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Development and validation of promoter-probe vectors for the study of methane monooxygenase gene expression in *Methylococcus capsulatus* Bath

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A series of integrative and versatile broad-host-range promoter-probe vectors carrying reporter genes encoding green fluorescent protein (GFP), catechol 2,3-dioxygenase (XylE) or β-galactosidase (LacZ) were constructed for use in methanotrophs. These vectors facilitated the measurement of *in vivo* promoter activity in methanotrophs under defined growth conditions. They were tested by constructing transcriptional fusions between the soluble methane monooxygenase (sMMO) σ^{54} promoter or particulate methane monooxygenase (pMMO) σ^{70} promoter from *Methylococcus capsulatus* and the reporter genes. Reporter gene activity was measured under high- and low-copper growth conditions and the data obtained closely reflected transcriptional regulation of the sMMO or pMMO operon, thus demonstrating the suitability of these vectors for assessing promoter activity in methanotrophs. When β-galactosidase expression was coupled with the fluorogenic substrate 4-methylumbelliferyl β-D-glucuronide it yielded a sensitive and powerful screening system for detecting cells expressing this reporter gene. These data were substantiated with independent experiments using RT-PCR and RNA dot-blot analysis.

INTRODUCTION

The construction of *in vitro* gene fusions with a promoter and a promoterless reporter gene has revolutionized genetic techniques and how they can be applied to analyse gene expression. Gene fusion systems were originally developed in *Escherichia coli*, where β-galactosidase, encoded by *lacZ*, was placed under the control of new or altered promoter signals and the activity of this enzyme was precisely quantified using a simple and sensitive colorimetric assay (Casadaban *et al.*, 1980). Gene fusion technology utilizing many different reporter genes has been widely used in bacteria to monitor *in vivo* gene expression, especially of those genes whose products are difficult to assay (Schweizer & Chuanchuen, 2001). Reporter genes have been used as both transcriptional (operon) and translational (protein) fusions (Silhavy & Beckwith, 1985). In transcriptional fusions, the transcription of a reporter gene is placed under the control of a promoter, where the reporter gene has its own ribosome-binding site (RBS). The activity of the reporter gene product is proportional to the level of transcription initiation from the promoter (Linn & St Pierre, 1990). In translational fusions, both the transcription and translation of a reporter gene are placed under the control of a promoter and RBS, and the activity of the reporter gene product can be correlated with the activity of the native gene product that the promoter and RBS normally regulate (Thomas *et al.*, 1982).

In recent years there has been increasing interest in carrying out genetic analysis of bacteria other than *E. coli*. One group of organisms that has received considerable interest over the past 35 years is the methanotrophs, for example *Methylococcus capsulatus*, due to their ecological significance in methane cycling and their considerable potential for biotechnological applications such as biotransformation and bioremediation (Smith & Dalton, 2004). To investigate regulation of methane oxidation, suitable genetic tools such as broad-host-range (BHR) and integrative promoter-probe vectors are required. Replicating vectors such as BHR vectors are particularly useful for identifying weak promoters and for the expression of genes from a heterologous host. However, in certain cases, BHR promoter-probe vectors may not reflect the natural situation since titration effects involving single-copy chromosomally encoded transcriptional regulators may lead to improper gene expression. In these cases, chromosomal integration is the best way of circumventing such problems. One way of achieving this

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Abbreviations: BHR, broad host range; DMF, dimethylformamide; MUG, 4-methylumbelliferyl β-D-glucuronide; ONPG, *o*-nitrophenyl β-D-galactoside; pMMO and sMMO, particulate and soluble methane monooxygenase.

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is integration of non-replicative plasmids, but selective pressure needs to be maintained.

The lack of well-developed genetic tools for methanotrophs is due to the obligate nature of these organisms, the limited number of antibiotic markers that can be used and restricted methods for transferring plasmids into methanotrophs (Barta & Hanson, 1993; Murrell, 1992, 1994). This greatly hampered earlier work on transcriptional regulation of methane monooxygenase (MMO), the enzyme responsible for oxidizing methane to methanol. In some methanotrophs, including *Mc. capsulatus*, two different MMO enzymes catalyse this reaction, a membrane-bound, particulate MMO (pMMO) and a cytoplasmic, soluble MMO (sMMO), and their expression is dependent on the copper-to-biomass ratio during growth (Prior, 1985; Stanley *et al.*, 1983). Regulation of the sMMO operon is thought to be tightly regulated at the level of transcription. Transcription of the sMMO operon proceeds via a σ^{54} promoter in a copper-independent manner (Csaki *et al.*, 2003; Nielsen *et al.*, 1996, 1997). Transcription of sMMO is repressed under high copper-to-biomass ratio; however, this repression is relieved when the copper-to-biomass ratio is low. Expression of sMMO requires additional regulatory elements: a σ^{54}-dependent transcriptional activator, MmoR, and a GroEL homologue, MmoG (Csaki *et al.*, 2003; Stafford *et al.*, 2003).

The regulation of pMMO does not appear to be regulated at the level of transcription by copper. However, an increase in the amount of pMMO protein and its activity is observed with increasing copper concentration (Choi *et al.*, 2003). With this in mind, the aim of this study was to develop a range of versatile genetic tools for methanotrophs utilizing the reporter genes *gfp*, *xylE* and *lacZ*. The promoter-probe vectors were validated for their usefulness as tools for assessing promoter activity under a defined condition using the well-characterized MMO expression systems utilizing either the sMMO σ^{54} or pMMO σ^{70} promoters. Furthermore, the resulting *Mc. capsulatus* reporter strains were assessed for their suitability as a host for carrying out transposon mutagenesis for the high-throughput detection of a particular class of mutants. For example, when a *Mc. capsulatus* LacZ reporter strain, where *lacZ* is under the transcriptional control of sMMO σ^{54}, is used for mutagenesis, sMMO-minus mutants or sMMO-constitutive mutants can be relatively easily screened for by performing the MUG assay on cells grown under low- or high-copper growth conditions, respectively.

