

读书报告

汇报人：赵卓丽 汇报时间：2018-8-18



ORIGINAL ARTICLE

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

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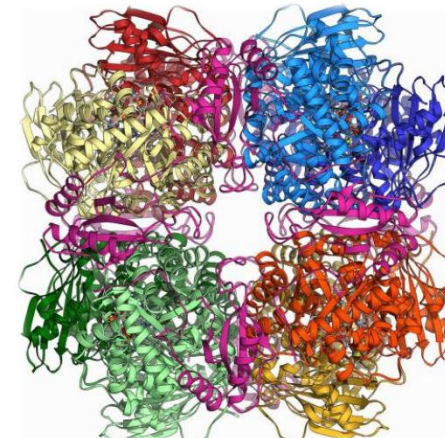
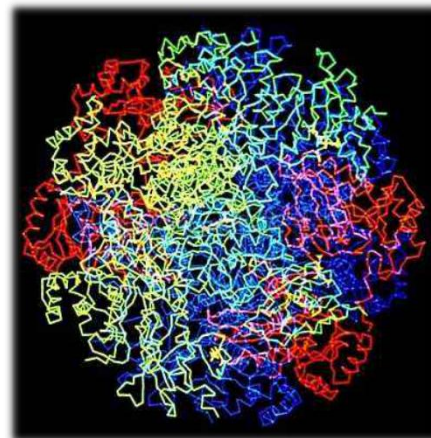
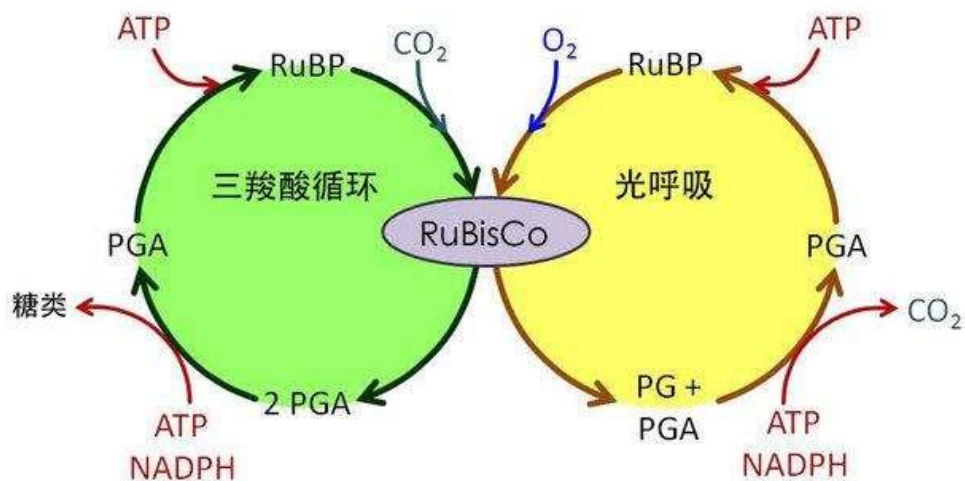
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IF=9.520

基于功能筛选从宏基因组中寻找RubisCO活性克隆：影响RubisCO活性的新酶

RubisCO

Ribulose-1,5-bisphosphate carboxylase/oxygenase 1,5-二磷酸核酮糖羧化酶/加氧酶



是光合作用C₃碳反应中重要的羧化酶，也是光呼吸中不可缺少的加氧酶。

EC 4.1.1.39，分子量约为53kD，由8个大亚基和8个小亚基组成，是光合作用中决定碳同化速率的关键酶。光合作用中卡尔文循环里催化第一个主要的碳固定反应，将大气中游离的二氧化碳转化为生物体内储能分子，比如蔗糖分子。



01 Introduction

02 Materials and methods

03 Results and discussion

04 Conclusion



01

Introduction

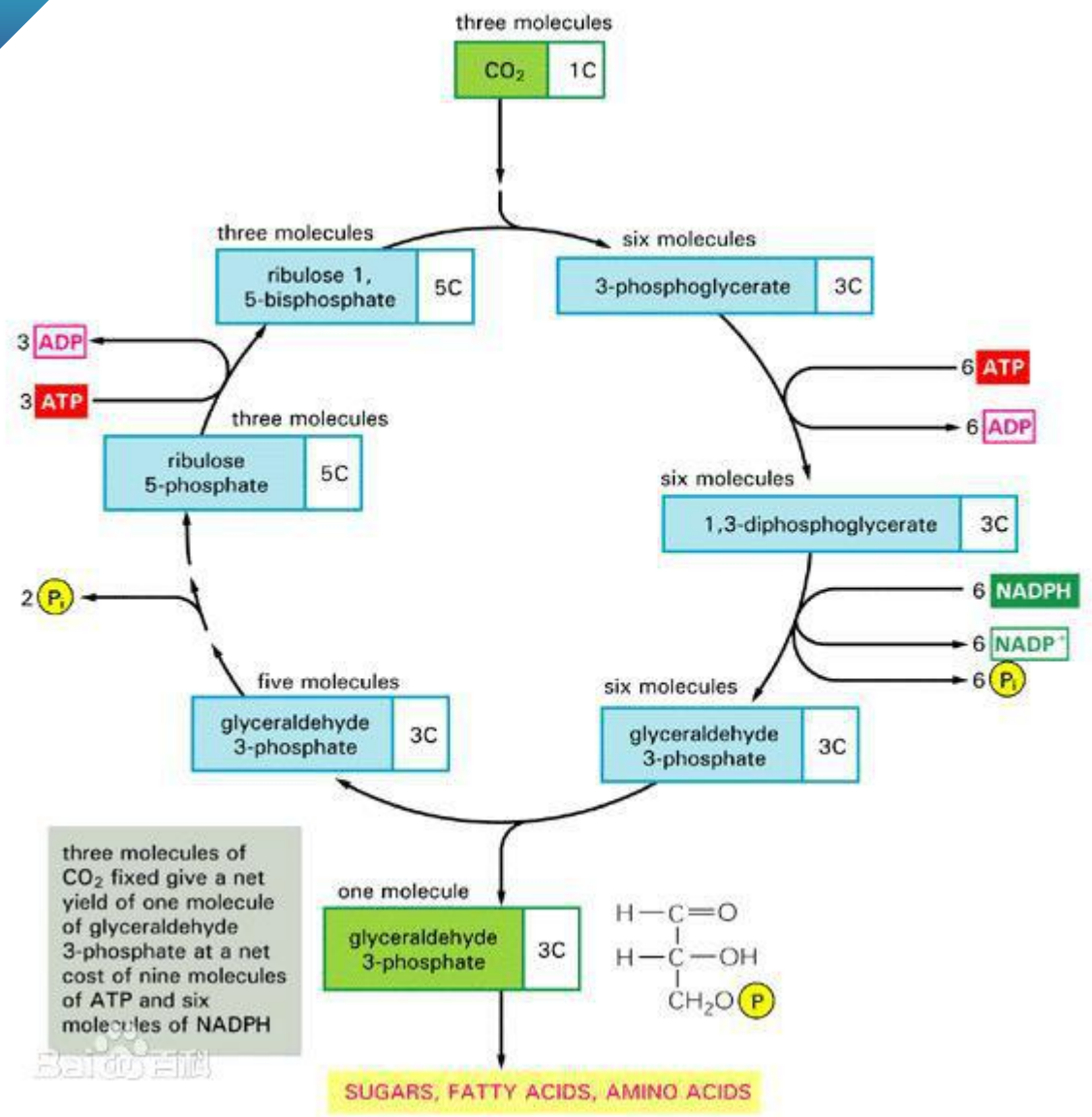


自养CO₂固定途径中，Calvin 循环占地球净初级产量的大部分。（每年105~109吨，> 99.5%）

RubisCO催化核酮糖-1, 5-二磷酸酯（RuBP）羧化成3-磷酸甘油酸。

RubisCO有四种结构，已知的Form I（CbbLS）和Form II（CbbM），在经典的自养Calvin 循环中起作用。

Calvin循环和RubisCO对全球初级生产具有根本重要性，更好地了解RubisCO的进化及其多样性至关重要。



Baidu Fint



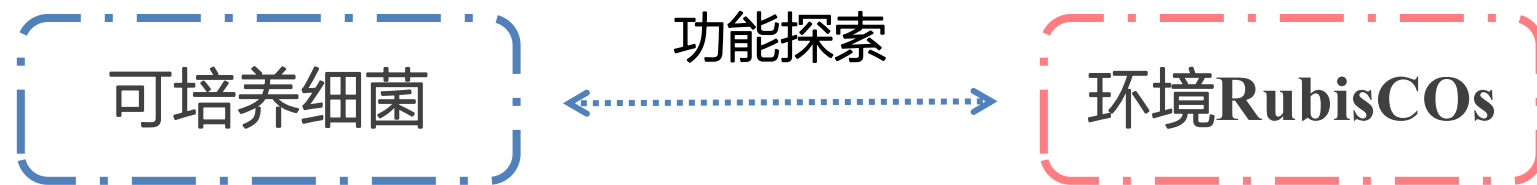
Why RubisCO?

