ORIGINAL ARTICLE

A function-based screen for seeking RubisCO active clones from metagenomes: novel enzymes influencing RubisCO activity

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Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) is a key enzyme of the Calvin cycle, which is responsible for most of Earth's primary production. Although research on RubisCO genes and enzymes in plants, cyanobacteria and bacteria has been ongoing for years, still little is understood about its regulation and activation in bacteria. Even more so, hardly any information exists about the function of metagenomic RubisCOs and the role of the enzymes encoded on the flanking DNA owing to the lack of available function-based screens for seeking active RubisCOs from the environment. Here we present the first solely activity-based approach for identifying RubisCO active fosmid clones from a metagenomic library. We constructed a metagenomic library from hydrothermal vent fluids and screened 1056 fosmid clones. Twelve clones exhibited RubisCO activity and the metagenomic fragments resembled genes from *Thiomicrospira crunogena*. One of these clones was further analyzed. It contained a 35.2 kb metagenomic insert carrying the RubisCO gene cluster and flanking DNA regions. Knockouts of twelve genes and two intergenic regions on this metagenomic fragment demonstrated that the RubisCO activity was significantly impaired and was attributed to deletions in genes encoding putative transcriptional regulators and those believed to be vital for RubisCO activation. Our new technique revealed a novel link between a poorly characterized gene and RubisCO activity. This screen opens the door to directly investigating RubisCO genes and respective enzymes from environmental samples.

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Introduction

Among the autotrophic CO₂ fixation pathways, the Calvin Benson (CB) cycle accounts for most of Earth's net primary production (> 99.5% of 105 × 10⁹ tons per year) (Field *et al.*, 1998; Raven, 2013). One of its key enzymes is the ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO, EC 4.1.1.39), which can catalyze the carboxylation of ribulose-1,5-bisphosphate to 3-phosphoglycerate. Four types of structural RubisCOs are currently distinguished, of which two types, form I (CbbLS) and form II (CbbM), are known to operate in the classical autotrophic CB cycle (Tabita *et al.*, 2007). RubisCO is present in all plants, cyanobacteria and many autotrophic bacteria (Berg, 2011) and is believed to be the most abundant protein on Earth (Ellis, 1979; Raven, 2009). Hence, the CB cycle and the RubisCO enzymes are of fundamental importance for global primary production and it is vital to better understand RubisCO evolution and its diversity.

Despite multiple years of research on RubisCO genes and enzymes (Li *et al.*, 1993; Portis, 2003; Mueller-Cajar *et al.*, 2011), still comparatively little knowledge exists about their regulatory mechanisms, assembly or activation. The role that CbbQ (AAA + ATPase domain) and CbbO (von Willebrand factor, type A) play in prokaryotic RubisCO activation is still enigmatic. In some cases, they are likely involved in post-translational RubisCO activation (Hayashi *et al.*, 1997, 1999), whereas in other cases they do not appear to influence RubisCO activity at all (Schwedock *et al.*, 2004). Also, the information on the mechanisms that regulate RubisCO expression is scarce: transcriptional regulators of the LysR family have been found in many genomes adjacent to the structural RubisCO genes (Kusian and Bowien, 1997; Scott *et al.*, 2006). In the *Hydrogenovibrio marinus* genome two of these regulatory proteins, namely CbbR1 and CbbRm, are encoded upstream of the RubisCO structural genes *cbbLS-1* and *cbbM*, respectively, and have been demonstrated to regulate adjacent RubisCO gene expression (Toyoda *et al.*, 2005).

Most of our understanding of RubisCO functioning is based on the studies conducted with cultured bacteria (Joshi *et al.*, 2009 and references therein;

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Tabita *et al.*, 2008 and references therein), but hardly any information exists about the function of environmental RubisCOs and the role of the enzymes encoded on the flanking DNA regions. Approaches that are currently used for gathering information on environmental RubisCO gene clusters rely on sequence searches (for example, Xie *et al.*, 2011). In some cases this has been advanced to subsequently determine the recombinant RubisCO activity in a surrogate host after having identified the RubisCO gene through sequence similarity first (Witte *et al.*, 2010). However, inherent limitations of sequence-based screening exist because only sequences with significant similarities to known genes can be detected. To date, there is no method available that allows seeking RubisCOs by a function-based approach alone on a large scale directly from environmental DNA (metagenomic libraries). Therefore, the large majority of RubisCO gene clusters from uncultured organisms (>99%) remains inaccessible and the questions whether the environmental RubisCOs are functional and how they are regulated and activated remain largely unanswered. We here describe a novel function-based approach whereby RubisCO active enzymes can be sought from the large majority of uncultured organisms inhabiting the environment. We constructed a metagenomic fosmid library from deep-sea hydrothermal fluids and screened the clones for recombinant RubisCO activity. The metagenomic DNA of fosmid clones exhibiting RubisCO activity was then further analyzed to elucidate the role of flanking genes and resulting gene products fundamental for expressing a fully functional RubisCO enzyme.