METHODS

Cultivation of bacterial strains and media. All bacterial strains and constructs are listed in Table 1. Methanotrophs were cultivated in nitrate mineral salt (NMS) medium (Whittenbury *et al.*, 1970). NMS agar plates were prepared with 1.5 % (w/v) Bacto agar. Antibiotics were added as required: kanamycin (15 μg ml^{-1}) and gentamicin (5 μg ml^{-1}). Low-copper medium was prepared for sMMO expression by omitting copper sulfate from NMS medium. For pMMO expression growth conditions, copper sulfate was added to a final concentration $\geqslant 1$ μM.

Methanotrophs were routinely cultivated in 250 ml conical flasks containing 50 ml NMS medium. Flasks were sealed with Suba-seals and gassed with 50 ml (i.e. ~20 %) methane/carbon dioxide (95/5, v/v mix). All *Mc. capsulatus* strains were incubated at 45 °C with shaking at 200 r.p.m. *Methylosinus sporium* was incubated at 30 °C. A typical methanotrophic culture took about 4–6 days to reach stationary phase (OD_{540} 0.8). Methanotrophs grown on NMS agar plates were incubated in a gas-tight container under a methane/air/carbon dioxide atmosphere (50/45/5, by vol.) at the appropriate temperature. The gas was replenished every 2 days until colonies formed, usually within 5–10 days depending on the strain.

Large-scale or high-density cultivation of methanotrophs was carried out in 5 l fermenters (Inceltech LH Series 210) supplied with air (1 l min^{-1}) and methane (140 ml min^{-1}). Dissolved oxygen concentrations were maintained above 5 % by adjusting the agitation speed and air flow rate. pH was maintained between 6.8 and 7.2 by addition of 0.5 M HCl or 0.5 M NaOH. Growth was monitored by measuring the OD_{540} using a Beckman DU-70 spectrophotometer. Cells were harvested during mid-exponential phase (OD_{540} 4–6) and were used immediately for enzyme assays, or stored at −80 °C by resuspending the cells in a minimal volume of 20 mM Tris/HCl (pH 7.0) and drop-frozen in liquid nitrogen.

Chemically competent *E. coli* TOP10F cells were used as host for genetic manipulation of plasmids and PCR products. Electrocompetent *E. coli* S17.1 *λpir* cells were transformed with integrative suicide plasmids containing an RP4 origin of transfer determinant in order to facilitate plasmid transfer into methanotrophs by conjugation using methods described previously (Ali *et al.*, 2006). *E. coli* transformants were typically selected on LB agar plates containing antibiotics: kanamycin (50 μg ml^{-1}); ampicillin (50–100 μg ml^{-1}); gentamicin (15 μg ml^{-1}); tetracycline (12.5 μg ml^{-1}).

Genetic procedures and manipulation. All general DNA cloning and manipulations were performed according to Sambrook *et al.* (1989). Small-scale plasmid preparation and DNA purification following gel extraction were performed using the Qiaprep Miniprep kit and QIAquick Gel Extraction kit (Qiagen). DNA from methanotrophs was extracted using the method described by Marmur (1961). All plasmids used in this study are listed in Table 1. PCR amplifications were performed in 50 μl total volume of reaction mixture using a Hybaid Touchdown Thermal Cycling System. *Taq* DNA polymerase and dNTPs were purchased from Fermentas and custom primers were obtained from Invitrogen. DNA sequencing reactions were performed by the Molecular Biology Service at the University of Warwick using a Dye Terminating kit (PE Applied Biosciences) and the DNA signals were analysed using a 373A automated sequencing system. DNA sequences were annotated using Chromas (Version 1.45) and Lasergene sequence analysis software tools.

Construction of integrative promoter-probe vectors. Plasmid pDAH350 was selected for constructing the promoter-probe vectors. This plasmid was based on pUC18/19, with a ColE1 origin of replication (*ori*) and a gentamicin-resistance (Gm^R) gene present on the backbone. To fulfil the criteria for an integrative plasmid, an RP4-based recognition site for plasmid mobilization (*oriT*) was cloned into pDAH350. This was done by amplifying *oriT* from plasmid pMJ153 by PCR. The primers used introduced *Eco*RI and *Xho*I restriction sites in the forward and reverse primer, respectively, to facilitate cloning via the same sites in pDAH350, yielding construct pMHA001. The *gfp*, *xylE* and *lacZ* reporter genes were obtained from pMJ153, pCM130 (Marx & Lidstrom, 2001) and pDAH274, respectively, by PCR amplification with introduced *Bam*HI and *Eco*RI restriction sites to facilitate cloning via the same sites in pMHA001. This yielded the

