

Supporting Information

Baani and Liesack 10.1073/pnas.0702643105

SI Methods

Generation of *pmo* Mutants. Suicide vectors used for targeted deletion of *pmo* operons in *Methylocystis* sp. strain SC2. The *pmo* suicide vectors were generated as follows:

(I) The regions flanking the site into which the Kan^r gene cassette would be inserted were amplified in the first round of PCR from the target *pmo* operons by using sequence-specific primers (see Scheme 1). The pU/pN and pC/pD primers [supporting information (SI) Table S1] were designed to generate restriction sites at the 5' (HindIII) and 3' (AdeI) termini of the amplified fragments. The primer sets pU1/pN1 and pC1/pD1 targeted regions within *pmoC1* and *pmoB1*, respectively. This primer positioning enabled simultaneous deletion of both copies of *pmoCAB1*. To delete specifically *pmoCAB2*, the target positions of the primer sets pU2/pN2 and pC2/pD2 differed from those of the primer sets pU1/pN1 and pC1/pD1, respectively. The Kan^r gene cassette was amplified by PCR by using conditions that introduced AdeI restriction sites at both termini.

(II) The gene disruption construct for deletion of either *pmoCAB1* or *pmoCAB2* was generated in a second round of PCR (fusion PCR) by using the Kan^r cassette plus the upstream and downstream flanking regions. The AdeI restriction sites inserted into the flanking regions during PCR differed in the variable sequence motif (CACNNGTG), so that the AdeI restriction sites generated with primers pN1 and pN2 were complementary only to the 5' (AdeI) termini of the Kan^r cassette, whereas the AdeI restriction sites generated with primers pC1 and pC2 were complementary only to the 3' (AdeI) termini of the Kan^r cassette (see Table S1).

(III) The constructs were inserted into the unique HindIII site of pUC18 and cloned into *Escherichia coli* JM109 (Promega). pUC18 cannot replicate in *Methylocystis* sp. strain SC2; thus, the plasmid is a suicide vector for this strain, and the constructs pUCP1 and pUCP3 (Table S2) introduced into wild-type strain SC2 by electroporation induced deletion of the target *pmo* operon(s) by homologous recombination. The construct pUCP4 was introduced into mutant strain SC2-P2 by electroporation. Electrocompetent strain SC2 cells were transformed by using a Gene Pulser (Bio-Rad) with the following parameters: 2.5 kV, 400 Ω , and 25 μ F in a 2-mm gap cuvette. To eliminate plasmid transformants, generated mutants were screened for their sensitivity to ampicillin. Resistance to kanamycin or, in the case of mutant SC2-P4, resistance to tetracycline, but sensitivity to ampicillin, indicated deletion of the target *pmo* operon(s) by double-homologous recombination. The double-homologous recombination event was verified by diagnostic PCR (data not shown) and Southern hybridization (Fig. S1).

Fusion PCR was used to construct mutant strains SC2-P1, SC2-P2, SC2-P3, and SC2-P4. The Kan^r gene cassette was used to construct mutant strains SC2-P1 ($\Delta pmoCAB1a::kan$),