Materials and methods

Growth of TH-55 and cloning of RubisCO genes

Thiomicrospira crunogena TH-55 (DSMZ No. 12353) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and was cultivated as described (Jannasch *et al.*, 1985; Dobrinski *et al.*, 2005). Cells were harvested after 24 h, DNA was extracted and RubisCO form I (*cbbLS*) and form II (*cbbM*) encoding gene regions were amplified (incorporating at least 500 bp upstream to include possible promoter regions) and cloned into the fosmid vector pCC1FOS according to standard procedures (see Supplementary Materials and Methods).

Sampling of hydrothermal fluids

The hydrothermal fluids were collected from the Nibelungen field at 8°18'S at a water depth of 2915 m along the Mid Atlantic Ridge (Melchert *et al.*, 2008). The investigated low-temperature hydrothermal fluids originated from the interface

between hot fluids emanating from a crater and the ambient seawater. During sampling the online-monitored fluid temperature was between 90 and 120 °C (Perner *et al.*, 2013). The fluids had a pH of 5.6, 22 µM hydrogen, 8 µM sulfide and 56 µM oxygen (Perner *et al.*, 2013). Sampling was done by a remote-operated vehicle (ROV 6000, GEOMAR, Kiel) during the MAR-SUED V cruise (March/April 2009) with the RV Meteor. Around 200 ml of hydrothermal fluids were concentrated onboard on a 0.2 µm polycarbonate filter and kept at –20 °C. Detailed sampling procedures and information on the sampling site are described elsewhere (Perner *et al.*, 2007, 2009, 2013).

Construction of fosmid libraries

Two fosmid libraries were constructed: (i) one genome library with DNA material from TH-55 and (ii) one metagenomic library with the hydrothermal fluids from Nibelungen. Treatment of TH-55 and environmental DNA prior to metagenomic fosmid library construction is reported in Supplementary Materials and Methods. The (meta)-genome libraries were constructed using the pCC1FOS fosmid vector of the CopyControl Fosmid Library Production Kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions. Fosmid clones were selected on Luria-Broth (LB) agar plates by blue–white screening and chloramphenicol (12.5 µg ml^{–1}). The quality of fosmid inserts was randomly checked and DNA inserts were sequenced (Supplementary Materials and Methods).

Transposon mutagenesis

Two fosmid transposon insertion libraries were constructed with RubisCO active metagenomic fosmid clones using the EZ-Tn5™ <KAN-2> insertion kit (Epicentre): (i) one with the fosmid clone 71C2 (35.2 kb) carrying the RubisCO gene cluster (*cbbQOM lysR2 lysR1 cbbLSQO*) and flanking DNA regions and (ii) one with the fosmid subclone 71C2II, comprising only the RubisCO gene cluster (*cbbQOM lysR2 lysR1 cbbLSQO*) (13 kb). The 71C2 and 71C2II transposon libraries consisted of 360 and 24 clones, respectively, which were sequenced with the manufacturer's designed KAN-2 FP-1 forward primer targeting the kanamycin cassette (5'-ACCTA CAACAAAGCTCTCATCAACC-3') to identify the insertion position. Transposon clones were selected by chloramphenicol (12.5 µg ml^{–1}) and kanamycin (50 µg ml^{–1}). Selected clones were tested for RubisCO activity by following the high-pressure liquid chromatography (HPLC) based activity assay described below.

Preparation of crude extracts

Pure cultures of TH-55 were harvested at the end of the exponential growth phase by centrifugation

(17 600 g, 20 min, 4 °C). *Escherichia coli* clones with pCC1FOS::cbbLS and pCC1FOS::cbbM as well as single metagenomic fosmid and transposon clones were grown at 28 °C on 200 ml preheated LB medium with chloramphenicol (12.5 µg ml⁻¹) (and for transposon clones additionally with kanamycin (50 µg ml⁻¹)) and autoinduction solution (1 × final concentration (Epicentre)) in 1 l flasks at 130 r.p.m. All these *E. coli* cells were harvested after 18 h by centrifugation (9800 g, 10 min, 4 °C). After centrifugation, cell pellets were washed twice with buffer A (100 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 1 mM EDTA, 25 mM NaHCO₃ and 1 mM DTT) and resuspended in 2 ml of the same buffer. Cells were fractured by French press, followed by centrifugation (19 580 g, 20 min, 4 °C). Supernatants were used for analyzing the specific activity of RubisCO. The concentration of total protein in the crude extracts was measured according to Bradford and Williams (1976).