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Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
Methylosinus sporium strain 5	Wild-type strain	Warwick Culture Collection
Methylococcus capsulatus strain Bath	Wild-type strain	Warwick Culture Collection
Methylococcus capsulatus [pMHA011]	GFP reporter strain, Gm^R	This study
Methylococcus capsulatus [pMHA021]	XylE reporter strain, Gm^R	This study
Methylococcus capsulatus [pMHA034]	LacZ reporter strain, Gm^R	This study
Methylococcus capsulatus [pMHA200]	Promoterless GFP reporter strain, Gm^R	This study
Methylococcus capsulatus [pMHA201]	GFP reporter strain, Gm^R	This study
Methylococcus capsulatus mmoG::Tn-pAG408	*Mc. capsulatus mmoG*::Tn transposon mutant (pAG408), Gm^R	R. Csaki, University of Szeged
Methylococcus capsulatus mmoR::Tn-pAG408	*Mc. capsulatus mmoR*::Tn transposon mutant (pAG408), Gm^R	R. Csaki, University of Szeged
Methylococcus capsulatus mmoG::Tn-pAG408 [pMHA012]	*Mc. capsulatus mmoG*::Tn-pAG408 GFP reporter strain, Gm^R Km^R	This study
Methylococcus capsulatus mmoR::Tn-pAG408 [pMHA012]	*Mc. capsulatus mmoR*::Tn-pAG408 GFP reporter strain, Gm^R Km^R	This study
Escherichia coli TOP10F	Chemically competent cells	Invitrogen
Escherichia coli S17.1 λpir	*recA1 thi pro hsdR*$^-$ RP4-2Tc::Mu Km::Tn*7* λpir	Herrero *et al.* (1990)
Plasmids		
pCR2.1-TOPO	Km^R Ap^R, PCR product cloning vector	TA TOPO Cloning kit (Invitrogen)
pK18*mob*	Km^R, RP4-mob, mobilizable cloning vector	Schafer *et al.* (1994)
pMJ153	Source of *gfp* and *oriT*	D. A. Hodgson, University of Warwick
pDAH274	LacZ promoter-probe vector	D. A. Hodgson, University of Warwick
pDAH350	Gm^R, cloning vector	D. A. Hodgson, University of Warwick
pMHA001	pDAH350 containing *oriT* from pMJ153, Gm^R	This study
pMHA010	pMHA001 containing *gfp* from pMJ153, Gm^R	This study
pMHA011	pMHA010 containing sMMO σ^{54} promoter from *Mc. capsulatus*, Gm^R	This study
pMHA012	pK18mob containing sMMO σ^{54} promoter and *gfp*, Km^R	This study
pMHA020	pMHA001 containing *xylE* from pCM130, Gm^R	This study
pMHA021	pMHA020 containing sMMO σ^{54} promoter from *Mc. capsulatus*, Gm^R	This study
pMHA030	pMHA001 containing *lacZ* from pDAH274, Gm^R	This study
pMHA034	pMHA030 containing sMMO σ^{54} promoter from *Mc. capsulatus*, Gm^R	This study
pCM130	BHR *xylE* promoter-probe vector, Tet^R	Marx & Lidstrom (2001)
pCM132	BHR *lacZ* promoter-probe vector, Km^R	Marx & Lidstrom (2001)
pMHA199	pCM130 containing km^R gene instead of tet^R	This study
pMHA200	pCM132 containing *gfp* from pMJ153 (no *lacZ*), Km^R	This study
pMHA201	pMHA200 containing pMMO σ^{70} promoter (operon 1) from *Mc. capsulatus*, Km^R	This study

general integrative *gfp*, *xylE* and *lacZ* promoter-probe vectors pMHA010, pMHA020 and pMHA030, respectively (Fig. 1a).

The *mmoX* σ^{54} promoter from *Mc. capsulatus* was PCR amplified with introduced *Pst*I and *Bam*HI restriction sites. The resulting promoter fragment was cloned into pMHA010, pMHA020 and pMHA030 via *Pst*I and *Bam*HI restriction sites, yielding the promoter-specific reporter constructs pMHA011, pMHA021 and pMHA034 (Table 1).

The promoter-probe vector pMHA011 was further modified to facilitate selection using an antibiotic marker other than gentamicin. The sMMO σ^{54} promoter fused with the *gfp* gene in pMHA011 was excised with *Pst*I and *Eco*RI and cloned into the suicide vector pK18mob (Schafer *et al.*, 1994), containing a kanamycin-resistance gene, via the same restriction sites (*Pst*I and *Eco*RI). This yielded the modified promoter-probe vector pMHA012.

Construction of BHR promoter-probe vectors. Improved versatile low-background BHR promoter-probe vectors pCM130 and pCM132 for use in methylotrophs are available (Marx & Lidstrom, 2001). To facilitate the application of these BHR promoter-probe vectors in methanotrophs, they were refined in this study as follows. The *tetR* and *tetA* gene in pCM130 conferring tetracycline resistance, a poor antibiotic selection marker in methanotrophs, was replaced by a kanamycin-resistance gene. The *tetR* and *tetA* genes were removed from pCM130 following restriction digestion with *Sex*AI and *Nar*I and a DNA fragment carrying a kanamycin-resistance gene was removed from pCM132 with the same restriction enzymes and cloned into pCM130 to give the new BHR promoter-probe vector pMHA199 (Fig. 1b). An alternative BHR promoter-probe vector utilizing *gfp* was constructed. The *lacZ* gene from pCM132 was removed by *Kpn*I and *Sph*I digestion and a

