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Heterologous expression of the *Halothiobacillus neapolitanus* carboxysomal gene cluster in *Corynebacterium glutamicum*

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ABSTRACT

Compartmentalization represents a ubiquitous principle used by living organisms to optimize metabolic flux and to avoid detrimental interactions within the cytoplasm. Proteinaceous bacterial microcompartments (BMCs) have therefore created strong interest for the encapsulation of heterologous pathways in microbial model organisms. However, attempts were so far mostly restricted to *Escherichia coli*. Here, we introduced the carboxysomal gene cluster of *Halothiobacillus neapolitanus* into the biotechnological platform species *Corynebacterium gluta-micum*. Transmission electron microscopy, fluorescence microscopy and single molecule localization microscopy suggested the formation of BMC-like structures in cells expressing the complete carboxysome operon or only the shell proteins. Purified carboxysomes consisted of the expected protein components as verified by mass spectrometry. Enzymatic assays revealed the functional production of RuBisCO in *C. glutamicum* both in the presence and absence of carboxysomal shell proteins. Furthermore, we could show that eYFP is targeted to the carboxysomes by fusion to the large RuBisCO subunit. Overall, this study represents the first transfer of an α-carboxysomal gene cluster into a Gram-positive model species supporting the modularity and orthogonality of these microcompartments, but also identified important challenges which need to be addressed on the way towards biotechnological application.

1. Introduction

Spatial organization is used by all living organisms for the optimization of cellular transport and metabolism. The overall concept of encapsulation of metabolic (sub-) pathways has been found in a variety of bacterial species and is realized, for example, by the formation of bacterial microcompartments (BMCs) (Bobik et al., 2015; Kerfeld and Erbilgin, 2015). The spatial separation of distinct cellular processes confers a selective advantage to the particular host, e.g. by avoiding metabolic interference with other cytoplasmic reactions, encapsulation of toxic intermediates, and increasing pathway efficiency by scaffolding of participating enzymes or increasing local substrate concentration.

A typical BMC consists of metabolic enzymes that are encapsulated by proteins forming a shell with selective permeability, which is controlled by specific protein pores within the shell structure. Different studies revealed the presence of more than seven different BMC types in 23 bacterial phyla (Axen et al., 2014; Kerfeld and Erbilgin, 2015). The genomic organization of BMC gene clusters suggests that these genetic modules are frequent targets of horizontal gene transfer (Gupta, 2012). The most extensively studied classes of BMCs are the carboxysomes (Kerfeld and Melnicki, 2016; Rae et al., 2013) as well as the metabolosomes involved in the catabolism of 1,2-propandiol (Pdu), and ethanolamine (Eut) (Bobik et al., 2015; Chowdhury et al., 2014; Frank et al., 2013; Kerfeld and Erbilgin, 2015).

CO_2-fixing carboxysomes are found in almost all cyanobacteria, where they enhance autotrophic growth *via* the Calvin cycle (Rae et al., 2013). In general, both types of carboxysomes (α and β), which show significant differences in components and assembly, consist of a protein shell of polyhedral shape encapsulating the RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) enzyme and the carbonic anhydrase (Kerfeld and Melnicki, 2016; Rae et al., 2013). Whereas one RuBisCO substrate, ribulose bisphosphate, is freely crossing the carboxysomal shell, CO_2 is produced from bicarbonate in the BMC lumen by carbonic anhydrase (Badger and Price, 2003). Thus, the protein shell of the carboxysome plays an important role in enhancing RuBisCO activity by increasing the local CO_2 concentration and by acting as a diffusion barrier for O_2, the substrate of photorespiration.

BMCs are nowadays highly interesting for synthetic biology and bioengineering approaches. Detailed insights into the molecular factors contributing to the functional assembly and organization are the key for

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an understanding of BMC function and transfer into a heterologous background. The formation of α- and β-carboxysomes follows different modes: whereas α-carboxysomes assemble concomitantly with the aggregation of RuBisCO (Iancu et al., 2010), the assembly of β-carboxysomes is initiated by the aggregation of RuBisCO and the CcmM-CcmN complex at the cell poles (Cameron et al., 2013; Kinney et al., 2012). Within the BMC core, RuBisCO is present in a paracrystalline state, which likely contributes to an optimal substrate channeling (Kaneko et al., 2006). The formation of empty compartment shells has been reported for carboxysomes in a *H. neapolitanus* strain lacking the RuBisCO enzyme (Baker et al., 1998; Menon et al., 2008).

Recent studies explored the use of modular BMCs as synthetic nanobioreactors enhancing small molecule production of microbial strains. As an important groundwork, the α-carboxysome of *H. neapolitanus* was expressed in *Escherichia coli*: correct assembly of the carboxysome and the presence of RuBisCO activity was demonstrated (Bonacci et al., 2012). Further studies reported on the heterologous production of Pdu and Eut metabolosomes in *E. coli* (Choudhary et al., 2012; Parsons et al., 2008). These studies emphasized BMCs, in general, as orthogonal modules for synthetic biology approaches. However, in all cases, BMC gene clusters where transferred from a γ-proteobacterial donor strain to *E. coli*, which is likewise a member of the Gammaproteobacteria. Only the study of Lin et al. provided a first proof-of-concept of the expression and assembly of β-carboxysomes in chloroplasts of the plant *Nicotiana benthamiana* (Lin et al., 2014).