RubisCO的**调节机制**、如何**装配与激活**? 在*Hydrogenovibrio marinus*基因组中, CbbR1和CbbRm调节蛋白分别在RubisCO结构基因*cbbLS-1*和*cbbM*的上游编码, 并且已被证明可调节邻近的RubisCO基因表达。(Toyoda et al., 2005)

原核生物CbbQ和CbbO在激活RubisCO中的作用仍然是神秘的。他们可能参与翻译后的RubisCO活化, 有时不影响RubisCO活性。(Hayashi et al., 1997, 1999; Schwedock et al., 2004)



Why Function-based screen?



序列搜索：仅可检测到与已知基因具有显著相似性的序列，存在固有限制。

无法涉及来自未培养生物体的RubisCO基因簇(>99%)，环境RubisCOs是否起作用以及如何被调节和激活，仍然没有答案。

基于**功能筛选**的方法可以从未培养生物中寻找RubisCO活性酶。

在深海热液中构建宏基因组fosmid文库，筛选克隆的重组RubisCO活性。进一步分析其宏基因组DNA，阐明侧翼基因和所得基因产物的作用，这些基因产物是表达完全功能性RubisCO酶的基础。



02

Materials and methods



1. Growth of TH-55 and cloning of RubisCO genes

Thiomicrospira crunogena TH-55 DSMZ No.12353 硫微螺菌属



RubisCO form I (cbbLS) and form II (cbbM) encoding gene regions
(incorporating at least 500 bp upstream to include possible promoter regions)

2. Sampling of hydrothermal fluids (热液)

Nibelungen大西洋海底 (2915m) 火山口与海水界面处的热流体
90-120°C pH=5.6 22 mM hydrogen 8 mM sulfide 56 mM oxygen

0.2毫米的聚碳酸酯过滤器过滤200毫升的热液流体, -20°C保存



3. Construction of fosmid libraries

CopyControl Fosmid Library Production Kit

one genome library with DNA material from TH-55

1152 fosmid clones 29 kb \pm 5 kb

metagenomic library with the hydrothermal fluids from Nibelungen

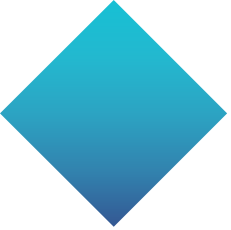
1056 fosmid clones 37 kb \pm 2.1 kb

4. Transposon mutagenesis (转座子诱变)

EZ-Tn5TM<KAN-2> insertion kit

one with the fosmid clone 71C2 (35.2 kb) carrying the RubisCO gene cluster (cbbQOM lysR2 lysR1 cbbLSQO) and flanking DNA regions

one with the fosmid subclone 71C2II, comprising only the RubisCO gene cluster (cbbQOM lysR2 lysR1 cbbLSQO) (13 kb)