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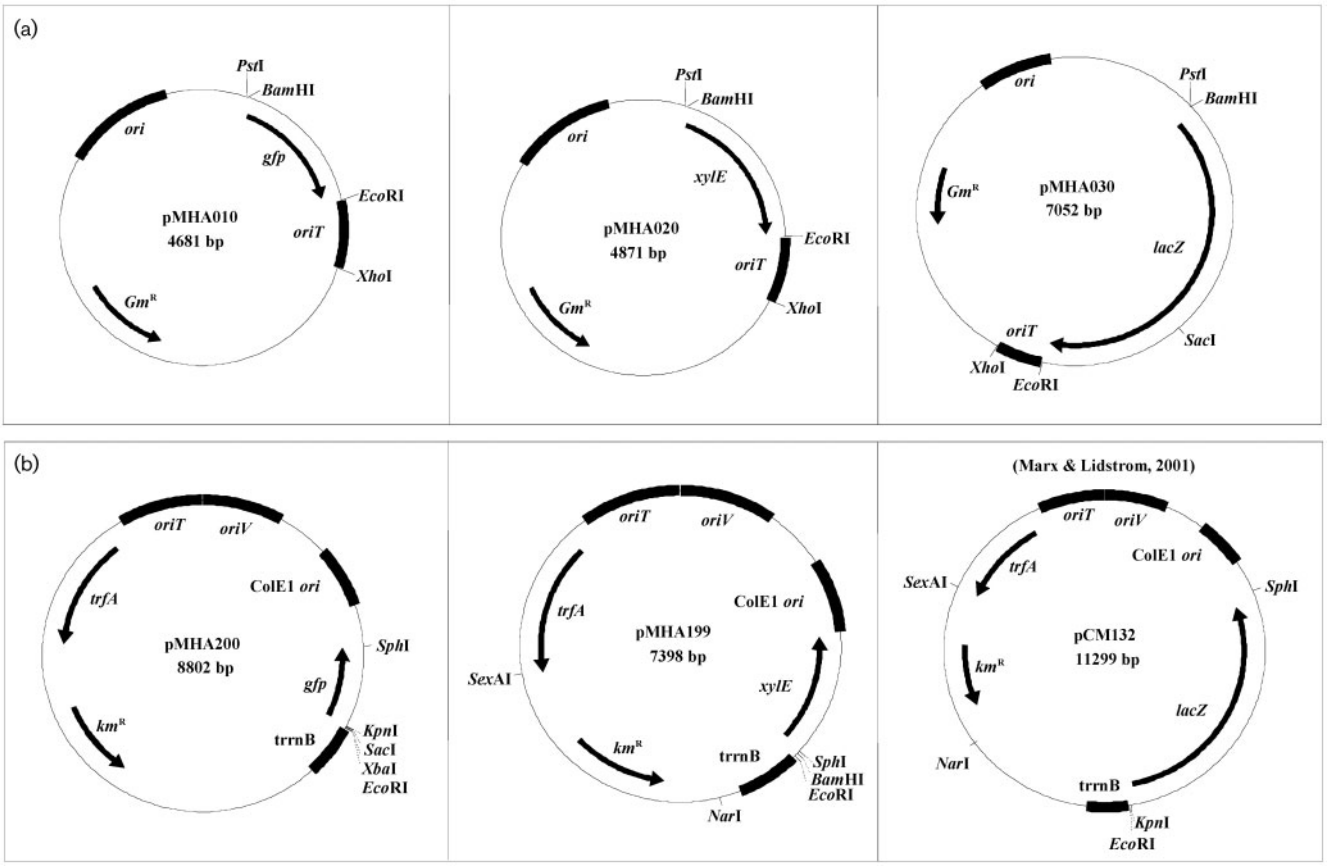

Fig. 1. Integrative promoter-probe vectors (a) and low-background BHR promoter-probe vectors (b) containing *gfp*, *xylE* or *lacZ* reporter gene.

gfp gene was cloned in via the same restriction sites to give the new vector, pMHA200 (Fig. 1b).

The pMMO σ^{70} promoter from operon 1 of the *Mc. capsulatus* Bath genome (NCBI accession no. NC_002977) was amplified by PCR with the introduced *Eco*RI and *Sac*I restriction sites. The resulting promoter fragment was cloned into pMHA200 via the same sites, yielding the promoter-specific BHR reporter construct, pMHA201.

Reporter gene assays. GFP fluorescence was measured using a fluorimeter at an excitation wavelength of 475 nm and an emission wavelength of 510 nm. When appropriate, samples with high GFP activity were diluted in deionized water to prevent saturation of the detector. The fluorescence intensity from samples was represented as arbitrary fluorescence units. All GFP fluorescence was measured from cell-free extracts whose protein content was quantified using the Bio-Rad reagent according to the manufacturer's instructions. The data obtained were normalized by expressing arbitrary fluorescence units as specific activity in units (mg protein)$^{-1}$. The mean specific activity from at least two independent assays is indicated in the results.

Catachol 2,3-dioxygenase (XylE) activity was measured using methods described previously (Kataeva & Golovleva, 1990; Zukowski *et al.*, 1983), with slight modifications. The qualitative detection and thus the functional expression of XylE was examined by either spraying or pipetting a solution of 0.1 M catechol (Sigma) directly onto colonies or liquid cultures, which become yellow/orange when expressing XylE. Quantitative assays for XylE were conducted in a 1 cm cuvette at 30 °C in a total volume of 1 ml containing the following: 0.88 ml 50 mM Tris buffer (pH 7.5); 0.02 ml 50 mM catechol dissolved in the Tris buffer; and 0.1 ml of cell-free extract or appropriate dilutions. XylE activity was calculated as the rate of change in A_{375} min^{-1} (mg protein)$^{-1}$ and finally expressed as nmol 2-hydroxymuconic semialdehyde formed min^{-1} (mg protein)$^{-1}$ (note: a change in A_{375} of 1 min^{-1} is equal to 1 unit and one milliunit corresponds to the formation at 30 °C of 1 nmol 2-hydroxymuconic semialdehyde min^{-1}). An average change of A_{375} min^{-1} over 3 min was used to calculate specific activities.

