Supplementary Information for

Unraveling RubisCO Form I and Form II Regulation in an Uncultured Organism from a Deep-Sea Hydrothermal Vent via Metagenomic and Mutagenesis studies

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Supplementary Results and Discussion

Polar effects caused by transposon insertions. Polar effects of the \(cbbM\) and \(cbbL\) gene deletions were tested for the downstream genes \(cbbO-m/cbbQ-m\) and \(cbbO-1/cbbQ-1\), respectively (Supplementary Figure 3). In the \(cbbM\) mutant \(cbbQ-m\) and \(cbbO-m\) transcription was disturbed. In \(\Delta cbbL\) only \(cbbQ-1\) transcripts were significantly downregulated. However, we consider the actual polar effects on downstream genes not of significant importance for understanding the regulatory machinery behind RubisCO expression on our metagenomic fragment for the following reasons: If indeed \(cbbQ-m/cbbO-m\) downregulation was detrimental to RubisCO activity then one would expect (i) a loss of activity in the \(cbbM\) mutant relative to the undeleted fragment, and (ii) no or little change in RubisCO activity when these genes were deleted. However, neither is the case. RubisCO activity rises 5-fold when \(cbbM\) is knocked out and deletion of \(cbbQ-m\) and \(cbbO-m\) results in a considerable RubisCO activity loss (Figure 2A). Since deletions in the \(cbbQ-m/cbbO-m\) analogues \(cbbQ-1\) and \(cbbO-1\) do not result in any change of activity, CbbQ-m/CbbO-m appear to be sufficiently present to activate the RubisCO enzyme. The same principle applies for the \(cbbQ-1\) gene, downregulated likely as a consequence of the insertion in the \(cbbL\) gene.
Supplementary Figures

Supplementary Figure 1

Over expression of RubisCO and RubisCO associated genes. The purified eluates of the over expressed RubisCO transcriptional regulators LysR1 and LysR2 and the RubisCO form I (CbbL) and form II (CbbM) are visualized on SDS-polyacrylamide gels (SDS) and with Western Blot (WB). The prestained protein ladder, 10-180 kDa (Thermo Fisher Scientific, Waltham, MA, USA) was used as a marker (M).
## Supplementary Figure 2

### IR1

|    | Sequence                                      | Description                     |
|----|-----------------------------------------------|---------------------------------|
| 1  | ATGTCACCTTG AGTTGACGAG GTTGGCGATG TTTCGGTGAA CAAAATAGT TCCTGATCC ATCTTAACCA GCTCGTTTGC | carbonic anhydrase |
| 81 | AGGAACTCGG CTATCGGAGC AACCTATCCA GAGATATTCG GTGGAGATAG GGTTCAAAAA AAGTCCGGGAC | carbonic anhydrase |
| 161| GAATGGGCATT GATTTCCATCC ACCCAATCAC GGTTGTGGT TAACAGCTGC TCGATTTGATG TATCGCAGTC ACATTGATGG |
| 241| CACATTAAGA ATCCTTTTGG AGAAGTGGTT TTGGGGGATAC AAAATGCC CTTAAATTGA AGGCGGATTA CTTAAAGCT |
| 321| TCTTCAAAT GGGAAATT A CACGTTTTTA TTGTGTGTATT TCCATAAAAGA CTTGAGGTAA TTTTTCTGGC AACTTGTTGA |
| 401| CATGGTCAAT GACGGTATAG TGGTTGGTCA AAATGGTCTC AAGCTATCCA TCGGCATTGC GATCTAAGGT AATGCAGTAA |
| 481| GAATAAGATCC CTTTGCTTTT AGGTTCTTGG ACCGCTTTAT GGGTAATTAAAACC TGTTGGGTCTT TTGTGTCAAT |
| 561| ATCCGCCGGC TCACCATCGG TTAATAATCAG CATCAGTTTT TTCTCAGCTTT GCTGTGGCTTC CAGATAGTGT GCGGCATGAC |
| 641| GCATGCGCGGC TCCATTGCA AGGCTTTTAC AGCAGTTCTT ATTCAGATAG ATTCGGAATG TATCGCAGTC ACATTGATGG |
| 721| GATAGGCTTT TGATATGCTT TAGCAGCGGT TCAGTTTCCC ATAATGGCTCT |
| 801| CTGTTCAATG GTCCACGCAG TGATGGCAAG GGGTTCTTCA CTTAATTTCG GTAAGGGTGT GCGCGGTTC TGATTTGGGT |