5. Preparation of crude extracts

6. RubisCO activity assay (Rulp→3-PGA HPLC定量)

7. Transcription experiments with TH-55 and transposon clones

8. Complementation experiments

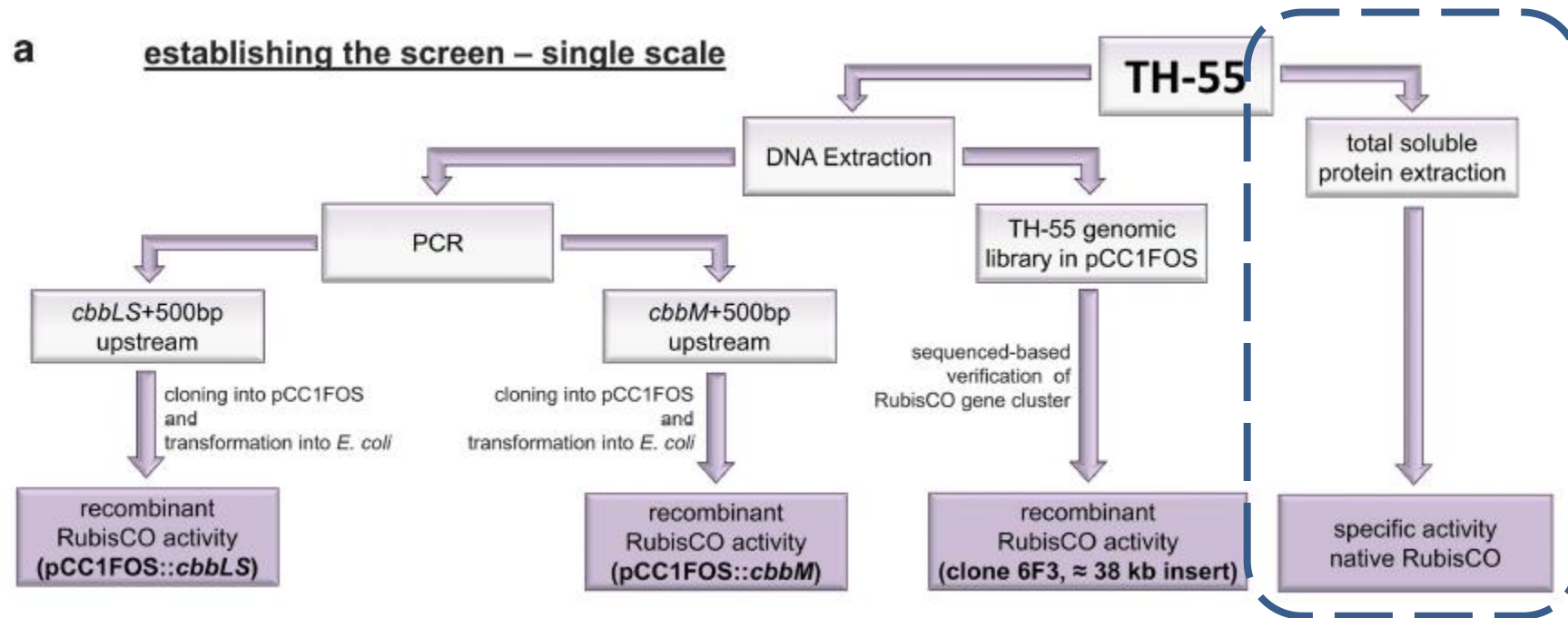
9. Sequence access



03

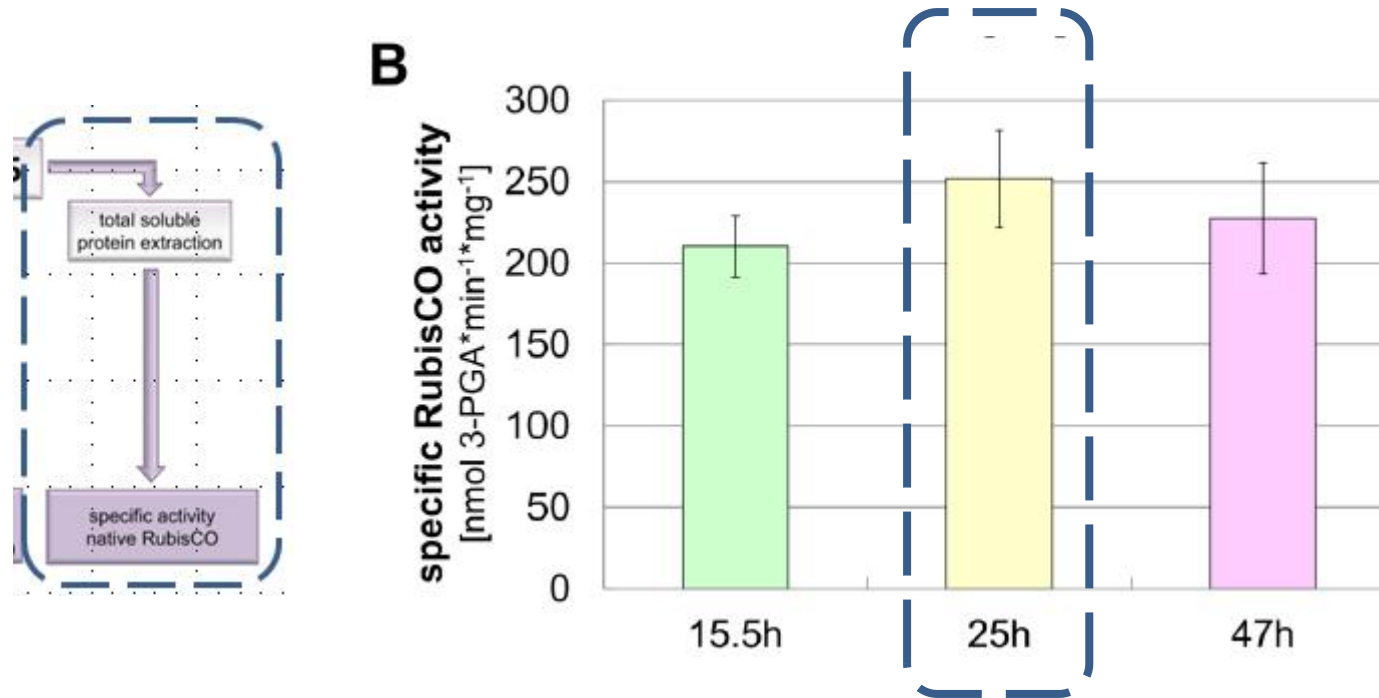
Results and discussion

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



pCC1FOS::cbbLS : 编码RubisCO form I的小、大亚基； pCC1FOS :: cbbM: 编码RubisCO form II的大亚基；
 pCC1FOS fosmid clone 6F3: 插入38.1kb DNA片段，包括RubisCO基因簇 (*cbbQOM lysR2 lysR1 cbbLSQO*)
 和侧翼区 (25.1kb)。

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

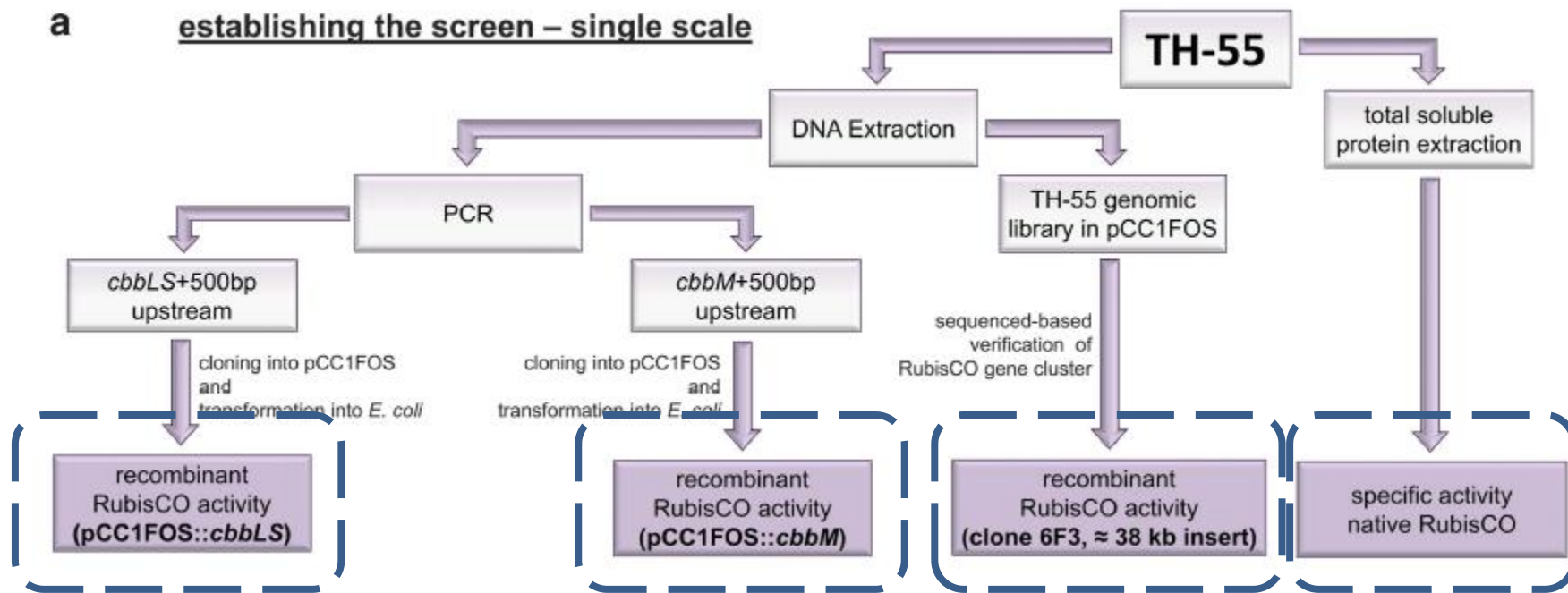


指数生长期: $252 \pm 19 \text{ nmol 3-PGA min}^{-1} \text{ mg}^{-1}$

TH-55基因组fosmid克隆6F3的特异性RubisCO活性高1.8倍 ($455 \pm 30 \text{ nmol 3-PGA min}^{-1} \text{ mg}^{-1}$)

原因: pCC1FOS载体高拷贝诱导, 因此表达的RubisCO比天然宿主TH-55中的更多。

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



$266 \pm 23 \text{ nmol 3-PGA min}^{-1} \text{ mg}^{-1}$ $55 \pm 9 \text{ nmol 3-PGA min}^{-1} \text{ mg}^{-1}$

59%

+

12%

≠

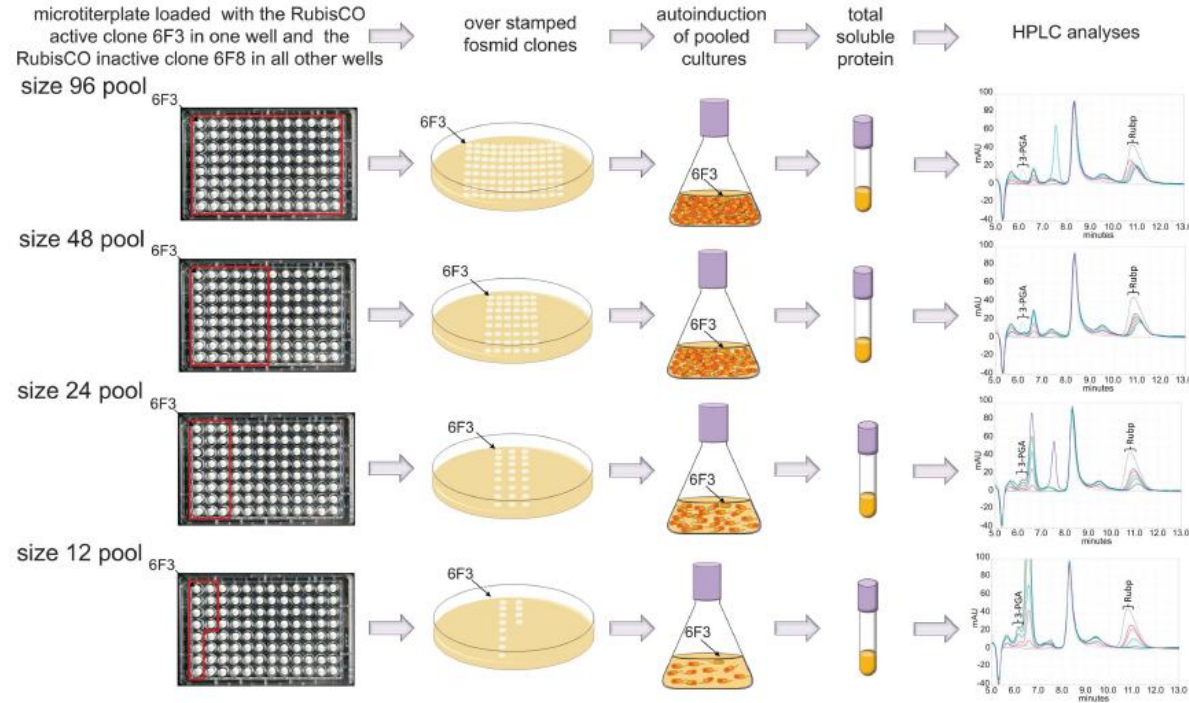
1

56%

相对于O₂, RubisCO Form I对CO₂特异性更高,比RubisCO Form II具有更高的耐氧性。(Tabita, 1999), 基因组片段上存在除结构基因之外的基因,有助于表达完全功能性的RubisCO,活性更高。因此完全有氧条件下进行重组的RubisCO活性测定, Form I的RubisCO活性远高于Form II。