Qualitative expression of *lacZ* genes was examined by the addition of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), dissolved in DMSO or dimethylformamide (DMF) to a final concentration of 40 μg ml^{-1}, either to the cooled medium just before pouring the plates or directly onto the surface of the agar. The formation of blue colonies indicated the expression of *lacZ*. 4-Methylumbelliferyl β-D-glucuronide (MUG) was used as an alternative substrate for detecting β-galactosidase activity. MUG solution (4 mg ml^{-1} in DMSO) was directly added onto the colonies and the plates were incubated at room temperature for 20 min. Colonies were then viewed under UV light for detection of fluorescence.

β-Galactosidase activity was measured quantitatively by following the conversion of *o*-nitrophenyl β-D-galactoside (ONPG) to *o*-nitrophenol using methods described by Miller (1977).

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Naphthalene assay. A naphthalene oxidation assay was routinely used for the qualitative detection of sMMO activity on NMS agar plates or in liquid cultures using methods described previously (Brusseau *et al.*, 1990; Graham *et al.*, 1992). sMMO is capable of oxidizing naphthalene to naphthol, which can react with a zinc complex (tetrazotized *o*-dianisidine) to form a deep purple colour, thus indicating sMMO expression and activity.

RT-PCR and RNA dot-blot analysis. A hot-phenol method (Gilbert *et al.*, 2000) was used to isolate total RNA from 4 ml of exponential-phase fermenter cultures of *Mc. capsulatus* expressing either pMMO or sMMO. DNA was removed from RNA samples using 2–4 units of DNase I (Promega) per μg total nucleic acid, according to the manufacturer's instructions. The first-strand cDNA synthesis for RT-PCR was performed with SuperScript II reverse transcriptase (RT) (Invitrogen). DNase-treated total RNA (0.5–1 μg) was added to 50 pmol gene-specific reverse primer and 1 μl dNTP mix (10 mM each) in a final volume of 12 μl. The mixture was heated to 65 °C for 5 min and then chilled on ice. To the reaction mixture, 4 μl 5× first-strand buffer, 2 μl 0.1 M DTT and 1 μl (200 units) of SuperScript II RT were added to give a 20 μl final volume before incubation at 42 °C for 50 min, followed by a 15 min incubation at 70 °C to inactivate the RT enzyme. The cDNA (1–2 μl) was used as a template for PCR amplification without further purification. cDNA synthesis of low-abundunce mRNA was achieved using the SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA polymerase (Invitrogen) according to the manufacturer's instructions.

Various amounts of RNA (0.5–3 μg), extracted from pMMO- or sMMO-expressing cultures, were blotted in parallel onto HybondN+ membrane according to Sambrook *et al.* (1989). A number of different DNA fragments amplified by PCR from *Mc. capsulatus* were used as probes (~25 ng) for hybridization. Hybridization by random priming was performed as described previously (Ali *et al.*, 2006).

RESULTS AND DISCUSSION

Application of integrative promoter-probe vectors in methanotrophs

A series of general integrative promoter-probe vectors utilizing *gfp*, *xylE* and *lacZ* were constructed (Fig. 1a). These were based on the stable integration of the promoter-probe vectors into the chromosome by allelic exchange via the sMMO σ^{54} promoter. This allowed the expression of the reporter gene to be independent of the plasmid copy number. Furthermore there were no obvious differences in growth between the wild-type (WT) and the reporter strains of *Mc. capsulatus*, suggesting that the integration of the various reporter genes at the recombination site had no disadvantage to the host strain.

To validate the usefulness of these vectors as genetic tools for investigating *in vivo* transcriptional regulation of gene expression, transcriptional fusions were constructed with the reporter genes and the well-characterized copper-repressible sMMO σ^{54} promoter from *Mc. capsulatus* (Csaki *et al.*, 2003; Stafford *et al.*, 2003). GFP, XylE and LacZ reporter strains were established by conjugative transfer of the sMMO σ^{54} promoter-specific reporter vectors into *Mc. capsulatus*. The effectiveness of the reporter strains for assaying transcriptional activity was assessed both qualitatively and quantitatively.

All three reporter strains of *Mc. capsulatus* were initially cultivated on NMS agar plates containing no added copper (low-copper conditions), to induce reporter gene activity, and on NMS agar plates containing 1 μM copper (high-copper conditions). Qualitative assessment of these reporter strains clearly showed that under high-copper conditions, no reporter gene activity could be observed (Fig. 2). However, under low-copper conditions, the *Mc. capsulatus* GFP reporter strain had only very weak GFP activity when visualized under a fluorescence microscope (data not shown). Due to the low reporter activity, the use of this reporter strain for the development of a high-throughput detection assay was discontinued. However, it is worth noting that the GFP promoter-probe vector is nevertheless excellent as a tool for assessing promoter activity since it can be used to assess the promoter activity under defined conditions where any changes in activity can be detected with high sensitivity.

The XylE reporter strain of *Mc. capsulatus* yielded higher levels of reporter gene activity under low-copper conditions. From the XylE plate assay, *Mc. capsulatus* cells expressing XylE could be differentiated from those that did not express XylE (Fig. 2a).