To upscale the RubisCO activity screen we tested whether one RubisCO active fosmid with TH-55 genomic DNA (clone 6F3) could still be detected among 11, 23, 47 and 95 inactive clones. The RubisCO inactive fosmid clone was 6F8 (also from the genomic TH-55 library). These RubisCO active and inactive clones were grown together on one LB agar plate (12.5 µg ml⁻¹ chloramphenicol) overnight at 37 °C. The metagenomic fosmid clones were then swamped off with 10 ml LB medium (12.5 µg ml⁻¹ chloramphenicol), and 200 µl of this cell suspension was used as inoculum for the working cultures, grown as described above. For identifying RubisCO active fosmid clones from the Nibelungen metagenomic library, pools of 24 metagenomic clones were screened and prepared as described for the other pooled samples. If RubisCO activity was confirmed in the pool of 24 fosmid clones, then two pools each consisting of 12 fosmid clones were prepared as described above and tested for RubisCO activity. The pool with activity was then further broken down into three pools each containing four fosmid clones and RubisCO activity was measured. The four remaining fosmid clones from the pool with the activity were then screened individually for activity.

RubisCO activity assay

The enzyme assay was performed at 25 °C for individual cultures or at 25 and 55 °C if testing pools for RubisCO activity. The assay mixture (150 µl final volume) contained buffer A, 0.2 mg unpurified, total protein and 5 mM ribulose-1,5-bisphosphate (Rubp)—Rubp addition is the initiation step. If RubisCO enzymes are in the sample Rubp is converted to 3-phosphoglycerate (3-PGA) over time. To monitor this conversion, subsamples (50 µl) were taken at different time points (0, 30 and 120 min for pools and 0, 10 and 30 min for single active clones) from the assay mixture. The reactions were stopped at 95 °C for 3 min and denatured

proteins removed by centrifugation (16 100 g, 20 min, 4 °C). The supernatant was used to quantify the concentration of Rubp and 3-PGA with HPLC (LaChrom Elite system, Hitachi, Tokyo, Japan) with a Lichrospher 100 RP-18e column (VWR International GmbH, Darmstadt, Germany). HPLC procedures were performed as described (Jakob and Saenger, 1985) with some modifications: detection was at 200 nm, with acetonitrile as an eluent and a flow of 0.6 ml min⁻¹.

Transcription experiments with TH-55 and transposon clones

Total RNA from (i) TH-55 was isolated with the Presto Mini RNA Bacteria Kit (Geneaid, New Taipei City, Taiwan) and (ii) from the metagenomic fosmid clones 71C2, the subcloned 71C2II and the transposon clones 96Δorf06, 6IIΔlysR1, 149IIΔlysR2 and 17IIΔcbbO-m using the UltraClean Microbial RNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). Genomic DNA was removed (RTS DNase Kit, MO BIO Laboratories, Inc.) and total cDNA synthesized (Invitrogen's SuperScript VILO cDNA Synthesis Kit, Life Technologies, Darmstadt, Germany). cDNA served as a template for the amplification of (i) different gene-to-gene interspaces to elucidate which genes are co-transcribed in TH-55 and (ii) small fragments of ~300 bp within cbbL and cbbM to examine the relative variations of transcript amounts of RubisCO form I and II structural genes in the above-mentioned transposon clones and respective intact versions. Primers used for amplifications are listed in Supplementary Table S1. Controls of the template lacking reverse transcriptase were performed by PCR. For details on culturing and RT-PCR conditions see Supplementary Materials and Methods.

Complementation experiments

To test whether the activity of the transposon clones could be restored, transposon clones 96Δorf06, 149IIΔlysR2, 6IIΔlysR1 and 17IIΔcbbO-m were complemented with pUC19::orf06, pUC19::lysR2, pUC19::lysR1 and pUC19::cbbO-m, respectively. For detailed procedures see Supplementary Materials and Methods.

Sequence access

The nucleotide sequence of the entire 35.2 kb metagenomic fragment has been submitted to the GenBank database under accession number KJ639815.