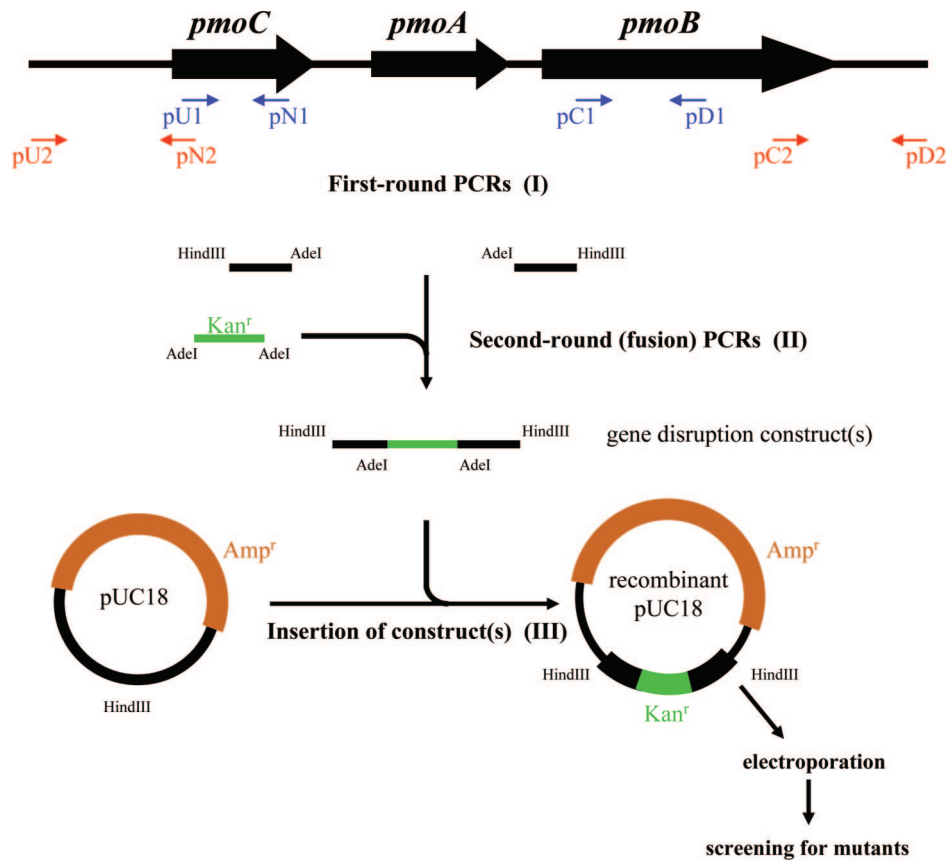
SC2-P2 ($\Delta pmoCAB1a::kan$, $\Delta pmoCAB1b::kan$), and SC2-P3 ($\Delta pmoCAB2::kan$). The Tet^r gene cassette was used to delete *pmoCAB2* in mutant strain SC2-P2, thereby constructing the *pmo* null mutant SC2-P4 (Table S2).

Methane Oxidation Kinetics [$K_{m(app)}$, $V_{max(app)}$, a_s^0]. The apparent kinetic parameters of conventional pMMO1 were determined with the mutant strain SC2-P3, and parameters of the pMMO2 were determined with wild-type strain SC2 and mutant SC2-P2 by using an experimental design described in ref. 1.