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

b upscaling the screen – metagenomic scale



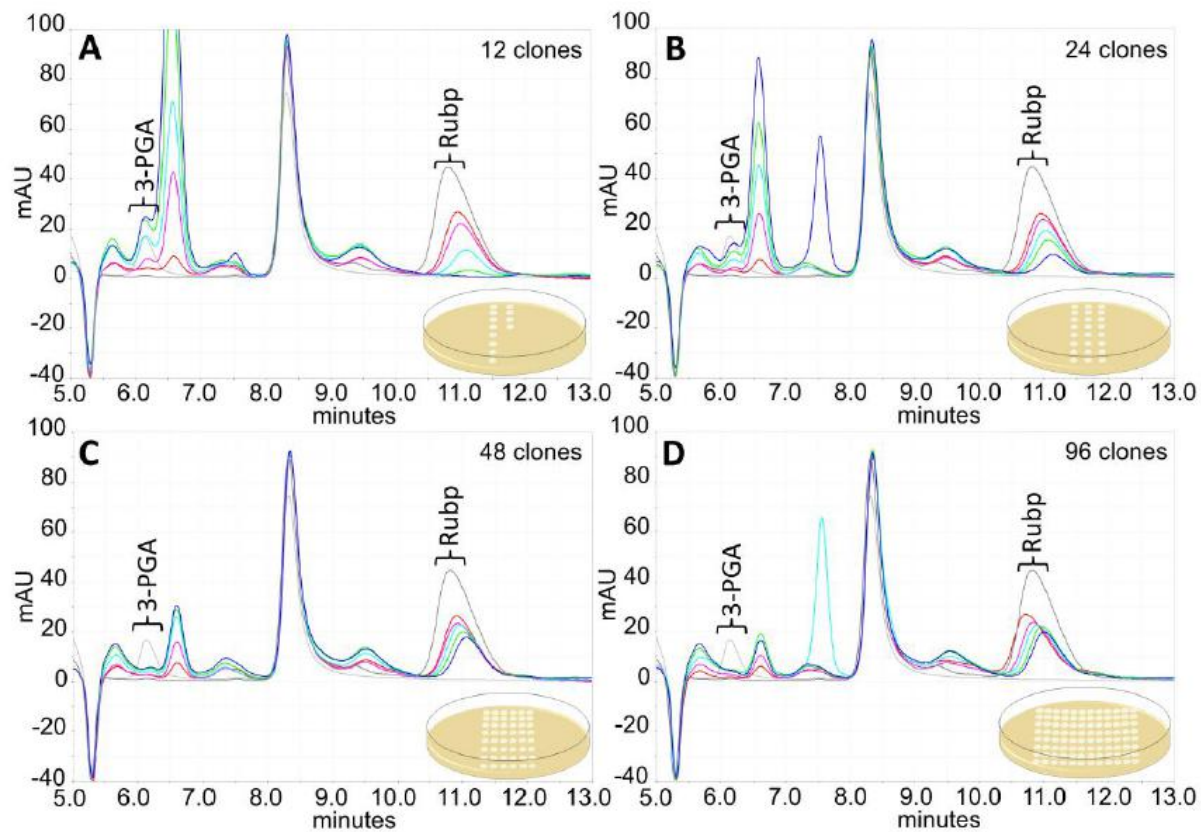
LB平板（12.5mg/ml氯霉素），37°C，过夜。

10ml LB（12.5mg/ml氯霉素）淹没fosmid clone，
200ml细胞悬浮液作接种液。

HPLC分析

determine whether the RubisCO activity of one active clone among 11, 23, 47 and 95 inactive clones is still detectable.

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

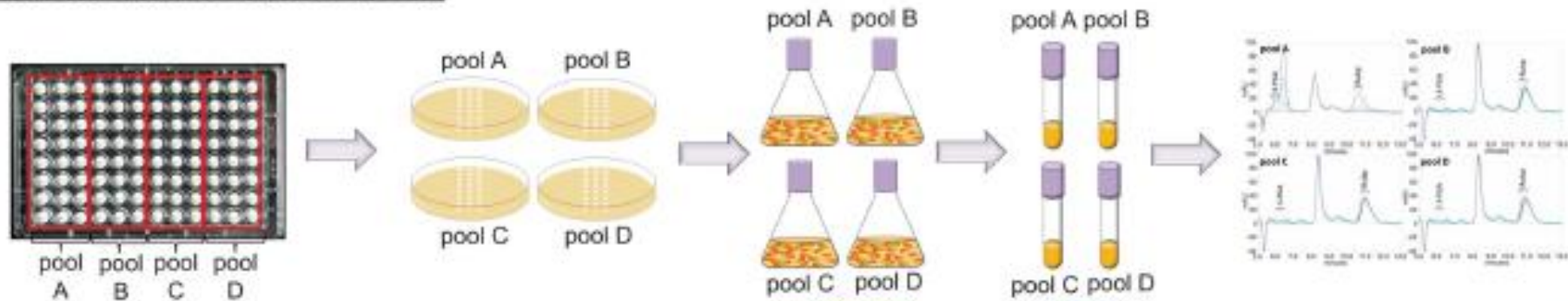


与其他11,23,47或95个非活性克隆一起进行测试，仍然可以识别。只有当不超过24个克隆合并用于RubisCO活性测量时，才能保证从所有克隆中清楚地检测到一个活性克隆中的RubisCO活性。