The growth and the sensitivity of detection of β-galactosidase expression in the *Mc. capsulatus* LacZ reporter strain was tested using X-Gal and MUG reagent as substrate. The growth of the *Mc. capsulatus* LacZ reporter strain on X-Gal dissolved in DMF was very poor, with only slight improvement when grown on X-Gal dissolved in DMSO (data not shown). Despite X-Gal being an excellent substrate for detecting recombinant *E. coli* clones, the use of X-Gal as a substrate for screening sMMO mutants in *Mc. capsulatus* was discontinued due to the relatively poor growth as a result of DMF and DMSO toxicity. However, since the MUG assay is performed on fully grown cells in the absence of DMSO, any such toxicity is avoided. Therefore using the MUG assay, the expression of β-galactosidase and thus sMMO expression could be detected with greater sensitivity and was reliably specific to sMMO-expressing cells (Fig. 2b). In addition, the background activity was minimal and cells were still viable after the MUG plate assay, despite the use of DMSO and the short exposure of cells to UV light.

In addition to the qualitative assay, all three reporter strains were cultivated in a 5 l fermenter where high cell densities were obtained, thus allowing more precise control of copper-to-biomass ratios. Cells were removed from high- and low-copper-containing cultures during the exponential growth phase and the reporter gene activity was measured quantitatively (Table 2). It is noteworthy that the integration of the promoter-probe vector upstream of the sMMO σ^{54} promoter did not create a polar effect on the transcription of sMMO, since the expression of the sMMO could be still detected using the naphthalene assay in a manner similar to the wild-type strain. With the LacZ reporter strain, once the medium was replaced with

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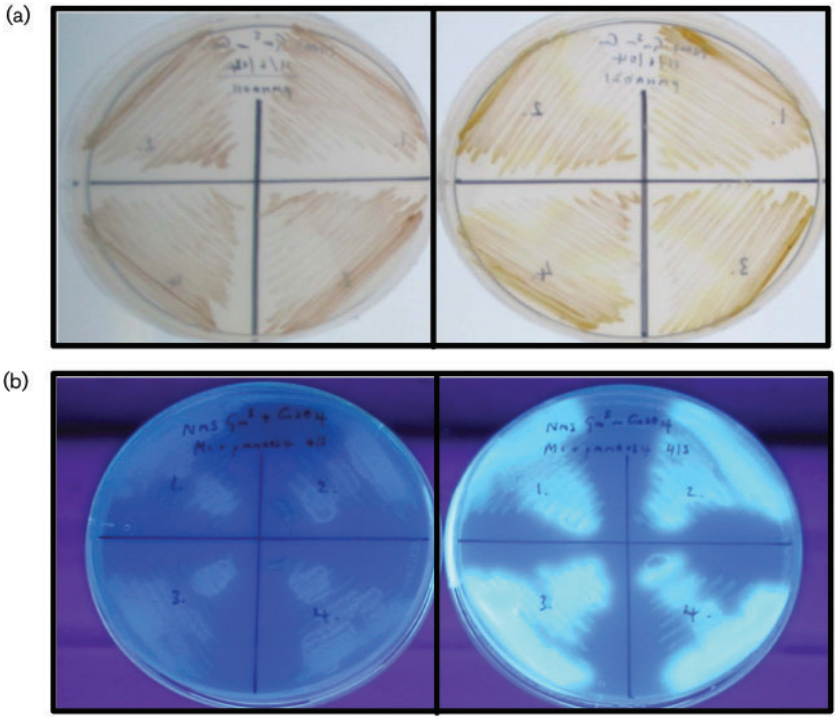

Fig. 2. Qualitative analysis of reporter gene activity in *Mc. capsulatus* reporter strains. (a) Catechol 2,3-dioxygenase plate assay performed on XylE reporter strain grown on high (left plate) and low (right plate) copper-containing NMS plates. The appearance of yellow/orange colour indicates the production of 2-hydroxymuconic semialdehyde and thus catechol 2,3-dioxygenase activity. (b) MUG assay performed on LacZ reporter strain grown on high (left plate) and low (right plate) copper-containing NMS plates. β-Galactosidase activity is indicated by fluorescing white colonies.

medium containing no added copper the β-galactosidase activity and sMMO expression using the naphthalene assay were monitored periodically with increasing biomass (Fig. 3). The data obtained clearly show that once the copper-to-biomass ratio is sufficiently low, an increase in β-galactosidase activity can be seen, concomitant with the detection of sMMO expression. The β-galactosidase activity continued to increase with increasing culture biomass and thus more closely reflects the induction of transcription from the sMMO σ^{54} promoter and hence sMMO expression under low-copper conditions.

All three reporter strains with single-copy integration of the promoter-probe vector confirmed the tight regulation of the transcription from the sMMO σ^{54} promoter with respect to copper concentration. In particular β-galactosidase activity in the *Mc. capsulatus* LacZ reporter strain was massively upregulated under low-copper growth conditions, with only negligible activity under high-copper growth conditions (Table 2).

Exploring the role of MmoR and MmoG using promoter-probe vectors

MmoR and MmoG have been shown to be involved in initiating transcription from the sMMO σ^{54} promoter by marker-exchange mutagenesis (Csaki *et al.*, 2003; Stafford *et al.*, 2003). To further substantiate the crucial role of MmoR and MmoG in initiating transcription from the sMMO σ^{54} promoter, pMHA012 was stably integrated into *Mc. capsulatus mmoR*::Tn-pAG408 and *mmoG*::Tn-

Table 2. GFP, XylE (substrate: catechol) or β-galactosidase (substrate: ONPG) activity in cell extracts prepared from wild-type (WT) *Mc. capsulatus* and reporter strains

The activities shown are means ± SD of two or three independent assays.

