-specific Tn5 mutagenesis, two suicide vectors were used: pSUP5011 for T707 and pSUP2021 for other mutants (Simon, Priefer and Pühler, 1983). These vectors were introduced into CXM1-188 from *Escherichia coli* strain S17-1 by conjugation (Chizhevskaya, Onishchuk and Simarov, 2011).

Cultivation conditions. *S. meliloti* strains were cultured at 28 °C in TY medium (Beringer, 1974). Antibiotics (mg/L) were added: Sm — 800, Km — 200. Mutants were stored at +4 °C on legume agar medium (Novikova and Simarov, 1979).

Strain growth analysis on diagnostic media. The salt tolerance of rhizobia was studied on TY or 79 media with NaCl (0.5–0.7 M). Acid resistance was determined on TY with pH = 5–9.

SE assay. Plants of alfalfa (*Medicago varia* variety Vega and *M. sativa* variety Agnia) were grown in sterile 60 ml glass tubes with Krasilnikov-Korenyako 0.6 % agar medium in a greenhouse at 20–22 °C, with illumination 20.000 lux, on a 16-hour photoperiod for 30 days; experiments were carried out in 2- or 6-fold replications (Fedorov and Simarov, 1987). SE was evaluated by the dry mass of the over-ground parts of inoculated seedlings compared to the control without inoculation (10^5 — 10^6 *S. meliloti* cells were added to each tube).

Identification of Tn5-tagged genes was carried out using the inverted polymerase chain reaction technique (Onishchuk et al., 2014).

Proteomic data analysis workflow has been described previously. For this work, we used the proteomic dataset of free-living cells and bacteroids described earlier (Antonets et al., 2018). The protein scores over 50 have been considered as significant.

Statistical data processing was implemented using analysis of variance and the Student's T-test (Lakin, 1990).

Results and discussion

Selection of mutants with an increased SE (Eff^{++} phenotype). We analyzed 650 Tn5-induced random Km^r transposants of *S. meliloti* strain CXM1-188 for SE with alfalfa variety Vega in 2-fold replications. We screened 100 mutants with the preliminary identified Eff^{++} phenotype exceeding the parental strain by more than 20 % for the dry mass of inoculated plants. After analyzing these mutants in a series of experiments with alfalfa varieties Vega and Agnia in 6-fold replications, seven mutants with the confirmed Eff^{++} phenotype were selected, which significantly exceeded CXM1-188 for SE in at least one experiment (Table 1). For five mutants, the increase in dry mass of inoculated plants was significant ($P_0 < 0.05$) on average for the entire set of experiments. Each mutant tested in 4–9 independent experiments exceeded the parental strain for SE by 16.2–21.8 %. The

Fig. 1. Alfalfa (*Medicago sativa*, variety Vega) plants inoculated with *Sinorhizobium meliloti* in the tube test. 1–3 — T802; 4–5 — T91; 6–7 — CXM1-188.

Table 1. Symbiotic efficiency (SE) in Tn5 mutants of *Sinorhizobium meliloti* strain CXM1-188

Mutants	Number of tube tests		SE increase (%) over parental strain	
	Total	Showed a significant increase of SE over parental strain CMX1-188	Mean with a standard error	T_{st} (P_0)
T802	5	1	21.8 ± 7.8	2.79*
T900	7	1	19.8 ± 5.8	3.41*
T813	5	1	19.6 ± 8.1	3.11
T749	4	1	18.4 ± 6.2	2.97
T91	5	1	17.6 ± 5.1	3.45*
T707	5	1	17.6 ± 4.0	4.0*
T796	9	2	16.2 ± 6.5	2.49*

T_{st} — values of Student T-criterion; *$P_0 < 0.05$.

preliminary screening of the Eff^{++} mutants may be done after a visual inspection of inoculated alfalfa plants (Figure 1).

Phenotypic analysis of mutants on diagnostic media. The resistances of mutants to stress factors — increased acidity and salinity — were tested using the diagnostic media. On the salinized 79 media, three mutants (T749, T707, T55) as well as the parental strain CXM1-188 grew with 0.7 M NaCl; weak growth was detected in T813, T91, T900, T802; no growth—in T796. There were no differences between the tested strains in pH sensitivity: all mutants did not grow at pH 5 and grew at pH 6–9, like the parental strain CXM1-188.