Results and discussion

Establishing a function-based screen for detecting RubisCO active fosmid clones

We used a HPLC-based approach to establish a new functional screen for seeking RubisCO active

recombinant enzymes from metagenomic fosmid clones (for experimental setup see Figure 1). First, we tested our screen by measuring RubisCO activity from crude extracts of TH-55 and from recombinantly expressed RubisCOs of TH-55. Three types of cloned RubisCO genes and gene clusters from TH-55 were used for the initial tests: one consisted of a large genomic DNA fragment containing 38.1 kb and included the RubisCO gene cluster (*cbbQOM lysR2 lysR1 cbbLSQO*) and flanking regions (25.1 kb) (pCC1FOS fosmid clone 6F3), the second encoded only the small and large subunit of the RubisCO form I (pCC1FOS::*cbbLS*) and the third encoded the large subunit of form II (pCC1FOS::*cbbM*). All specific RubisCO activities are summarized in Figure 2a and Supplementary Table S2. Details of genes present on inserts in pCC1FOS clones are shown in Figure 2b.

The specific RubisCO activity of TH-55 was 252 ± 19 nmol 3-PGA min⁻¹ mg⁻¹ in the exponential growth phase (Supplementary Figure S1), which is in the same magnitude of what has been measured for other *Thiomicrospira* isolates (Takai *et al.*, 2005; Scott *et al.*, 2006). In contrast, the specific RubisCO activity of the TH-55 genomic fosmid clone 6F3 was 1.8-fold higher (455 ± 30 nmol 3-PGA min⁻¹ mg⁻¹). However, this can be explained by the copy number of the pCC1FOS vector: after autoinduction, ~10–200 plasmid copies are present in the cell and thus more RubisCO is expressed than in the natural host TH-55. Under the provided conditions, recombinant *cbbLS* from TH-55 expressed individually in *E. coli* exhibited a specific RubisCO activity (266 ± 23 nmol 3-PGA min⁻¹ mg⁻¹), which was 59% of what we measured for the fosmid clone 6F3 containing the genomic insert from TH-55. The individual expression of the recombinant *cbbM* from TH-55 in *E. coli* (55 ± 9 nmol 3-PGA min⁻¹ mg⁻¹) only reached 12% of the total specific RubisCO activity of fosmid clone 6F3. Since RubisCO form I has a higher specificity for CO₂ relative to oxygen and thus a higher tolerance toward oxygen than RubisCO form II (Tabita, 1999), it is not surprising that in our experiments, performed under fully oxic conditions, RubisCO activity of the recombinant RubisCO form I was considerably higher than the activity of RubisCO form II. However, when we added up the individual RubisCO activities measured from pCC1FOS::*cbbLS* and pCC1FOS::*cbbM*, still only 71% of the total RubisCO activity of genomic fosmid

clone 6F3 could be reached. This suggests that genes other than the structural genes exist on the genomic fragment that contribute to expressing a fully functional RubisCO. Nevertheless, these experiments showed that the function-based screening can be successfully applied to genomic fosmid clones for identifying recombinant RubisCO active enzymes on a single scale.

Given that single sample screening of thousands of fosmid clones for a specific function is somewhat cumbersome and does not allow searching through a high number of metagenomic clones, we tested whether one active fosmid clone, namely clone 6F3 (455 ± 30 nmol 3-PGA min⁻¹ mg⁻¹), can still be identified if tested together in a pool with other 11, 23, 47 or 95 inactive clones. A clear detection of RubisCO activity from one active clone among other clones is only guaranteed when no more than 24 clones are pooled for RubisCO activity measurements (Supplementary Figure S2).

Recovering RubisCO active fosmid clones from metagenomic hydrothermal fluid samples

We then applied our newly established screen for seeking RubisCO active fosmid clones to a metagenomic library that we constructed with DNA from deep-sea hydrothermal vent fluids. From 1056 screened fosmid clones, 12 fasmids exhibited RubisCO activity (427 ± 16 to 484 ± 44 nmol 3-PGA min⁻¹ mg⁻¹) (Figure 2a; Supplementary Table S2). Restriction analyses of the corresponding fasmids showed that these 12 clones had ~35.2 kb DNA inserts and shared the same restriction pattern (data not shown). Double-stranded sequencing of three metagenomic fragments including the RubisCO gene cluster for all 12 clones demonstrated a 100% DNA identity (for genes encoded on the fragments see Figure 2b and Supplementary Table S3) and resembled genes from *T. crunogena* XCL-2 (90–96%) (Scott *et al.*, 2006). Sequencing of one representative clone illustrated that the genes encoded on the DNA metagenomic insert of the fosmid clone followed the arrangement of the homologous genes from XCL-2 (Tcr0411–Tcr0446) (Scott *et al.*, 2006), but with four genes missing (Tcr0415–Tcr0418), namely those encoding an ApbE-like lipoprotein (Tcr0416), a hypothetical protein with a FMN-binding domain (Tcr0417) and two hypothetical proteins with unknown function (Tcr0415 and Tcr0418) (Supplementary Figure S3).