Strain and growth conditions	Reporter gene activity in *Mc. capsulatus*		
	GFP [fluorescence units (mg protein)$^{-1}$)]	XylE (milliunits min^{-1} (mg protein)$^{-1}$)	LacZ (Miller units)
WT (high Cu)	5.9 ± 0.2	0	0
WT (low Cu)	6.1 ± 0.3	0	0
Reporter strain (high Cu)	8.1 ± 0.4	9 ± 0.4	3.9 ± 0.2
Reporter strain (low Cu)	127.6 ± 3.2	1400 ± 7	6922.5 ± 311.5

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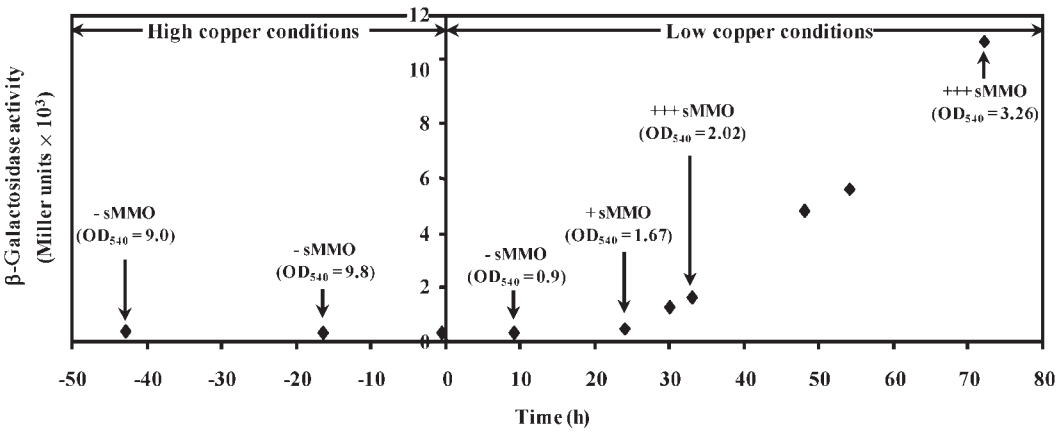

Fig. 3. β-Galactosidase activity curve. The *Mc. capsulatus* LacZ reporter strain was initially cultivated in NMS medium containing high copper (1 μM $CuSO_4$) represented by minus time points. At time zero, cells were harvested and NMS medium with no added copper (low-copper conditions) was added. At the time points indicated, a sample of cells was removed and β-galactosidase activity was measured quantitatively by following the conversion of ONPG to *o*-nitrophenol. The OD_{540} and sMMO expression status are indicated for some of the time points. sMMO activity was detected using the naphthalene assay; − indicates no activity, + weak activity and +++ very strong activity.

pAG408 transposon mutants. Construct pMHA012 contains *gfp* under the conditional control of the sMMO σ^{54} promoter and confers kanamycin resistance, making it suitable for selecting its stable integration in *Mc. capsulatus* transposon mutants. Transconjugants and thus the reporter strains were selected using medium containing gentamicin and kanamycin. However, analysis of cell extracts prepared from exponentially growing cultures expressing pMMO or sMMO revealed no GFP activity under either growth condition. This indicated that both MmoR and MmoG were essential for initiation of transcription from the sMMO σ^{54} promoter. As previously suggested (Stafford *et al.*, 2003), a possible role for MmoG could be in assembly of MmoR to form an open promoter complex with the RNA polymerase, which is required for transcription to proceed from the sMMO σ^{54} promoter (Oguiza *et al.*, 1999; Reitzer & Schneider, 2001).

Application of BHR promoter-probe vectors in methanotrophs

The availability of the low-background BHR promoter-probe vector pCM130 (Marx & Lidstrom, 2001) gave us the opportunity to modify this vector for use in methanotrophs. The tetracycline-resistance gene in pCM130 was removed and replaced with a kanamycin-resistance gene, which is a more suitable antibiotic marker for methanotrophs. This gave the modified BHR vector pMHA199. The *lacZ* gene from pCM132 was removed and replaced with *gfp* to give the new BHR vector pMHA200. This gave rise to a number of suitable BHR promoter-probe vectors for methanotrophs utilizing *gfp*, *xylE* and *lacZ* (Fig. 1b).

In a previous study, we demonstrated the usefulness of these BHR promoter-probe vectors by showing the copper-independent nature of the sMMO σ^{54} promoter from facultative methanotroph *Methylocella silvestris* (Theisen *et al.*, 2005). Here we further explored the usefulness of these BHR vectors for studying transcriptional activity of promoters. Unlike the sMMO enzyme, which is tightly regulated at the level of transcription by copper, the pMMO σ^{70} promoter is not regulated at the level of transcription. However, it is known that pMMO activity is abolished and no polypeptide corresponding to pMMO can be detected under low-copper or sMMO-expressing conditions (Stanley *et al.*, 1983). Using the BHR promoter-probe vector pMHA200, we reinvestigated the transcription of the *Mc. capsulatus* pMMO operon under high- and low-copper conditions and whether it is linked to the transcriptional 'copper-switch' of the sMMO operon. A transcriptional fusion was constructed with *gfp* in the BHR promoter-probe vector pMHA200 and pMMO σ^{70} promoter (operon 1) (Stolyar *et al.*, 1999, 2001) from *Mc. capsulatus* to give construct pMHA201. All the BHR vectors contain a transcriptional terminator (*trrnB*) located upstream of the promoter region and the effectiveness of this terminator was demonstrated in *Methylobacterium extorquens* AM1 (Marx & Lidstrom, 2001). Since the pMMO σ^{70} promoter was recognized by *E. coli* RNA polymerase, it gave a convenient means to further investigate the low background activity of the reporter gene in *E. coli*. The GFP activity was quantified in cell-free extracts obtained from exponentially growing *E. coli* S17.1 λ*pir* cells containing pMHA200 (contains no promoter) and pMHA201 (contains pMMO σ^{70} promoter), respectively. In *E. coli* containing pMHA201, where *gfp* is under the transcriptional control of the pMMO σ^{70} promoter, high levels of GFP activity could be detected. However in *E. coli* containing pMHA200, no GFP activity could be