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

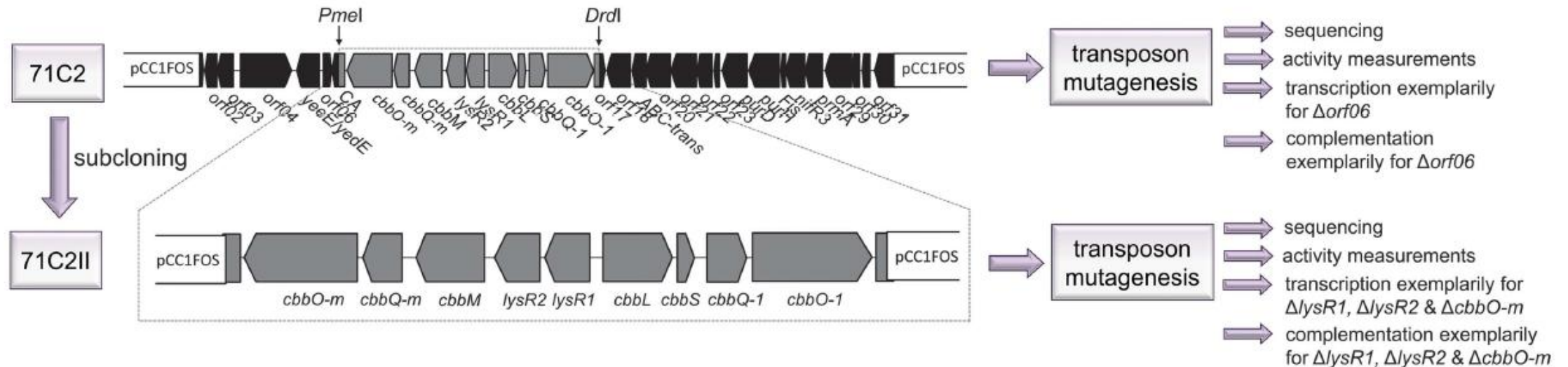
C using the metagenomic screen

size 24 pool



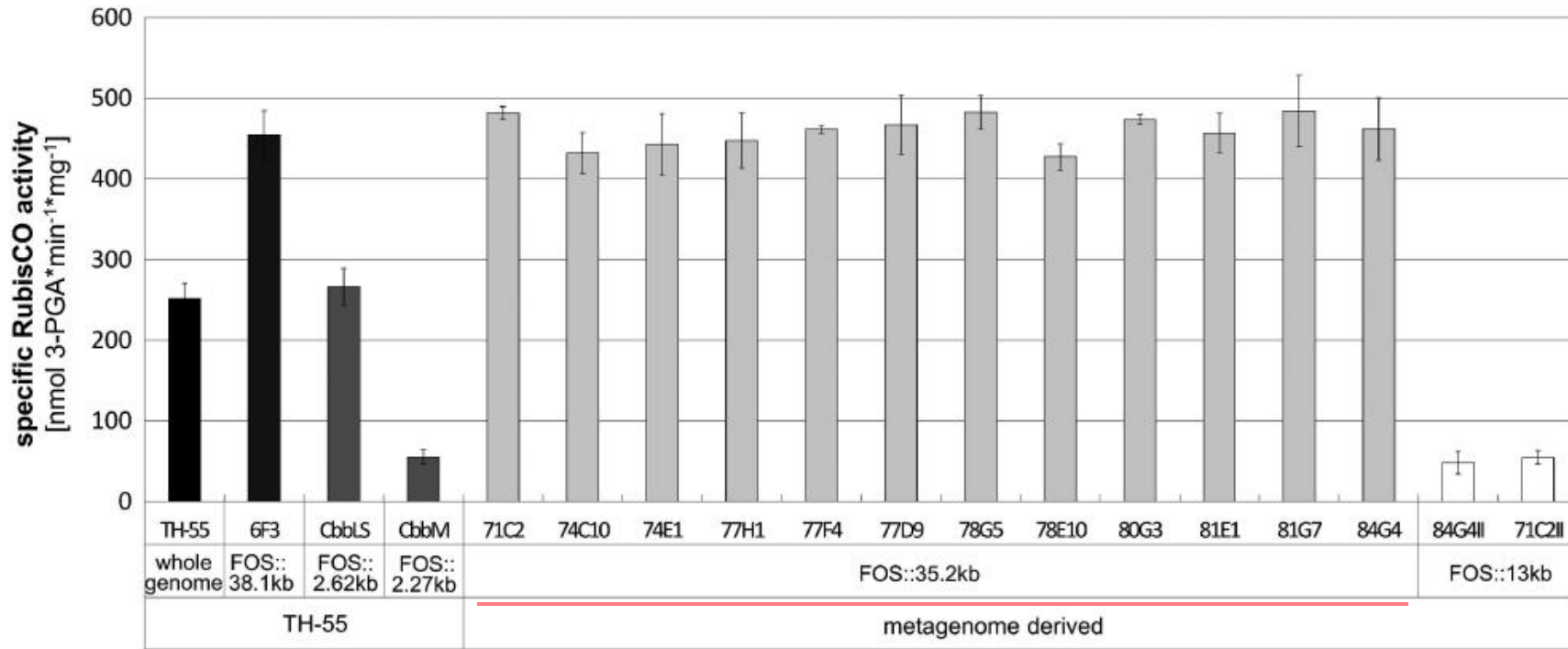
positive tested pools were disrupted and broken down into pools of 2 x 12, 3 x 4 and 4 x 1 respectively, to identify the RubisCO active clone

d investigating metagenome derived RubisCOs



Recovering RubisCO active fosmid clones from metagenomic hydrothermal fluid samples

a



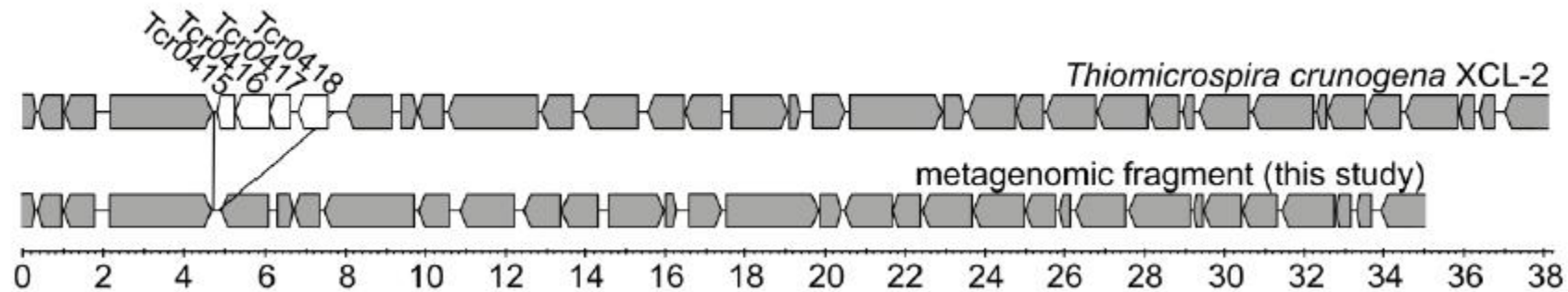
12 Active fosmid clones

$427 \pm 16 - 484 \pm 44 \text{ nmol 3-PGA min}^{-1} \text{ mg}^{-1}$

> 35.2kb DNA插入物 具有相同的限制性模式

three metagenomic fragments including the RubisCO gene cluster for all 12 clones demonstrated a 100% DNA identity and resembled genes from *T. crunogena* XCL-2 (90–96%) (Scott et al., 2006).

Recovering RubisCO active fosmid clones from metagenomic hydrothermal fluid samples



一个代表性克隆的测序表明，DNA插入物上编码的基因遵循来自XCL-2 (Tcr0411-Tcr0446) 的同源基因的排列，但缺失了4个基因 (Tcr0415-Tcr0418)。

Tcr0416-编码ApbE样脂蛋白

Tcr0417-具有FMN结合结构域的蛋白质

Tcr0415, Tcr0418-两个功能未知的蛋白质

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

metagenomic fosmid clones with RubisCO gene cluster (13 kb) + 22.2 kb flanking

DNA	
71C2	481 ± 8
74E1	443 ± 38
74C10	432 ± 25
77H1	448 ± 34
77F4	461 ± 4
77D9	467 ± 37
78G5	483 ± 21
78E10	427 ± 16
80G3	473 ± 6
81G7	484 ± 44
81E1	457 ± 24
84G4	462 ± 39