Figure 1 Overview of the experimental setup. The experimental setup includes an overview of the experiments conducted to establish the function-based screen, its upscaling and its application to metagenomic libraries. It further illustrates the steps to elucidate the functions of genes recognized on metagenomic fragments recovered by the function-based screen. (a) First, a schematic view of major points successively processed to establish a functional screen to seek RubisCO active clones from crude extracts, and genomic libraries on a single scale is given. (b) The process of upscaling is shown, where pools of 96, 48, 24 and 12 clones were initially applied to test whether one RubisCO active fosmid with TH-55 genomic DNA (clone 6F3) could still be detected among 11, 23, 47 and 95 inactive clones (6F8 from the genomic TH-55 library). Enlargements of the HPLC chromatograms are shown in Supplementary Figure S2. (c) The methodological procedure utilized to screen metagenomic libraries for RubisCO active fosmid clones is shown. (d) Finally, the experiments performed with the recognized RubisCO active metagenomic fragment to elucidate the roles of distinct genes and respective gene products are summarized. A detailed magnification of the 35.2 kb and 13 kb gene cluster is shown in Figure 2.

Figure 2 Specific RubisCO activities of TH-55 and recombinant RubisCOs as well as respective gene arrangements. **(a)** Specific RubisCO activities measured from crude extracts of TH-55 (TH-55, grown for 25 h), clone 6F3 (38.1 kb insert size) derived from a genomic fosmid library constructed with TH-55 DNA (FOS::38.1 kb), a pCC1FOS clone containing TH-55's *cbbLS* structural genes (FOS::cbbLS), a pCC1FOS clone containing TH-55's *cbbM* structural gene (FOS::cbbM), fosmid clones derived from the Nibelungen metagenome library (71C2, 74C10, 74E1, 77H1, 77F4, 77D9, 78G5, 78E10, 80G3, 81E1, 81G7 and 84G4, summarized as FOS::35.2 kb) and fosmid subclones (84G4II and 71C2II) containing the RubisCO gene cluster (fragment B; 13 kb) (summarized as FOS::13 kb). **(b)** Schematic representation of the gene arrangements of the DNA inserts. Identified open reading frames are indicated as gray arrows and structural RubisCO genes are indicated in black or light gray, for *cbbLS* or *cbbM*, respectively, in the direction of transcription. ORFs were numbered serially from *orf01* to *orf32* (for details on annotations of respective ORFs see Supplementary Table S3). Standard gene abbreviations were used (see Supplementary Table S6). Fragments A, B and C denote regions that were sequenced for all 12 RubisCO active metagenome-derived fosmid clones sought from the Nibelungen library through the function-based RubisCO screen.

Transposon insertion libraries to elucidate the role of metagenomic gene products on RubisCO activity

To investigate whether additional enzymes other than the structural enzymes CbbLS and CbbM may be required for a fully functional RubisCO, we subcloned the RubisCO gene cluster (13 kb) of two metagenomic clones (71C2 and clone 84G4) and measured their specific RubisCO activity. The specific RubisCO activities of these two subclones 71C2II and 84G4II were roughly fivefold lower (55 ± 8 and 48 ± 14 nmol 3-PGA min⁻¹ mg⁻¹) than the activity measured for the respective clones 71C2 and 84G4 with the original metagenomic DNA fragment containing flanking regions of additional

22.2 kb DNA (Figure 2; Supplementary Table S2). This is suggestive of genes up- and/or downstream of the cloned RubisCO gene cluster contributing to the expression of a fully functional RubisCO enzyme.

To unravel which genes and respective products in and outside of the RubisCO gene cluster contribute to a fully functional RubisCO in our metagenomic clones, two transposon mutant libraries were constructed: one with the fosmid clone 71C2 (with the entire metagenomic DNA fragment including the RubisCO gene cluster and flanking DNA regions: 35.2 kb) and one with the fosmid clone 71C2II (carrying only the RubisCO

gene cluster: 13 kb). In total, we sequenced 384 transposon clones with a primer targeting the transposase-inserted kanamycin cassette to establish the position of the insertion and measured RubisCO activity for 45 selected transposon clones (Supplementary Tables S4 and S5). Additionally, we performed complementation experiments with four transposon clones exhibiting the most dramatic activity changes (not related to structural RubisCOs), to show that the original RubisCO activity could be restored. In the complementation experiments, some discrepancies to the full activity of the intact fragments are visible. However, they are most likely related to the diverging and irregular copy number of pCC1FOS (after auto-induction, 10+ fosmid copies) and pUC19 (without any induction, 100+ plasmid copies). We further linked the most dramatic activity change of four transposon clones with transcription of RubisCO forms I and II to elucidate whether these deleted genes are involved in transcription or rather in subsequent cellular processes.