Identification of transposon tagged genes in Eff^{++} mutants. Using the technique of inverted polymerase chain reaction, DNA fragments adjacent to Tn5 insertions were identified in the mutants. It was demonstrated that in four mutants, Tn5 insertions occurred in the chromosome (Table 2). In T802, a transposon was inserted into gene SMc04399, encoding for the short-chain acetate-CoA transferase, which catalyzes the transfer of acetyl group to acyl-CoA, producing a fatty acid and acetyl-CoA. In T749, the gene inactivated by Tn5 insertion is homologous to *hip*O1 (SMc00682) of strain 1021 and encodes for hippurate hydrolase enzyme, involved in the metal-dependent cleavage of the peptide bonds. In T707, the inactivated gene is homologous to SMc04292 of strain 1021, which encodes for a protein containing the adenylate/guanylate cyclase domain (tetratricopeptide repeat). In mutant T900, Tn5 insertion was identified in the Smc01755 gene, encoding for an MFS-type protein transporter.

Table 2. Identification of *Sinorhizobium meliloti* genes labelled by Tn5 insertions

Tn5-mutants	Gene homologs in strain 1021*	Putative gene products	Functions
T749	*hip*O1; SMc00682	Hippurate hydrolase	Metabolism of small molecules
T802	SMc04399	Short chain acyl-CoA transferase (Acyl-CoA acetate/3-ketoacid Coa transferase)	Metabolism of small molecules
T707	*cya*F3; SMc04292 (Chr.KH35)	Adenylate/ guanylate cyclase domain-containing tetratricopeptide	Metabolism of small molecules
T900	SMc01755	MFS type transporter	Metabolism of small molecules
T796	SMb20767	Dihydroxyacetone (glycerone) kinase	Metabolism of small molecules
T813	Identified on SMa of RU11/001, SM11 and Rm41 strains	NAD(P)-dependent oxidoreductase dTDP-4-dehydrorhamnose reductase	Metabolism of small molecules
T91	SMa0774-SMa0775	NoeB synthesized in the *Medicago trucatula* infected root hairs	Host-specific nodulation

*Gene localization: SMc — chromosome, SMa — megaplasmid 1 (Sym plasmid), SMb — megaplasmid 2.

The insertion of Tn5 into Sym plasmid (megaplasmid 1) was detected in two mutants. For T813, the tagged gene does not have a homologue in strain 1021, but is found in some other *S. meliloti* strains encoding for dTDP-4-dehydro-ramnose reductase, involved in biosynthesis of nucleotide-activated L-ramnose — hexose, which is often found in the rhizobial extracellular polysaccharides. In T91 mutant, Tn5 insertion was detected between genes SMa0774 and SMa0775, having homologs of strain 1021 encoding for a NoeB hypothetical protein involved in host-specific nodulation.

On megaplasmid 2, a transposon insertion was revealed in T796 mutant for the dihydroxy-acetone kinase gene involved in the energy turnover.

Among the Tn5-tagged genes, the acetate-CoA transferase encoding gene SMc04399 is of special interest for addressing the metabolic integration of plants and bacteria since the disturbed enzyme catalyzes the formation of acetyl-CoA from acyl-CoA. In alfalfa rhizobia, CoA is involved in more than 60 reactions of the small molecules metabolism (Contador, Lo, Chan and Lam, 2020). We demonstrated that six out of seven analyzed genes are involved in this metabolism. It seems possible that wild-type bacteroids are capable of either producing acetyl-CoA by themselves or importing it from the host plant cell. Our data suggest that turning off some pathways of acetyl-CoA production leads to its increased import, which simplifies the metabolism of bacteroids, allowing them to allocate more energy to N_2-fixing machinery.