含有侧翼序列 活性约为9倍

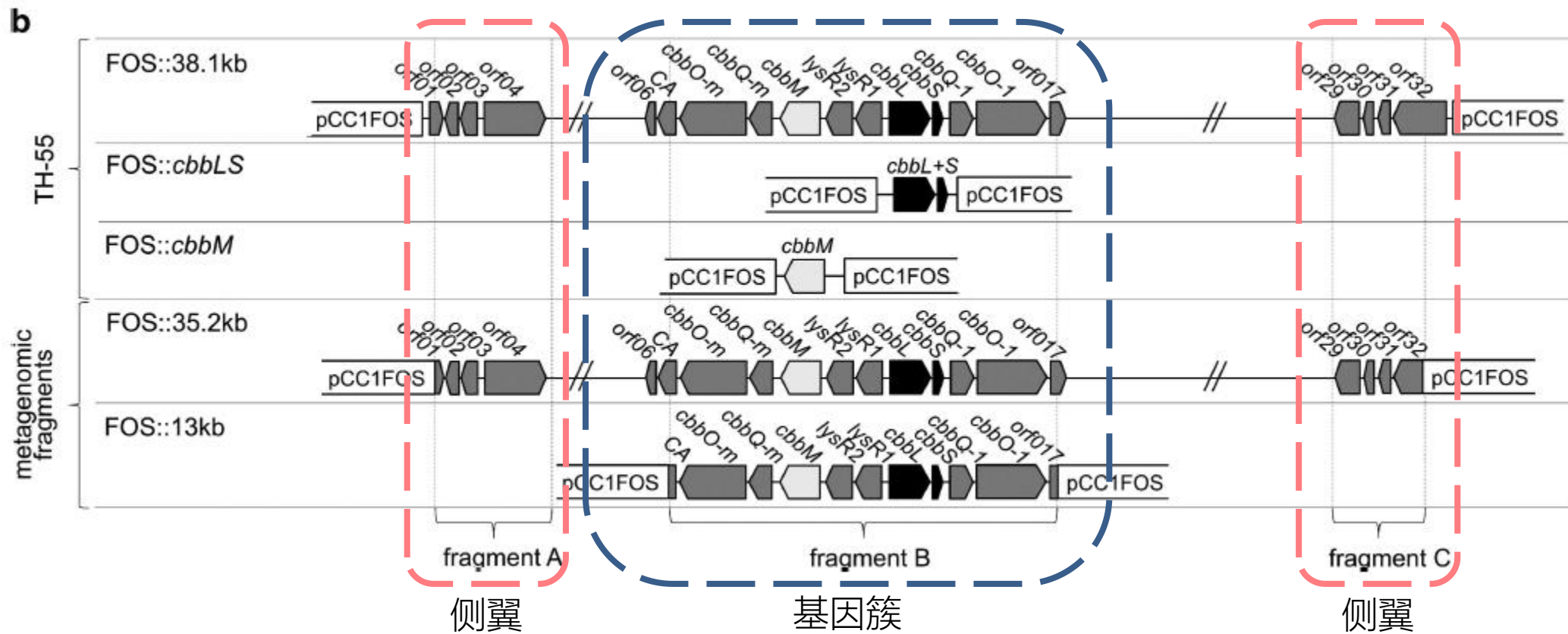
RubisCO基因簇的上游和/或下游基因有助于表达完全功能性的RubisCO

fosmid subclones only containing the RubisCO gene cluster (13 kb)

(<i>cbbOQM lysR2 lysR1 cbbLSOO</i>)	
71C2II	55 ± 8
84G4II	48 ± 14

为了研究完全功能性RubisCO可能需要除CbbLS和CbbM之外的其他酶，亚克隆了两个 metagenomic fosmid clones (71C2, 84G4) 的RubisCO基因簇 (13kb) 并测量了它们的特异性 RubisCO活性。

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



两个转座子突变体文库：揭示RubisCO基因簇内外的哪些基因和相应产物作用于完全功能性RubisCO

一个具有fosmid clone 71C2（整个宏基因组DNA片段包括RubisCO基因簇和侧翼DNA区域：35.2kb）

和一个具有fosmid克隆71C2II（仅携带RubisCO基因簇：13kb）

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

Supplementary Table S4. Insertion positions of tested transposon clones with 35.2 kb inserts.

ORF	clone number	insertion position [aa]	total orf length [aa]	specific RubisCO activity [nmol 3-PGA ⁺ min ⁻¹ mg ⁻¹]
TH-55	/	/	/	252 ± 19
pCC1FOS::35.2 kb ¹	/	/	/	481 ± 8
<i>orf02</i>	11	28	206	470 ± 12
<i>orf03</i>	81	52	270	433 ± 14
<i>orf04</i>	86	246	491 ± 28	491 ± 28
	88	290	854	450 ± 30
	16	499	451 ± 0.1	451 ± 0.1
<i>ncr orf04-05</i>	236	448 ²	450 ³	384 ± 3
<i>orf05</i>	58	237	385	351 ± 28
<i>ncr orf05-06</i>	231	34 ²	200 ³	311 ± 28
<i>orf06</i>	96	6	135	81 ± 20
<i>orf07</i>	138	139	218	522 ± 27
	207	212	437 ± 40	437 ± 40
<i>orf12 (lysR1)</i>	169	220	308	432 ± 27
	161	285	409 ± 56	409 ± 56
<i>orf14 (cbbS)</i>	38	111	116	418 ± 23
<i>orf15 (cbbQ-1)</i>	3	180	272	455 ± 19
<i>orf16 (cbbO-1)</i>	8	62	511 ± 21	511 ± 21
	4	157	482 ± 7	482 ± 7
<i>orf17</i>	100	143	177	460 ± 1
<i>orf18</i>	44	329	408	450 ± 1
<i>orf19</i>	117	98	233	447 ± 9
<i>orf20</i>	10	128	425	435 ± 13
<i>orf21</i>	5	245	431	508 ± 10
<i>orf22</i>	46	18	260	463 ± 37
<i>orf23</i>	89	52	91	380 ± 25
<i>orf24</i>	68	10	430	405 ± 11
<i>orf25</i>	180	397	520	444 ± 10
<i>orf26</i>	69	87	87	449 ± 8
<i>orf27</i>	179	22	320	459 ± 18
	45	151	438 ± 10	438 ± 10
<i>orf28</i>	91	147	294	401 ± 9
<i>orf29</i>	77	21	449	444 ± 32

¹ DNA fragment from TH-55 genomic library containing RubisCO gene cluster and flanking DNA

² insertion positions in non-coding regions (ncr) given in nucleotides

³ total length of non-coding region (ncr) given in nucleotides

Supplementary Table S5. Insertion positions of tested transposon clones with 13 kb inserts.

ORF	clone number	insertion position [aa]	total orf length [aa]	specific RubisCO activity [nmol 3-PGA ⁺ min ⁻¹ mg ⁻¹]
TH-55	/	/	/	252 ± 19
pCC1FOS::13 kb ¹	/	/	/	55 ± 8
<i>orf08 (cbbO-m)</i>	17II	189	757	40 ± 5
	19II	333	41 ± 9	41 ± 9
	14II	569	37 ± 2	37 ± 2
	21II	578	32 ± 13	32 ± 13
<i>orf09 (cbbQ-m)</i>	12II	46	55 ± 5	55 ± 5
	8II	94	266	59 ± 3
	11II	109	63 ± 11	63 ± 11
<i>orf10 (cbbM)</i>	22II	171	459	338 ± 7
<i>orf11 (lysR2)</i>	149II	271	314	102 ± 19
<i>orf12 (lysR1)</i>	6II	264	308	31 ± 12
<i>orf13 (cbbL)</i>	24II	41	472	8 ± 5
<i>orf15 (cbbQ-1)</i>	23II	229	272	113 ± 15
<i>ncr orf15-16</i>	20II	85 ²	105 ³	66 ± 9
<i>orf16 (cbbO-1)</i>	4II	303	777	63 ± 7