### IR2

|    | Sequence                                      | Description                     |
|----|-----------------------------------------------|---------------------------------|
| 321| TCTTCAAAT GGGAAATT A CACGTTTTTA TTGTGTGTATT TCCATAAAAGA CTTGAGGTAA TTTTTCTGGC AACTTGTTGA |
| 401| CATGGTCAAT GACGGTATAG TGGTTGGTCA AAATGGTCTC AAGCTATCCA TCGGCATTGC GATCTAAGGT AATGCAGTAA |
| 481| GAATAAGATCC CTTTGCTTTT AGGTTCTTGG ACCGCTTTAT GGGTAATTAAAACC TGTTGGGTCTT TTGTGTCAAT |
| 561| ATCCGCCGGC TCACCATCGG TTAATAATCAG CATCAGTTTT TTCTCAGCTTT GCTGTGGCTTC CAGATAGTGT GCGGCATGAC |
| 641| GCATGCGCGGC TCCATTGCA AGGCTTTTAC AGCAGTTCTT ATTCAGATAG ATTCGGAATG TATCGCAGTC ACATTGATGG |
| 721| GATAGGCTTT TGATATGCTT TAGCAGCGGT TCAGTTTCCC ATAATGGCTCT |
| 801| CTGTTCAATG GTCCACGCAG TGATGGCAAG GGGTTCTTCA CTTAATTTCG GTAAGGGTGT GCGCGGTTC TGATTTGGGT |

- **carbonic anhydrase**
- **cbbO-m**
| Sequence | Start | End | Description |
|----------|-------|-----|-------------|
| 1761 CCATTCTTTG TGC GCCATCA TATGC GCCAG CATG GCCGGA TAGCGATCCA AGCCGCTAAC ACCATT TTCA TCT CATACAA |
| 1841 CATCTGGCAC GGCAATGAGT TCTTCATCCA AATACGGGAAC GGGTTTACGA AGTTGATCGA ATGCGGTGGA GAAAACCGAA |
| 1921 AAAGGGCCGT CACAATGCCA CATGCAGTCT TTTAACAT TCCACGTCT CTGAAACGTTG TGGCATCGTG CGATTCTAAG CTGAAATAGG CAATTTGCTG ATCGGGC |
| 2001 TTCACGTGGA ATGATGCTTT TGGC ATCGT C GACC TTAAGG CTGAAATAGG CAATTTGCTG ATCGGGC |
| 2081 TACCGCCGCC ATAATCAATA AAATTACGGGA TTCCGGCCAG GCTTAATTTG GAAATCAGTT GGGGCATGGA TTCCAGTAAA |
| 2161 GGAATCATGC GCCGACTTTC ATACAAAGAG TGATGACCAT GAATGACGGT TTGCGTTTTG TCGACGTATT CATCAATGATGCTAG |
| 2241 GTCTGATAG TGCTGAAGGT GGT TCCAGCGT ATCGGATGCGT CGGCAGACAC TGCTGAGTGA GGCCAAAAAA GGAATTAAGG |
| 2321 CTTTTTTGTT AGGGCTGCCAG CTTAATCCAT ATACAAAGAG TGATGACCAT GAATGACGGT TTGCGTTTTG TCGACGTATT CATCAATGATGCTAG |
| 2401 ATTTCCGGCA TGATCTCAAG GATGACCAGA ACCGGCTCCTA CGCCCATGCC TATCTTACAA AGGAAGTTGG CGCCATCAAT |
| 2481 ATAGGCAGTC ACCCTTCGT CTGAAAGTTG CTGGCCAGGCC TCTTGCATAC AGCTGGAAGA AACTTCAAAC GCTTTGGGA |
| 2561 AAT TACAGGT GAATTTGTCT TGATATCACG AAACGCCTTC TGTTATGACG TCTGCATTCA TAATCTAACT CATTTGATTG |