The effect of gene deletions outside of the RubisCO gene cluster on RubisCO activity

Among the 25 transposon clones with deletions up- or downstream of the 13 kb RubisCO gene cluster, seven clones had significantly lower RubisCO activity than the intact 71C2 clone (Figures 3a and b). One transposon clone, namely clone 96, exhibited a dramatic activity loss. The activity was comparable to the reduced RubisCO activity measured for the 13 kb clones, which had the RubisCO gene cluster but lacked the flanking DNA regions (Figure 2a; Supplementary Table S2). The gene deleted in transposon clone 96 (insertion at 6 of 135 aa) was designated *orf06* and resembled the gene Tcr0420 of XCL-2 (97% aa similarity) (Supplementary Table S3). In XCL-2 this gene product has been assigned a hypothetical protein with unknown function (Scott *et al.*, 2006). Interestingly, the closest related protein of known function to *orf06*'s gene product was the ArsR regulatory protein of *Micromonospora aurantiaca* ATCC 27029 (Bioproject: PRJNA42501) (Supplementary Figure S4) (30% aa identity), which functions as a metal-sensing transcriptional repressor (Wu and Rosen, 1991). Transcription experiments with the transposon clone 96 (Δ *orf06*) where the relative abundance of *cbbL* and *cbbM* gene products was determined, demonstrated that transcription for both genes is downregulated relative to the metagenomic fragment with the intact *orf06* (Supplementary Figure S5). Conclusively, the *orf06* gene product is likely involved in directly or indirectly regulating the structural RubisCO forms I and II. Complementation experiments of transposon clone 96 (pCC1FOS:: Δ *orf06*) with puc19::*orf06* managed to restore most of the original RubisCO activity (Supplementary Figure S6), evidencing that

orf06 is responsible for the dramatic activity loss. We also conducted experiments with TH-55's RNA to identify which genes were co-transcribed. They showed that in TH-55 the *orf06* homolog was transcribed separately from the carbonic anhydrase and other neighboring RubisCO-associated genes (Figure 4; Supplementary Figure S7). The activity loss of transposon clone 231, with an insertion in the non-coding region upstream of *orf06* (Figures 3a and b), may be related to difficulties in the transcription process of *orf06* due to an impaired promoter region.

Other transposon clones with insertions outside of the RubisCO gene cluster included clones 58, 89, 68 and 91 (for details see Supplementary Results). Despite RubisCO activity loss, none of these four enzymes have ever been linked to the expression of a fully functional RubisCO and current annotations do not imply that these genes encode functions associated with RubisCO regulation, assembling or activation mechanisms. However, the significant RubisCO activity loss may be explained if gene products interplay with RubisCO-associated genes encoded on the entire metagenomic fragment or if the activity of encoded enzymes has an impact on intercellular conditions such as CO₂ concentrations, pH values or ATP/ADP ratio, which are important to maximize RubisCO activity (Portis, 1990; Toyoda *et al.*, 2005) and which might be applicable in heterologous hosts like *E. coli* as well.

The effect of gene deletions within the RubisCO gene cluster on RubisCO activity

Among the 20 transposon clones exhibiting deletions in the RubisCO gene cluster, 10 displayed a significantly lower and two clones a significantly higher RubisCO activity relative to the respective intact fosmid clone (Figure 3). An insertion in the transposon clone 149II resulted in a significant increase in RubisCO activity (Figures 3c and d). Sequencing of this gene (*orf11*) revealed a similarity to *lysR2* of the LysR family, which is classified as a transcriptional regulator (Maddocks and Oyston, 2008). The insertion in *orf11* was located at base 553 (of 945 nt), that is, 185 aa (of 315 aa). Blastp of the translated intact *orf11* of the metagenomic fosmid clone demonstrated that the LysR substrate binding domain is located between 99 and 303 aa, and thus a considerable impact on the LysR binding ability can be expected through the deletion, which can explain the significant change in RubisCO activity. According to the transcription experiments with transposon clone 149II (Δ *lysR2*), it appears that the transcription level of *cbbL* remained unchanged but that transcription was upregulated for *cbbM* relative to the respective intact metagenomic fragment (Supplementary Figure S5), suggesting that Orf11 acts on *cbbM* transcription as a repressor. In complementation experiments with the transposon clone 149II (pCC1FOS:: Δ *lysR2*) and pUC19::*lysR2*