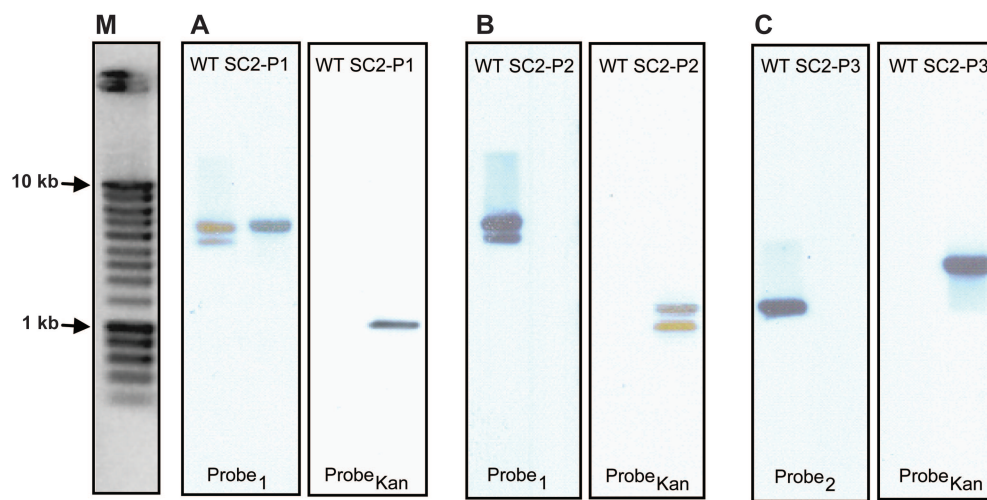
For measurement of $V_{max(app)}$, cultures were pregrown at different methane concentrations. Cultures of mutant SC2-P3 were grown under a headspace of either 1,000 ppmv or 20% (vol/vol) CH₄. When cells were grown with 1,000 ppmv, the methane concentration in the headspace was continuously re-installed, so that it never decreased to <700 ppmv. Cells of wild-type strain SC2 and mutant SC2-P2 were cultured for 4 weeks with methane concentrations constantly <500 ppmv, thereby avoiding expression of the conventional *pmoCAB1* in wild-type strain SC2. The cell density of the cultures was determined in a Helber counting chamber. To ensure that measurements of $V_{max(app)}$ were not biased by diffusion limitation in the liquid phase of the cultures or across the air–water interface, two series of measurements were carried out. Cultures were adjusted to starting concentrations of 5×10^7 cells ml⁻¹ (series 1) and 2.5×10^7 cells ml⁻¹ (series 2) with 0.5 mM phosphate buffer. Chloramphenicol (1.25 mg·liter⁻¹) was added to inhibit further cell growth. Three-milliliter aliquots were transferred to 28-ml tubes, which were sealed with gas-tight butyl rubber septa. Methane was added to final mixing ratios of 500 ppmv CH₄ (wild-type strain SC2 and mutant SC2-P2) or 0.4–0.5% CH₄ (vol/vol) (wild-type strain SC2, and mutants SC2-P2 and SC2-P3). The linear decrease of methane over time in triplicate incubations was used to estimate $V_{max(app)}$ of the two isozymes. The tubes were fixed in a horizontal position on a rotary shaker and agitated at 250 rpm. The decrease of methane in the headspace was monitored over time by using a gas chromatograph equipped with a flame ionization detector (GC-FID).

Incubation of wild-type strain SC2 and mutant SC2-P2 under 50–100 ppmv CH₄ resulted in an exponential decrease of methane, from which the specific affinity (a_s^0) of the pMMO2 was calculated. The a_s^0 value of the pMMO1 was derived from an exponential decrease of methane during incubation of mutant SC2-P3 under 600–700 ppmv CH₄. The a_s^0 values were calculated based on four measurements (duplicates of cell suspensions with 5×10^7 cells ml⁻¹ and 2.5×10^7 cells ml⁻¹). The $K_{m(app)}$ values were calculated as $K_{m(app)} = V_{max(app)}/a_s^0$. Multiplication by the Oswald constant (0.03395 at 25°C) gave the $K_{m(app)}$ as the methane concentration in water.

1. Knief C, Dunfield P-F (2005) Response and adaptation of different methanotrophic bacteria to low methane mixing ratios. *Environ Microbiol* 7:1307–1317.



Scheme 1. Generation of *pmo* mutants



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      10      20      30      40      50
, GCGGCGGGCGGCGTCTGGCTCTGAGCTTTTCGCGAAAAACCAGAGAGCTT
  :: :      :      :      :      ::
, TGAGCTGAACCGCCCAAATGCGGATGCGTGATCGAATCCGAAAAGTTAGAG
   10      20      30      40      50

      60      70      80      90     100
, TTGCCTGTGCGCTTGGCGCGTGGGTTGGCTTGTTCGGTTCGAGCAGGGTC
  :: :      :      :      :      ::
, CGCGCTCCAAGCAAAAAGCCGGTTCCCACTTTTTCGCGCGCGCGCCAAC
   60      70      80      90     100