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IR6

3521 AACGTTTATG GTAGGTATTT TCAACAGAG TGTG^AAATA^ CCGATGAATA ^AAATTA^ ACG ATATTAAGGT TATTTAGGTT

LysR bs2_{cbbQ-m}

8

TnA - n7 - TnA

3601 TCTGTTTTAC CGTAAGACAG GTAAAAGAGA AAACCTAAATA ACCTTGTGTT TGGCTAGGTT TTTGTTTGG GTGTAAAAAA

3681 AACCCCTCGC CGAACGCAGA GGTTCCTTGG GTGGTTAAT TAAACATCGTG TAAAGAATGG CTTATTTGTG TACGCCCAAC

TnA - n7 - TnA

3761 TTCTCTCCAC AACCTGGGTA GATTTGTCTGT GCATCTGCAG GGAAAGATTC GAATGCGCGA GCGAACTTCTTG TGTGAATTTT

TnA - n7 - TnA

3841 TGCGAATTCTG ATGGGATCTG CACCTGATTT CCAGCACTCG TAAGATTGAC GAAGTGACGT TGCACCTGCC GCTGGAGAAT

4001 AGTGCGTTCA TACCACCCGA GTAGATAGGA GCATTTGGCT TCATAACCATT CCATTTCCTGG TAGAAAAGTG GCTCTTGACA

4081 CTCATCAGGC TCGATCATGT ATGGGATCTG CTTATCGAGA GCATACCTTG CCATTTCACC GATAACCATT GTGCCACAGT

4161 GGATACCAGA CGCACCTTGT AGACGAGAGA TTTTAGCAAG AACG AATGCC GTGTAACCAC GTTTCGCTGA AGGCGACGTG

4241 ATCGCACCCAT GACCTGCACG GTGATAATGT AAGTACTGTG AAGGATAGTT ACGACGTGCT TGCATATCA TACCACGGACC

IR7

4321 ACCAACATAA CCATCAACTA GGAAGCAGAC CTGAGGGCAG TCAGCACCAGA AAGTTTCTAG GATGTAATCT GCACGGTGAGC


-10 box cbbQ-m

-35 box cbbQ-m

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7041 TTTTTTCCG ATCAATCGA TCAATTTAAT TTCGTTATTT TCTTCCAGAC GCTTAACTTG AACGGATACG GCAGGTTGAG
------------ -------------- -------------- ------ lysR1 ------ -------------- --------------

7121 AAAGGTTGAG TTTTTCGGCT GCTTTTGTGT AGCCTAACTG CCTGGAAAAC GCCTGAAAAC TCTTATCTTG CTGGGCGGGT
------------ -------------- -------------- ------ lysR1 ------ box cbbL -------- lysR bs3

7201 ATATGTAAGT TTTGCGTTTG TTTTTCCTTG TTGTTAACTG CATAAATGTT TGGATTATAA AGTAAATGAG AGGAATCATA
------------ lysR1 ---- 35 box lysR1 T nA - n8 - TnA IR9 LysR bs2lysR1

7281 TACATTGTGT TATGAAATTG ATAAACAATA CTCAATATG CACGGATTA TTTTAAATGA GATACGAATC ACTGAAAGCG
------------ IR9 TnA - n7 - TnA

7361 GAAGTGGTTT TAAAACGCAA TTNTAAGAATC GCTTCTCTTA ACTCTTTTCA TGATTAAACT AAAACGAAGA GGTAAACCATA
------------

7441 GGCTAAAGACT TATAACCGCG GTGTTAAAGA ATACCCCGCAA AGTATTGGG TGGCGCAAATA TGAGCTA AATAGACTATG
------------ cbbL -------- -------------- --------------