Previously we demonstrated (Onishchuk, Vorobyov, Provorov and Simarov, 2009) that a significant increase in nitrogenase activity and SE (Eff^{++} phenotype) can be gained in alfalfa rhizobia by amplification of *dct* genes encoding for the transport of malate, which is involved in the Krebs cycle and can also be used for pyruvate production in bacteroids (Dunn, 1988; Liu, Contador, Fan, and Lam, 2018). However, a deletion of some domains from malic enzyme, which catalyzes the production of pyruvate from malate, leads to increased N_2-fixing activity (Mulley et al., 2018), suggesting that this reaction interferes with the energy supply to nitrogenase. Our data demonstrate that reorganizations of metabolic pathways involved in formation of acetyl-CoA in bacteroids can be useful to construct the Eff^{++} rhizobia strains.

Proteomic analysis. In order to reveal the enzymes which may be considered as the targets for symbiotically beneficial modifications of the bacteroid metabolism, we used the previously obtained dataset (Antonets et al., 2018), in which proteomic profiles of free-living cells and bacteroids of *S. meliloti* were compared. We found that four enzymes for pyruvate formation and catabolism are present in bacteroids (Dme, PdhA, PpdK, Tme) and are absent or identified with much lower scores in the free-living cells (Table 3). These data suggest that the rhizobia cells can broadly modify the concentrations of pyruvate, which is a major precursor for acetyl-CoA production. Moreover, the AccA enzyme, responsible for carboxylation of acetyl-CoA, is active in bacteroids, not in free-living *S. meliloti* cells, suggesting an improved intensity of acetyl-CoA utilization under symbiotic conditions.

Table 3. Proteins involved in symbiotically essential biochemical reactions in the *Sinorhizobium meliloti* bacteroids

ID*	Gene	Product	Protein score**	
			Bacteroids	Free-living cells
O30807	*dme*	malic enzyme	449.32	N/I***
O30808	*tme*	malic enzyme	156.23	60.31
Q59754	*ppd*K	pyruvate phosphate dikinase	99.83	N/I
Q9R9N5	*pdh*Aa	pyruvate dehydrogenase α-subunit	572.24	101.58
Q9R9N4	*pdh*Ab	pyruvate dehydrogenase β-subunit	351.14	85.54
Q92ME2	*acc*A	acetyl-CoA carboxylase α-subunit	75.37	N/I
Q52893	*noe*B	host-specific nodulation protein	70.56	N/I

*Protein identifier in the UniProt database (http://www.uniprot.org/).

**The "Score" parameter for the Mascot database mass-spectrometric identification.

***N/I — protein has not been identified or protein score was less than 50.

Surprisingly, NoeB protein has been identified in *S. meliloti* bacteroids but not in the free-living culture (Table 3), while inactivation of *noe*B resulted in an increased SE (Table 1). This gene is located in the flavonoid inducible operon down stream of the *nod* box n5 promoter. The NoeB protein is predicted to possess sulfuric ester hydrolase activity and membrane localization according to UniProt database (https://www.uniprot.org/). Its specific function remains unknown, nevertheless, sulfation of the lipo- and glucosamine oligosaccharides is known to be important for host specificity during plant–microbial interactions (Lerouge et al., 1990; Roche et al., 1991). The loss-of-function mutants in *noe*B cause weak nodule-forming activity and a drastic decrease in the infection thread formation in *Medicago littoralis*, while in *M. truncatula* or *Melilotus alba* these phenotypes were not revealed (Ardourel et al., 1995). Participation of the sulfatase enzymes in symbiotic interactions (e.g., in sulfating the Nod factors typical for *S. meliloti*) should be expected. Interestingly, *noe*B expression is controlled by the *fix*LJ operon, which encodes for the kinase pair operating as a regulator of symbiosis-associated nitrogenese synthesis (Gao, Nguyen, González and Teplitski, 2016). Thus, our data obtained by transposon mutagenesis and

proteomic analysis suggest some unknown functions for *noe*B gene related to regulation of symbiotic N_2 fixation.

In summary, we suggest a previously unknown mechanism of plant–microbe interaction based on cooperative metabolism of acetyl-CoA, which plays a central role in the energy support of nitrogenase reaction. Mutational and proteomic analyses of symbiotically specialized bacteroids and free-living cells of alfalfa rhizobia (*Sinorhizobium meliloti*) allows us to reveal the cooperative enzymatic pathways involved in production and catabolism of acetyl-CoA, which may be modified to develop highly effective, agronomically perspective microbial strains for sustainable agriculture.

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