¹ DNA fragment from TH-55 genomic library containing only RubisCO gene cluster

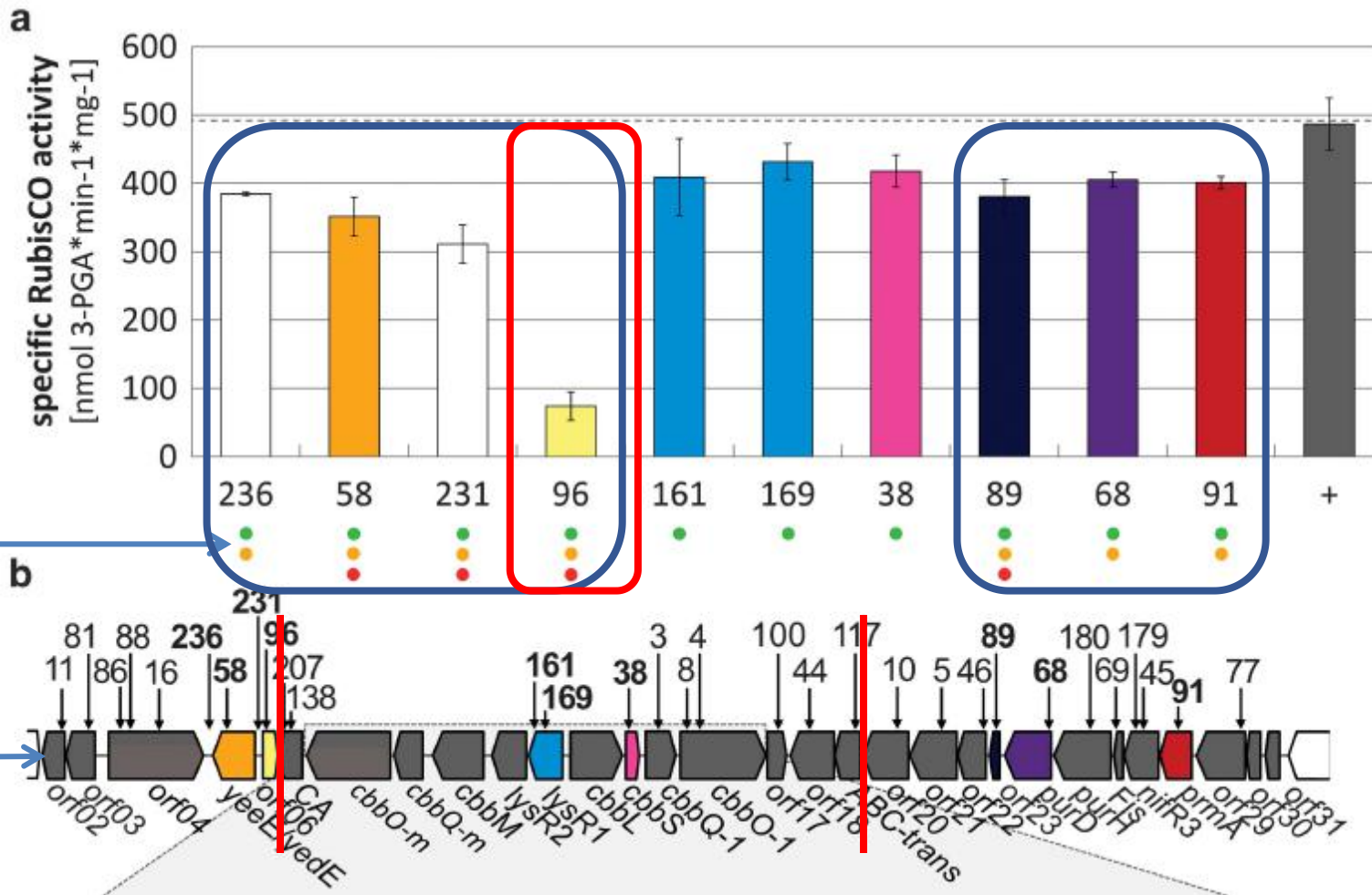
² insertion positions in non-coding regions (ncr) given in nucleotides

³ total length of non-coding region (ncr) given in nucleotides

用靶向插入转座酶的引物对384个转座子克隆进行测序以确定插入位置并测量45个选择的转座子克隆的RubisCO活性。

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

显著差异水平
 绿色-0.05 (95%)
 黄色-0.01 (99%)
 红色-0.001 (99.9%)



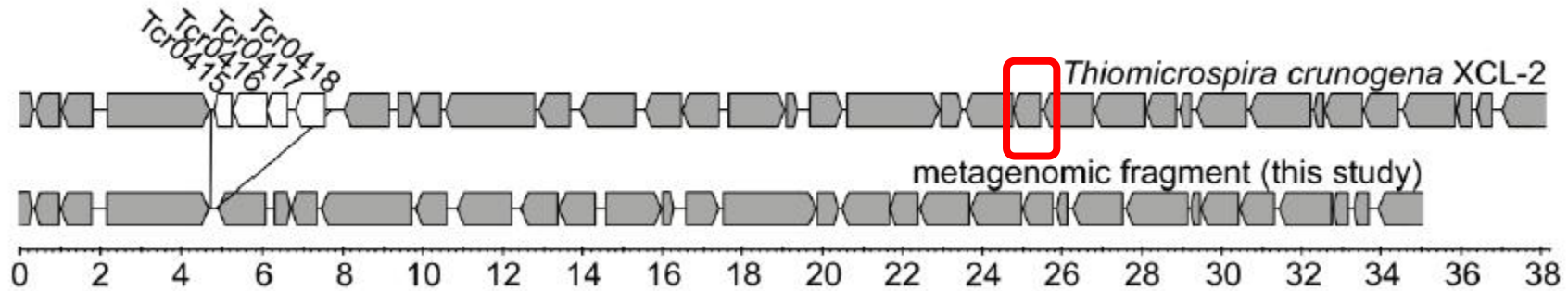
RubisCO基因簇外的25个转座子克隆中，7个克隆的RubisCO活性显著低于完整的71C2克隆。

Clone 96缺失表现出显著的活性丧失。该活性与13kb克隆测得的降低的RubisCO活性相当，后者具有RubisCO基因簇但缺少侧翼DNA区域。

衍生自71C2的完整35.2kb插入片段的转座子克隆（显著差异）的特异性RubisCO活性和示意性基因排列

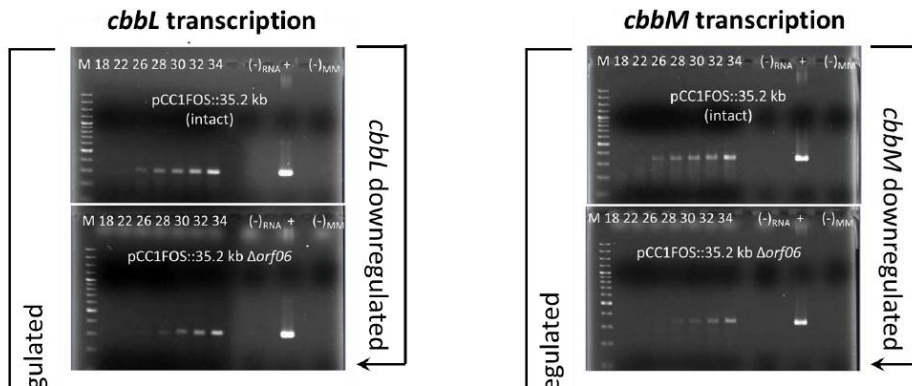
The effect of gene deletions outside the RubisCO gene cluster on RubisCO activity

Clone 96 *orf06* 135 hypothetical protein Tcr0420 97 YP_390690



Clone 96缺失的基因 (*orf06*) 与XCL-2的基因的Tcr0420类似。(97% aa相似性)

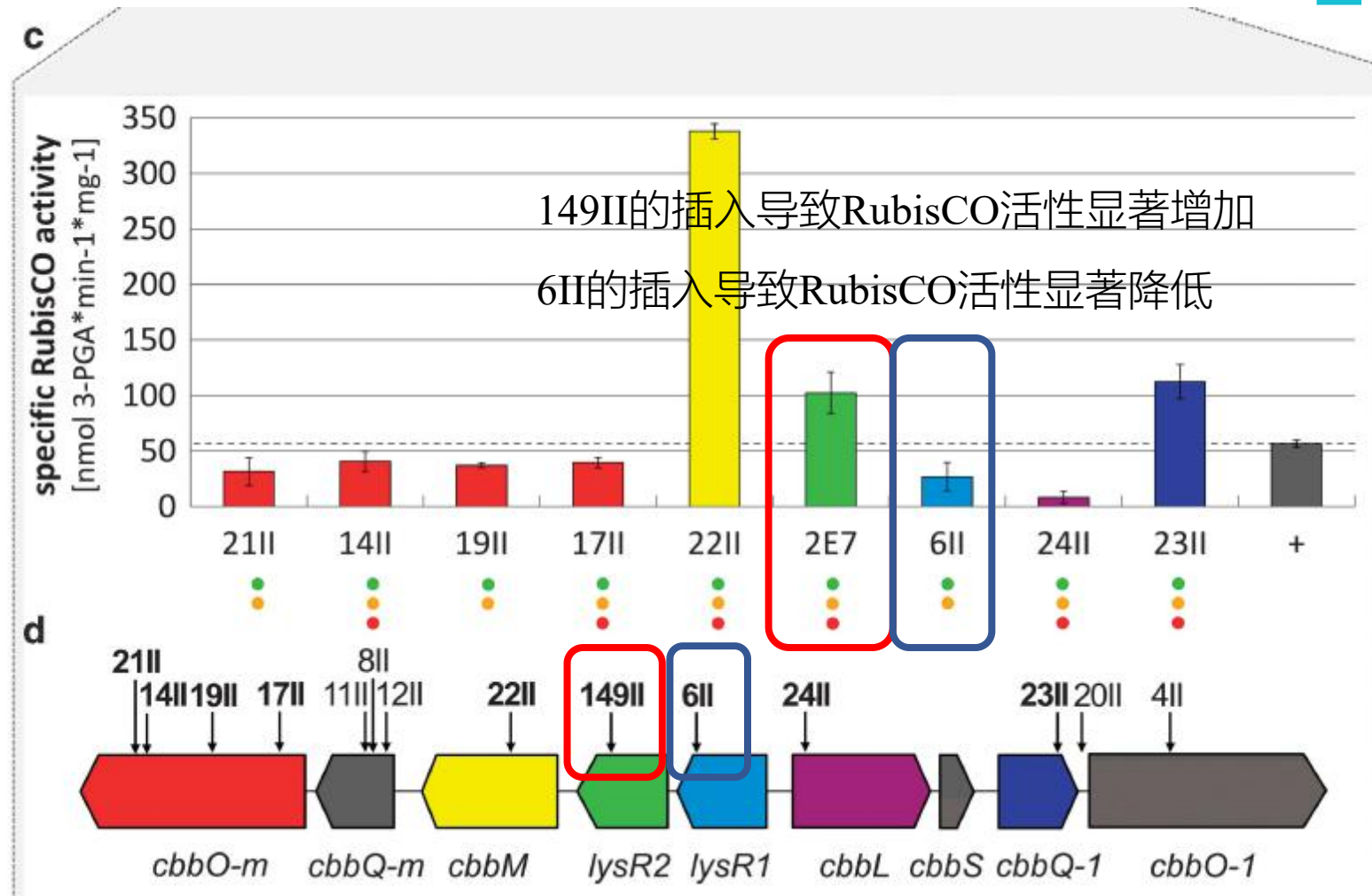
与*orf06*基因产物具有已知功能的最接近的相关蛋白是小单孢菌 (*Micromonospora aurantiaca* ATCC 27029) 的ArsR调节蛋白(30% aa同一性), 其作为金属感应转录抑制因子起作用。