7521 TTCTAGCATG TTTTAAAGTA ATCCCCAGGA ATGGTTTTCC AGTGAAGAA ATCGACGAGT CGGCTGCGG TGAACAGTTC
------------ cbbL -------- -------------- --------------

7601 ACGGGTACAT GGACAACGTG TTGGACTGAC TTGTTAACAG ATCTCTGACT TAATTTAAGGT CGGCATACA AAATGGAAGA
------------ cbbL -------- -------------- --------------

7681 CGTTTCTGTT GACGATGCAG CATTGTTATGC TTTCATCGCA TACCCAATCG ATCTATTGGA AGAAGGTTCT GTCGTTTCTG
------------ cbbL -------- -------------- --------------

7761 TAATGACATC TTTAGTTTGG AACGTATTGC GATTAAAAGC ACTACGAGTG TGCTGCTAG AAGATATCGG TTTCTGCTCTA
------------ cbbL -------- -------------- --------------

7841 GCGTATGTGA TGACGTTGG TGACCACCA CAGGTAGAGCG TGACTAAATG YTAAAGATTA GTCGTTCCAATT
------------ cbbL --------
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| Sequence | | | | |
|----------|---|---|---|---|
| 7921 GCTAGGTTGTC ACTATCAAGC CTAAGCTAGG TCTATCGGCT AAAAACTACG GTCGTCGTCTG ATATGAGTGT CTAGCTGGTG | | | | |
| 8001 GTCTTGACCTT CACGAAAGAT GATGAAAACG TTACTTCTCA GCCGTTCATG CGTTGAGAGAG ATCGTTTCCCT ATTCTGTCAAA | | | | |
| 8081 GATGCAATCG AAAAATCACA AGCAGAAACG GGTGAGGGC AAGTCACTA CCTAAACTGT ACTACGTGGTG | | | | |
| 8161 AATGTACGAG CGTGCTGAGT TCGCTAAAGA AATCGGTACT CGGATCATCA TGCACGATTA CCTAACAGGT GGGTTCACGGT | | | | |
| 8241 CTAACACAGGG TCTTGCAAC TACTGTCGTA AGAATGGTCT GTGTTTACAC ATTCACCACG CAAATCGACGG TGTAATTGAC | | | | |
| 8321 CGTAAACCAC ATCACTGCTA TCTTCCGTG AAGTAAAGAC AAGCCTACGG TCTATCGGCT GGTGATCATG ATTACCACTG | | | | |
| 8401 TACTGTTGTT TCCGAAAGAT AAGTACGGCG TGGTGGTCACTCA TCGATCTTTA GGTGATTTTC GCATTTTCCG | | | | |
| 8481 AAGATCGTTC ATGGAATTCA AAGCTTCCG AAGCTTCCG ATGCGATCCT GGTGGATTCG CAGTTGCTTC TGTTGATCTTC | | | | |
| 8561 CACGTATGCC ATATGCCCAGC ACTTCTTTCT ATCTTGCTGG ACTAGCTCTGT TCTTCAGTTC GGTGGTGGA CGCTAGGTCA | | | | |
| 8641 CCCAGGGTGG AAGGCTTCCG GGTGGCTGTC GAACGCTGTTT GCTTTGGAGG CGTGTGTACA AGCAGTAAAG TGAAGCTGGTT | | | | |
| 8721 AAGTCCAGAAA AGAAGCTTCTA CTAACGCTTC TAAACATAGC CCAGAACTTA AGATCTGCAAT GGAAGCTTGG | | | | |
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LysR bs1<sub>cbbS</sub>

8801 AAAGAAATCA AGTTCGAATT CGACACAGTT GATAAGCTAG ATGTAAATAG TAAATTTAGG AGAATATAAT

--- ------- --- cbbL ------- ------- --- cbbS

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8881 TATGAGTATT CAAGATTACC CATCTCGTCT TTCAGATCCA CAATCTCGTA AGGCTGAGAC GTTCTCTTAC CTTCCAAAAA