      110
, TTCGGGCGCTG  Probe1
  ::
, GGCATCGACC  Probe2
      110

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Fig. S1. Validation of *Methylocystis* sp. strain SC2 *pmo* mutants by Southern hybridization. The deletion of the target *pmo* operon(s) in mutants SC2-P1 ($\Delta pmoCAB1a::kan$) (A), SC2-P2 ($\Delta pmoCAB1a::kan, \Delta pmoCAB1b::kan$) (B), and SC2-P3 ($\Delta pmoCAB2::kan$) (C) is demonstrated in relation to wild-type strain SC2. The DNA size markers (Smart Ladder, Eurogentec) are shown on the left for size comparison (M). The genomic DNA isolated from wild-type and *pmo* mutants was digested with XhoI and separated on an agarose gel. After transfer to a nylon membrane, the blot was cut into subblots for separate hybridizations by using Probe₁ and Probe₂, two polynucleotide probes that exhibit low sequence identity as indicated. Probe₁ specifically targets the *pmoC1-pmoA1* intergenic regions of the two copies of *pmoCAB1* in the wild type. The region is deleted in the mutants SC2-P1 (single deletion) and SC2-P2 (double deletion). Accordingly, Probe₂ specifically targets the *pmoC2-pmoA2* intergenic region in the wild-type strain, which is deleted in mutant SC2-P3. The probes were labeled during PCR amplification with digoxigenin by using the PCR DIG probe synthesis kit (Roche). After hybridization with Probe₁ (A and B) or Probe₂ (C), the membranes were stripped and rehybridized with Probe_{Kan}, which specifically targets the Kan^r gene cassette inserted in place of the deleted *pmo* operons.

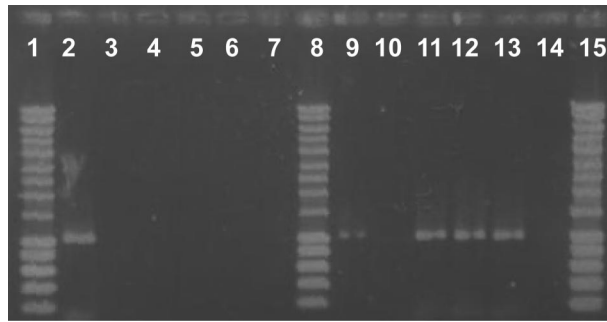


Fig. S2. Detection of *pmo* mRNA transcripts in wild-type strain SC2 and mutant strains SC2-P1, SC2-P2, and SC2-P3 after a 3-month incubation under atmospheric (1.75 ppmv) methane. RT-PCR was carried out to specifically detect mRNA transcripts of either *pmoCAB1* or *pmoCAB2*. The expected size of the mRNA RT-PCR products was 1,075 bp for *pmoCAB1* (lanes 2–7) and 1,056 bp for *pmoCAB2* (lanes 9–14). Lanes 1, 8, and 15, DNA size markers (Smart Ladder, Eurogentec); lanes 2–7, detection of *pmoCAB1* mRNA transcripts; lane 2, positive control (wild-type strain SC2 grown under a headspace of 20% CH₄); lane 3, negative control (same extract of total RNA as used for the positive control in lane 2 but without reverse transcription); lanes 4–7, wild-type strain SC2, and mutants SC2-P1, SC2-P2, and SC2-P3, respectively, maintained under atmospheric methane; lanes 9–14, detection of *pmoCAB2* mRNA transcripts; lane 9, positive control (wild-type strain SC2 grown under a headspace of 20% CH₄); lane 10, negative control (same extract of total RNA as used for the positive control in lane 9 but without reverse transcription); lanes 11–14, wild-type strain SC2, and mutants SC2-P1, SC2-P2, and SC2-P3, respectively, maintained under atmospheric methane (same extracts of total RNA as used for the detection of *pmoCAB1* mRNA transcripts in lanes 4–7).

Table S1. Oligonucleotide primers used to generate gene disruption constructs for deletion of either *pmoCAB1* or *pmoCAB2*.