转录实验证明, 相对于具有完整*orf06*的宏基因组片段, *cbbL*与*cbbM*基因的转录均下调。

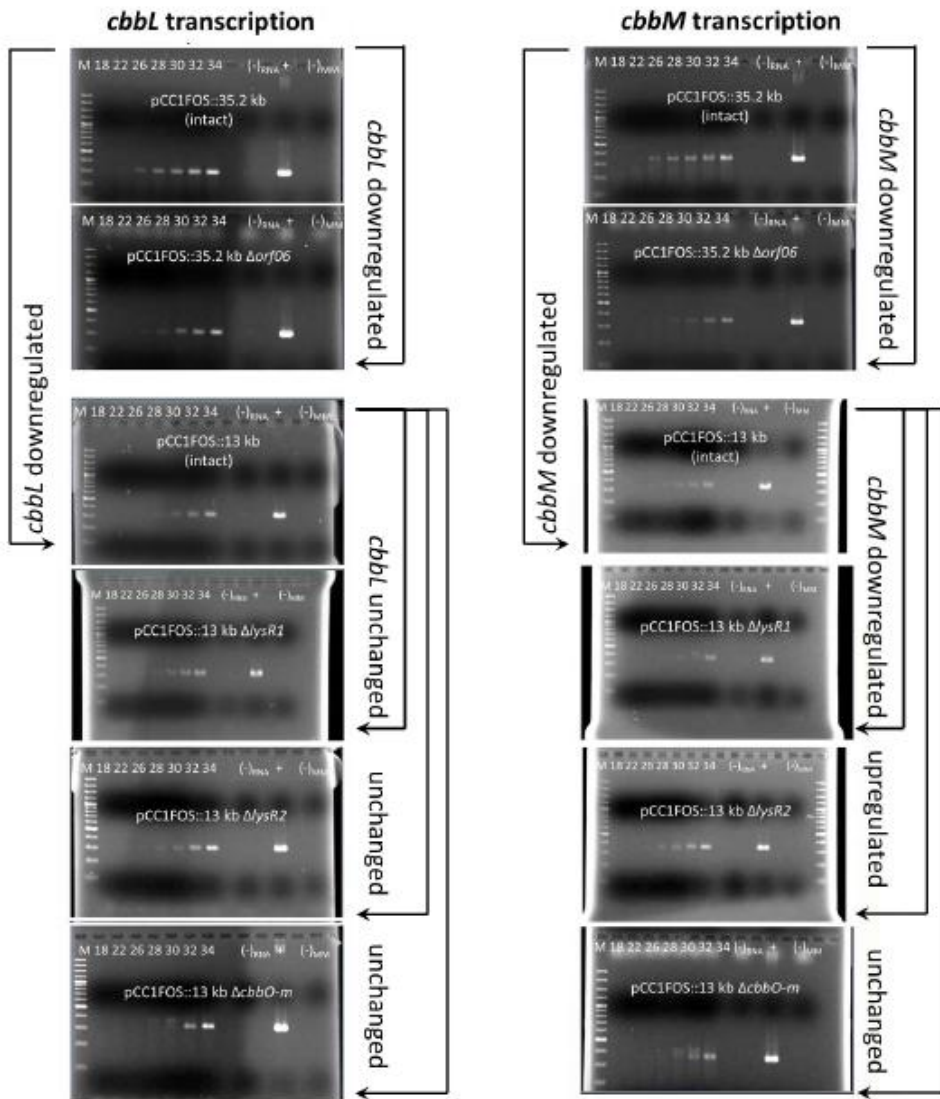
Orf06基因产物可能参与直接或间接调节结构RubisCO form I, form II。

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



衍生自71C2II的13kb插入片段的转座子克隆（显著差异）的特异性RubisCO活性和示意性基因排列

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



转座子克隆149II ($\Delta lysR2$) 的转录实验, 相对于各自完整的宏基因组片段, *cbbL*的转录水平保持不变, *cbbM*的转录被上调。

Orf11作为阻遏物作用于*cbbM*转录

转座子克隆6II ($\Delta lysR1$) 的转录实验, 相对于各自完整的宏基因组片段, *cbbL*的转录水平没有变化, *cbbM*的转录被下调。

orf12 (*LysR1*同系物) 激活*cbbM*转录

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*.

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

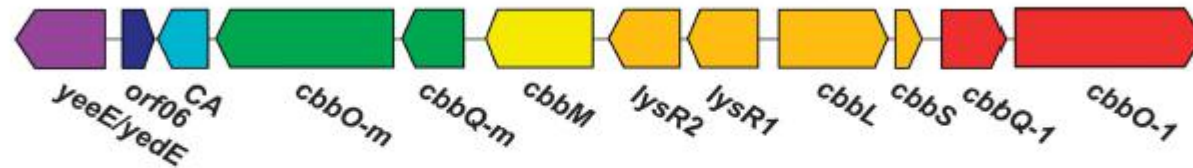


Figure 4 Transcription of RubisCO encoding genes and flanking gene regions from TH-55. Genes are indicated as arrows. Co-transcribed genes are displayed in the same color. Abbreviations are listed in Supplementary Table S7.

TH-55的转录实验表明 $lysR2$ 、 $lysR1$ 和 $cbbLS$ 是共转录的。由于 $lysR2$ 和 $lysR1$ 基因与 $cbbLS$ 并列，因此只有 $lysR2$ 和 $lysR1$ 或 $cbbLS$ 基因产物才会产生功能性酶。



04

Conclusion



通过功能筛选发现新型酶，在可培养的替代宿主中异源表达来自未知生物的环境DNA存在固有限制，并且命中率低。只有30-40%的外源DNA在*E. coli*中成功表达。重组蛋白的表达面临的一些问题：识别内在启动子和相关因子，分歧密码子使用，翻译，正确折叠，成熟过程和酶的激活等。

由于使用宏基因组DNA大片段（fosmid克隆平均插入大小为 37 ± 2.1 kb），可以预期靶基因的启动子和核糖体结合位点位于宏基因组片段上。

尽管功能筛选可能存在一些限制，但它可以成功用于从宏基因组文库中鉴定RubisCO活性fosmid克隆，用来阐明侧翼基因和各种酶对功能性RubisCO的重要性。

功能筛选避免了耗费时间的自养微生物的纯培养，规避目前的培养技术的缺陷，以及测序技术存在的固有偏差。它使我们能够利用环境中可用的巨大宏基因组资源，从而发现之前未发现的与RubisCO活性相关的物种。



敬请批评指正!