--- ------- ------- ------- ------- ------- ------- ------- ------- ------- ------- --- cbbS

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Unraveling RubisCO Regulation

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| Sequence | | | |
|----------|---|---|---|
| GAGCAATGA | TGGTGTGTCG | GGAACGTTTC | CATATGAAA | TTGAAGAAAA | TCTGGACAAC | GAGAAATG | CTGGGATTTT |
| GGGATTCTG | CTTTACAATG | TCTGAATAA | AGCGACTTCT | AAGTGGGAAT | CTCTTACGGA | GATCAGTCAA | CAACGTTTTG |
| GCTATCGTGA | TGACAACCGT | TTGGTTTGGG | CATCCGACGA | ATGGGCCGAA | ATGGAAGGGG | GCGGTGCACC | ACATCAAGAA |
| ACCGTTCGTA | AAAATGTTTC | ATGGATGGGA | ATGATCAATG | AAATTGATTC | TGAACTGGTG | GATGGGATC | ATGAAGAAAGT |
| TGGGTACTA | GGCTCGGAGC | TTTATCCTTA | CGAGATAAT | GACGTCTTT | ATATGAGAT | GGAAGGCATT | GAGCCCGTTT |
| CAGATCTCTT | CCATTATCAT | GAGTGGGATT | ACCCGTTCCA | GGGTTAATG | CCAACTGGG | TCACGCTGTA | TGAGCACCCTG |
| GCGAAAAAGG | GCGACCCTCA | GGTATATAAC | CGAATTCTAG | ATCAGAATAA | AGGAATTGCC | CATCGTATTA | AGCAAATCGT |
| TGATAAGTTG | CAAGCGGTTG | GTTGTCAGCG | TATTCGCCGA | ATGGAGATGG | GAGACGAAC | GGATTGAAT | GCCTGGGTTG |
| AAGCAATTAC | GTCAATTCGGT | ATGCGGCGATG | AACCGCGGATC | TGGCATTACG | ATGAAAATG | TGATTCGAGC | CCGTGAAGTA |
| TCGGTTGGTCG | TGGTGCTTGA | TTTATCGGAA | TCGACTAATG | AATGGTTGGA | TGGTGCCGAT | AAAACTGTC | TAGAAGTAAC |
| GCAGGAGGCCG | GCAATCTTGGG | TGTCGCATGC | CATCAATGGT | ATCCGGCGATA | AGTTTGCTG | TCATGGTTTC | TCATCAGATG |
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IR15

IR16

IR17

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11441 1501 1505 1509 1513 1517 1521 1525 1529 1533 1537 1541 1545 1549 1553 1557 1561 1565 1569 1573 1577
Structural features predicted for the DNA of the metagenome derived RubisCO gene cluster. Exact positions of predicted promoter boxes are indicated by black dashes over each associated base and a heading with -10 box or -35 box. Putative LysR binding sites (bs) are highlighted in green. Inverted repeats (IR) are numbered consecutively from IR1 to IR18. Each IR is highlighted in grey and red, whereby the grey part shows the repeat and its reverse complement, putatively forming the stem, and the bases in between, in red, represents the putative loop forming area. Only IR with at least 8 bp in the potential stem region and loops with a maximum of 100 nt were considered.
Fold changes of \(cbbO-m\), \(cbbQ-m\), \(cbbQ-1\) and \(cbbO-1\) transcription. Fold changes \((2^{\Delta \Delta C_t})\) of A) \(cbbO-m\) (red) and \(cbbQ-m\) (orange) transcripts expressed from the transposon clone \(\Delta cbbM\) (22II) and of B) \(cbbQ-1\) (blue) and \(cbbO-1\) (pink) transcripts expressed from the transposon clone \(\Delta cbbL\) (24II). The data shown is normalized to three different reference genes, the \(cat\) gene, which is encoded on the fosmid vector and reflects its copy number, the 16S rRNA and \(rpoD\) housekeeping genes. RubisCO transcription is relative to the clone 71C2II, containing the intact RubisCO gene cluster (\(cbbO-mQ-mM\) lysR2lysR1 \(cbbLSQ-1O-1\)). Bars and error bars denote mean values and +/- standard errors. Black dots denote that values are significantly different (p-value \(\leq 0.05\)).
## Supplementary Tables