Figure 3 Specific RubisCO activities and insertion positions of tested transposon clones. Specific RubisCO activities (**a**) and schematic gene arrangement (**b**) of transposon clones carrying the entire 35.2 kb insert derived from the metagenomic fosmid clone 71C2. Specific RubisCO activities (**c**) and schematic gene arrangement (**d**) of transposon clones carrying the 13 kb insert derived from the fosmid clone 71C2II. Only specific RubisCO activities for transposon clones with significant differences to the intact 35.2 kb (**a**) or 13 kb (**c**) fosmid clones (intact fosmid clones indicated as +) are displayed. Identified ORFs were numbered consecutively, designated *orf01*–*orf32* (for detailed abbreviations see Supplementary Table S3) and indicated as ORFs in the direction of transcription. Insertion sites are denoted by vertical black arrows and transposon clone numbers. Clones exhibiting significant changes in RubisCO activity relative to the intact clones are highlighted in bold. Genes with significant activity change are color coded according to the bars in **a** or **c**. The level of significance is denoted by dots under the activity, where green is 0.05 (95%), yellow is 0.01 (99%) and red is 0.001 (99.9%).

Figure 4 Transcription of RubisCO encoding genes and flanking gene regions from TH-55. Genes are indicated as arrows. Co-transcribed genes are displayed in the same color. Abbreviations are listed in Supplementary Table S7.

the RubisCO activity dropped down near the level of the intact metagenomic fragment (Supplementary Figure S6).

In contrast, three insertions in *orf12* (*lysR1*) at the nucleotide positions (i) 660, (ii) 790 and (iii) 855 (of 927 nt), conferring to aa positions (i) 220, (ii) 264 and (iii) 285 (of total 308 aa), led to significant lower activities for clones (i) 169, (ii) 6II and (iii) 161, respectively, relative to the intact metagenomic clones (Figures 3a and c). Complementation experiments with transposon clone 6II (pCC1FOS::*ΔlysR1*) and pUC19::*lysR1* managed to restore the original RubisCO activity of the intact metagenomic fragment (Supplementary Figure S6). Blastp searches of the translated, intact metagenomic *orf12* gene suggest that the LysR substrate binding domain is located between 93 and 298 aa and thus the insertions affect the last (i) 78 aa, (ii) 34 and 13 aa of the LysR substrate binding domain of the metagenomic transposon clones. Transcription experiments with transposon clone 6II (*ΔlysR1*) suggest an unchanged transcription level for *cbbL* but a downregulation of *cbbM* relative to the respective intact metagenomic fragment (Supplementary Figure S5). We thus propose that *orf12* (LysR1 homolog) activates *cbbM* transcription. However, Toyoda *et al.* (2005) suggested that in *H. marinus* transcriptional regulators of the lysR family, namely CbbR1 and CbbRm, were required to activate the expression of *cbbLS-1* and *cbbM*, respectively, which in our case only holds true for *orf12* (LysR1 homolog) and *cbbM*. More experiments will be needed to better understand the interaction and interconnection between LysR regulators and the structural RubisCOs. Transcription experiments with TH-55 suggest that *lysR2*, *lysR1* and *cbbLS* were co-transcribed (Figure 4; Supplementary Figure S7). Yet, since the *lysR2* and *lysR1* genes are juxtaposed to *cbbLS*, either only the *lysR2* and *lysR1* or the *cbbLS* gene product would result in a functioning enzyme. Nevertheless, if the promoter regions of the *lysRs* and the structural gene *cbbL* overlap, which is typical for *lysR* genes, which regulate their own transcription and that of adjacent structural genes (Schell, 1993; Maddocks and Oyston, 2008), the one acquired transcript (*lysR2R1cbbLS*) might mask two actual transcripts (that is, *lysR2R1* and *cbbLS*).