In this study, we transferred the α-carboxysomal gene cluster of *H. neapolitanus* to the Gram-positive soil bacterium *Corynebacterium glutamicum*. This member of the actinobacteria is used for the large-scale industrial production of amino acids and proteins (Eggeling and Bott, 2005; Wendisch, 2014). Metabolic engineering revealed its strong potential for the production of a variety of further value-added compounds including organic acids, polymer building blocks or plant natural products (Eggeling and Bott, 2015; Heider and Wendisch, 2015; Kallscheuer et al., 2016; Wendisch, 2014). Expression and presumably correct assembly of carboxysomes in *C. glutamicum* was demonstrated by purification of the microcompartments and identification of carboxysomal proteins by mass spectrometry. Different microscopy techniques and the measurement of RuBisCO activity provided further hints for the functionality of these compartments. This study provides important groundwork for the use of modular BMCs in bioengineering approaches for the improvement of small molecule production.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

All bacterial strains used in this study are listed in Table 1. *E. coli* DH5α and Stellar™ were used for cloning procedures and cultivated at 37 °C in lysogeny broth (LB, (Sambrook and Russell, 2001)). *E. coli* DH5α was also used as a reference strain for carboxysome production. *C. glutamicum* ATCC 13032 was used as wild type and all derivatives thereof were constructed as indicated in Table 1. *C. glutamicum* was cultivated either in brain heart infusion broth (BHI, Difco Laboratories, Detroit, MI, USA) or in CGXII minimal medium (Keilhauer et al., 1993) supplemented with 3,4-dihydroxybenzoate (30 mg/l) as iron chelator and 2% (w/v) glucose as carbon source. As appropriate, kanamycin (50 µg/ml for *E. coli* or 25 µg/ml for *C. glutamicum)* and/or chloramphenicol (34 µg/ml for *E. coli* or 10 µg/ml for *C. glutamicum*) were added to the medium. Chromosomal DNA of *H. neapolitanus* was obtained from the DSMZ and used as PCR template. For the growth experiment a colony from a fresh agar plate was used to inoculate the preculture of 20 ml BHI in baffled shake flasks. The preculture was incubated at 30 °C and 120 rpm overnight. On the following day the cells were washed twice with phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4, pH 7.3) and used to inoculate the 750 µl main cultures to a starting OD_{600} of 1. The main cultivation was performed in 48-well flower plates (m2p labs, Baesweiler, Germany) in a BioLector microbioreactor system (m2p labs) at 30 °C and 1200 rpm.

2.2. Recombinant DNA work and construction of chromosomal insertion strains

Routine methods such as PCR, DNA restriction and ligation were performed using standard protocols (Hanahan, 1983; Sambrook and Russell, 2001; van der Rest et al., 1999). The oligonucleotides used in this study were obtained from Eurofins Genomics (Ebersberg, Germany) and are listed in Table S1. The QIAquick Gel Extraction Kit, or the MinElute Gel Extraction Kit (both Qiagen, Hilden, Germany) were used for gel purification of digested plasmids and PCR fragments, respectively. The In-Fusion ® HD Cloning Kit was obtained from Clontech Laboratories (Mountain View, Ca, USA) and used according to the manufacturer's instructions. All plasmid sequences were confirmed by sequencing (Eurofins Genomics, Ebersberg, Germany). Details about plasmid constructions are provided in the supplemental material (Text S1). Integration of carboxysomal gene clusters into the chromosome of *C. glutamicum* was performed by double-crossover as described previously (Baumgart et al., 2013).

2.3. Cell cultivation for carboxysome purification

For carboxysome purification 50 ml BHI with kanamycin in a baffled shake flask were inoculated with a single colony from a fresh transformation of ATCC 13032 carrying pAN6-HNC and cultivated at 30 °C and 120 rpm overnight. For the main culture, 500 ml BHI with kanamycin in a baffled shake flask were inoculated with 20 ml of the preculture and cultivated at 30 °C and 100 rpm. After 1.5 h the production of the carboxysomes was induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and the cells were further cultivated at 20 °C until they were harvested on the following morning. The co-purification experiment was performed with the same cultivation procedure but chloramphenicol was added additionally to the medium as the strains were carrying two plasmids. For production of carboxysomes in *E. coli* 50 ml LB with kanamycin in a baffled shake flask were inoculated with a single colony of a fresh transformation of DH5α carrying pAN6-HNC and cultivated at 37 °C and 120 rpm overnight. The main culture (500 ml LB with kanamycin in a baffled shake flask) was inoculated with 20 ml of the preculture and cultivated at 37 °C and 100 rpm. At an OD_{600} of 0.5 the production of the carboxysomes was induced with 50 µM IPTG. The cells were further cultivated at 20 °C until they were harvested on the following morning.

2.4. Purification of carboxysomes by sucrose gradient centrifugation

In principle, the carboxysome purification was performed as described previously (So et al., 2004), except for some adaptations to *C. glutamicum*. The cells of 1 l of *C. glutamicum* cell culture expressing carboxysomes were harvested and washed once with PBS. The pellet was suspended in 25 ml TEMB (5 mM Tris-HCl pH 8.0, 1 mM EDTA, 10 mM $MgCl_2$, 20 mM $NaHCO_3$). Phenylmethylsulfonyl fluoride (PMSF, 0.5 mM final concentration) and Lysozyme (1 mg/ml final concentration) were added and the cell suspension was incubated slightly tilting for 1 h at room temperature. The cells were harvested and the supernatant was discarded. The pellet was suspended again in 25 ml TEMB containing 0.5 mM PMSF, 5 mM $CaCl_2$ and 50 µg/ml DNAseI. The cells were disrupted by passing five times through a French pressure cell at 15.000 psi. Subsequently, the suspension was further treated 1 × 1 min and 4 × 30 s with two minutes of cooling in between with a Branson Sonifier 250 (Duty Cycle 40%, Output Control 1). Cell debris and intact cells were separated by centrifugation at 4300*g* for 15 min at 4 °C. The solution was further clarified by centrifugation in a JA-25.50 rotor at 12,096*g* and 4 °C for 20 min. The supernatant was subsequently

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