PCR amplification of *pmoCAB1a,b* regions flanking the Kan^r resistance cassette

pU1	ATCATCAAGCTTGCTGCTGGCACGGAAGCTGTAGTT
pN1	ACTCCACACTTTGTGGGATGATCATGAACGGGCCGATCG
pC1	TGGAGACACCGCGTGGCCGTGTCGCCACCGGCAAGGTTGA
pD1	TCATCAAAGCTTCGAGAAGAACATCAAGAGGCCGCC

PCR amplification of *pmoCAB2* regions flanking the drug-resistance marker gene*

pU2	ATCATCAAGCTTTTGGCGTTAGATCGTCGCTGC
pN2	ACTCCACACTTTGTGATCGTATCGGTCTCGCCGGCTTGC
pC2	TGGAGACACCGCGTGGCGGACTGCTGTTCTTCTTCAGC
pD2	TCATCAAAGCTTACGCTGCGCTTCTCCATCGGACTG

PCR amplification of drug-resistance marker genes

Kan^r gene (source: pGEM, Qiagen)

Kan _f	TGGAGTCACAAAGTGGCATTGGTAACTGTCAGACC
Kan _b	ACTCCACACGCGGTGGATCTTTTCTACGGGGTCTG

Tet^r gene (source: pBR322, Promega)

Tet _f	TGGAGTCACAAAGTGTATGCGGTAGTTTATCACAGT
Tet _b	ACTCCACACGCGGTGTGGCTCCAATTCTTGGAGTG

*Mutant SC2-P3 was generated by using Kan^r as the resistance marker, whereas the *pmo* null mutant SC2-P4 was generated using Tet^r as the resistance marker.

Color code: blue, HindIII restriction site; green, AdeI restriction site; red, sequence-specific target regions in the *pmo* operons and drug-resistance marker genes.

Table S2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant trait(s)	Source or reference
Strains		
<i>Escherichia coli</i> JM109 (1)		Promega, Mannheim (2)
<i>Methylocystis</i> sp. SC2	Wild-type	
SC2-P1	$\Delta pmoCAB1a::kan$	This study
SC2-P2	$\Delta pmoCAB1a::kan, \Delta pmoCAB1b::kan$	This study
SC2-P3	$\Delta pmoCAB2::kan$	This study
SC2-P4 (<i>pmo</i> null mutant)	$\Delta pmoCAB1a::kan, \Delta pmoCAB1b::kan, \Delta pmoCAB2::tet$	This study
Plasmids		
pDrive	Source of the Kan ^r cassette	Qiagen, Hilden
pBR322	Source of the Tet ^r cassette	Promega, Mannheim
pUC18/Apr (3)	<i>lacZ</i> isolated from <i>E. coli</i> (<i>dam</i> ⁺ , <i>dcm</i> ⁺)	Fermentas, St. Leon-Rot
pUCP1	pUC18 with <i>pmoCAB1a::kan</i> , used for deletion of <i>pmoCAB1a</i> and/or <i>pmoCAB1b</i> in the wild-type	This study
pUCP3	pUC18 with <i>pmoCAB2::kan</i> , used for deletion of <i>pmoCAB2</i> in the wild-type	This study
pUCP4	pUC18 with <i>pmoCAB2::tet</i> , used for deletion of <i>pmoCAB2</i> in strain SC2-P2	This study

- Messing J, Crea R, Seeburg P-H. (1981) A system for shotgun DNA sequencing. *Nucleic Acids Res* 9:309–321.
- Dunfield P-F, et al. (2002) Isolation of a *Methylocystis* strain containing a novel *pmoA*-like gene. *FEMS Microbiol Ecol* 41:17–26.
- Yanisch-Perron C, Vieira J, Messing, J (1985) Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13 mp18 and pUC19 vectors. *Gene* 33:103–119.

Table S3. Primers used for RT-PCR detection of *pmoCAB1* and *pmoCAB2* mRNA transcripts

Primer	5'→3' sequence	Source
PmoB _{conv/nov} *	CTGGCTGTCGGTGTGTA	This study
PmoA1 _f †	GAGAGCTTTGCCTGTCGCC	This study
PmoA1 _b †	GTCTCTCGCAGCGGACCTTC	This study
PmoA2 _f ‡	GCATCTGAGCTGAACCGCCC	This study
PmoA2 _b ‡	CACTCCCTTGCGTTTCTCG	This study

*Used for reverse transcription (targets both *pmoCAB1* and *pmoCAB2* mRNA transcripts).

†Used to specifically amplify cDNA from *pmoA1a,b*.

‡Used to specifically amplify cDNA from *pmoA2*.