As the role that CbbO and CbbQ play for a fully functional RubisCO remains unclear, we selected 12 transposon clones with insertions scattered across *orf08* (*cbbO-m*), *orf09* (*cbbQ-m*), *orf16* (*cbbO-1*) and *orf15* (*cbbQ-1*) of our metagenomic fragment for RubisCO activity measurements. Four transposon clones with insertions at different positions in *cbbO-m* had a significant activity loss (Figures 3c and d). The full RubisCO activity could be restored when transposon clone 17II (pCC1FOS::*ΔcbbO-m*) was complemented with pUC19::*cbbO-m* (Supplementary Figure S6). In the transposon clone 17II (*ΔcbbO-m*), transcription of *cbbL* and *cbbM* was maintained at an unchanged level relative to the

intact metagenomic clone (Supplementary Figure S5). Therefore, it appears highly likely that CbbO-m is not involved in regulating transcription of the structural RubisCO genes but rather in post-transcriptional processes, as has been suggested (Hayashi *et al.*, 1997, 1999; Scott *et al.*, 2006). No changes in RubisCO activity were evident in *ΔcbbQ-m* (8II, 11II and 12II) and *ΔcbbO-1* (4, 8 and 4II) transposon clones compared to the intact metagenomic clone 71C2II (pCC1FOS::13 kb) or 71C2 (pCC1FOS::35.2 kb). Mutations in CbbQ-m and CbbO-1 may be able to be substituted by CbbQ-1 and CbbO-m functioning, respectively, or these enzymes may simply not be as detrimental for producing a fully functional RubisCO. Curiously, two transposon clones with deletions in *orf15* (*ΔcbbQ-1*) exhibited contrasting results for the RubisCO activity. Transposon clone 23II carrying the RubisCO gene cluster (13 kb) had significantly higher activities relative to the intact metagenomic clone, but the RubisCO activity of transposon clone 3 with the entire metagenomic DNA fragment (35.2 kb) remained stable (Figure 3). Although these results appear contrasting at first glance they can be explained: *cbbQ-1* may need to interact with proteins encoded on the flanking DNA regions. Interestingly, in TH-55 neither *cbbQO-m* nor *cbbQO-1* was co-transcribed with *cbbM* or *cbbLS* (Figure 4; Supplementary Figure S7).

In transposon clones with insertions in the structural genes *cbbS* (clone 38) and *cbbL* (clone 24II), RubisCO activity diminished significantly relative to the intact metagenomic clone 71C2II (pCC1FOS::13 kb) or 71C2 (pCC1FOS::35.2 kb) (Figure 3). On the contrary, an insertion in the RubisCO form II structural gene *cbbM* (clone 22II) led to an increased RubisCO activity (6.2-fold higher) relative to the intact version of clone 71C2II (pCC1FOS::13 kb), indicating that RubisCO form II (*cbbM*) may repress RubisCO form I (*cbbL*). According to transcription experiments with RNA from TH-55, *cbbLS* was transcribed separately from *cbbM* (Figure 4; Supplementary Figure S7).

Conclusion

Although novel enzymes can only be discovered by function-based screening approaches, the imperative of heterologously expressing environmental DNA from an unknown source organism in a culturable surrogate host is an inherent limitation itself and often associated with low hit rates (Perner *et al.*, 2011 and references therein). It has been illustrated that only 30–40% of foreign DNA are successfully expressed in *E. coli* (Rondon *et al.*, 1999; Gabor *et al.*, 2004), the host we used for our screen. Some of the problems faced in recombinant protein expression in *E. coli* include recognition of intrinsic promoters and associated factors, diverging codon usage, translation, correct folding,

maturation processes and activation of the enzyme (cf. Perner *et al.*, 2011 and references therein). Since we work with large metagenomic DNA fragments (average insert size of our fosmid clones 37 ± 2.1 kb), it can be expected that promoters and ribosomal binding sites of the targeted genes are located on the metagenomic fragment. All the RubisCO active clones we discovered resembled genes of *Thiomicrospira*, which could indicate the high abundance of *Thiomicrospira* in the analyzed sample. However, these findings may also indicate the difficulties of our screen in expressing RubisCOs from other organisms. For example, RubisCOs of taxonomically distinct species or anaerobic organisms may not be detectable in the host *E. coli* under the provided experimental conditions. Further experiments will be needed to recognize the full extent of RubisCOs that can be targeted. Despite possible limitations that may exist when applying this screen, we show that it can be successfully used to identify RubisCO active fosmid clones from a metagenomic library and that these clones can be utilized to elucidate the importance of flanking genes and the respective enzymes for a fully functional RubisCO. Our approach circumvents time-consuming cultivation of autotrophic microorganisms, some of which may be resilient to current cultivation techniques, and the inherent bias accompanying sequence-dependent methods. It allows us to tap the tremendous metagenomic resource available in the environment and hence enables the discovery of species not previously associated with RubisCO activity.

Conflict of Interest

The authors declare no conflict of interest.

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