### Supplementary Table 1

**Supplementary Table 1.** Insertion positions of tested double transposon clones with 13 kb inserts.

| inserted genes | clone designation | insertion position [aa] | total orf length [aa] | specific RubisCO activity [nmol 3-PGA*min⁻¹*mg⁻¹] |
|-----------------|-------------------|-------------------------|-----------------------|--------------------------------------------------|
| l               | 71C2II            | /                       | /                     | 49 ± 6                                           |
| ΔcbbM           | 22II              | 167                     | 459                   | 265 ± 48                                        |
| ΔcbbM ΔcbbO-m   | 22I1IIA3          | 208                     | 757                   | 130 ± 22                                        |
|                 | 22I1I1G1          | 34                      |                       | 178 ± 24                                        |
| ΔcbbM ΔcbbQ-m   | 22I2I2H4          | 135                     | 266                   | 81 ± 7                                          |
| ΔcbbM ΔlysR2    | 22I1IIA3          | 28                      | 314                   | 126 ± 39                                        |
| ΔcbbM ΔlysR1    | 22I1IIB2         | 79                      | 308                   | 163 ± 29                                        |
| ΔcbbL           | 22I1IC5           | 15                      | 472                   | 0.5 ± 7                                         |
|                 | 22I1I2B4         | 377                     |                       | -1.5 ± 11                                       |
| ΔcbbM ΔcbbQ-1   | 22I2I2C9         | 159                     | 272                   | 205 ± 48                                        |
| ΔcbbM ΔcbbO-1   | 22I1IIA10        | 75                      | 777                   | 235 ± 32                                        |
|                 | 22I1I4H6         | 503                     |                       | 234 ± 10                                        |
| ΔcbbL           | 24II              | 38                      | 472                   | 18 ± 7                                          |
| ΔcbbL ΔcbbO-m   | 24I1I2H3         | 482                     |                       | 17 ± 5                                          |
|                 | 24I1I2G7         | 212                     | 757                   | 13 ± 5                                          |
|                 | 24I1I1H11        | 82                      |                       | 14 ± 5                                          |
| ΔcbbL ΔcbbQ-m   | 24I1I1F9         | 147                     | 266                   | 17 ± 5                                          |
| ΔcbbL ΔcbbM     | 24I1I2F3         | 301                     | 459                   | -4 ± 5                                          |
| ΔcbbL + ΔnccrbbM-lysR2 | 24I1I2G2 | 103¹                   | 214²                  | 8 ± 3                                           |
| ΔcbbL ΔlysR2    | 24I1I1H7         | 46                      | 314                   | 48 ± 8                                          |
| ΔcbbL ΔlysR1    | 24I1I2F11        | 263                     | 308                   | 16 ± 7                                          |
|                 | 24I1I1H1         | 234                     |                       | 17 ± 7                                          |
| ΔcbbL + ΔnccrbbS-cbbQ-1 | 24I1I2G1 | 222¹                   | 245³                  | 9 ± 2                                           |
| ΔcbbL ΔcbbQ-1   | 24I1I6H6         | 10                      | 272                   | 2 ± 2                                           |
|                 | 24I1I5G11        | 43                      |                       | 6 ± 1                                           |
| ΔcbbL ΔcbbO-1   | 24I1I6G12        | 367                     |                       | 17 ± 3                                          |
|                 | 24I1I5G9         | 416                     | 777                   | 19 ± 4                                          |
|                 | 24I1I5D3         | 618                     |                       | 13 ± 1                                          |

¹ insertion positions in non-coding regions given in nucleotides
² total length of non-coding region given in nucleotides