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detected. This further substantiated the effectiveness of the transcriptional terminator in blocking any background transcription (Table 3).

A similar experiment was done where constructs pMHA200 and pMHA201 were conjugated separately into *Mc. capsulatus*. GFP activity was assayed using cell-free extracts obtained from cells grown under high- and low-copper conditions. The GFP assay from *Mc. capsulatus* conclusively demonstrated that transcription initiated from the pMMO σ^{70} promoter was not affected by copper concentrations ranging from 0 to 1 μM $CuSO_4$ (Table 3).

Exploring the copper-dependent reciprocal regulation of MMO

To further substantiate the data obtained using these new promoter-probe vectors, RT-PCR (Fig. 4a) and RNA dot-blot analysis (Fig. 4b) were performed on RNA extracted from exponentially growing fermenter cultures of *Mc. capsulatus*. As expected, no transcripts for *mmoX* could be detected by RT-PCR or by RNA dot-blot analysis under high-copper growth conditions. Furthermore no noticeable difference in the abundance of *pmoA* transcript could be seen under high- and low-copper conditions. Similar data were obtained when dot-blot analysis was done using RNA extracted from *Methylosinus sporium* (Fig. 4c). These data complemented those obtained using reporter strains, thus confirming the utility of the promoter-probe vectors as a genetic tool for testing *in vivo* promoter activities in methanotrophs.

It is clear that the expression of sMMO is tightly regulated by a 'copper-switch' at the level of transcription, via an unknown mechanism involving the σ^{54} promoter, MmoR and MmoG. The data we obtained using our promoter-probe vectors confirmed the absolute requirement of MmoR and MmoG for initiating transcription. Since both these regulatory elements are essential for initiating transcription, we further investigated whether the transcription of these elements themselves was subject to copper-dependent repression. This was done once again using RT-PCR (Fig. 4d) and RNA dot-blot analysis (Fig. 4e), the results of which suggested that at least transcription of these genes is independent of copper. However, a repression effect regulated by copper at the post-transcriptional level, such that the expression of MmoR and MmoG is directly inactivated by copper, cannot be ruled out.

Our data reconfirm that transcription from the pMMO σ^{70} promoter is constitutive. Further evidence exists to support the constitutive nature of pMMO transcription, as in our previous study where *Ms. sporium* sMMO-minus mutants (Δ*mmoX*) were able to grow as well as the wild-type strain when grown under low-copper conditions (Ali *et al.*, 2006). Earlier expression studies performed on cell extracts showed that pMMO activity decreased with increasing sMMO activity when the copper-to-biomass ratio was low and vice versa (Prior, 1985). This is consistent with the observation of significantly reduced amounts of pMMO polypeptides on SDS-PAGE when cells were grown under sMMO-expressing conditions (Choi *et al.*, 2003). In light of this information, it can be concluded that the transcription of the pMMO operon is not repressed under low-copper conditions and the expression of pMMO is likely to be regulated at the post-transcriptional level.

Table 3. GFP activity present in cell extracts prepared from *E. coli* and wild-type (WT) *Mc. capsulatus* and reporter strains containing the BHR promoter-probe vector pMHA200 or pMHA201

The activities shown are means ± SD of two independent assays.

Strain	GFP activity [fluorescence units (mg protein)$^{-1}$]
E. coli S17.1 *λpir* (WT)	0
E. coli S17.1 *λpir*+pMHA200	0
E. coli S17.1 *λpir*+pMHA201	11 317.3 ± 271.6
Mc (WT) (low Cu)	0
Mc (WT) (high Cu)	0
Mc+pMHA201 (low Cu)	248.9 ± 9.2
Mc+pMHA201 (high Cu)	230.3 ± 22.1

Future prospects

Despite advances in the development of genetic tools for methanotrophs, some basic questions still remain unanswered. It is still unclear how copper independently but in parallel regulates the expression of both pMMO and sMMO. The exact level at which copper exerts repression of transcription of sMMO is not known; however, from this study several lines of investigation can be pursued. For example, the study of post-transcriptional regulation of expression of proteins such as pMMO requires additional vectors suitable for constructing translational fusions. The suite of promoter-probe vectors developed in this study can be modified in the future to make such translational fusions to investigate protein expression profiles.

The data presented in this study highlight the complexity of the molecular regulation of MMO expression, and extend our initial perception of the 'copper-switch' as an on–off switch from pMMO to sMMO expression. Thus the wider question of the parallel MMO regulation by copper may require a global analysis for identification of an as yet unknown regulatory gene such as an activator/repressor or a master gene regulator as seen in other bacterial systems (Domian *et al.*, 1999). In light of this, it is clearly necessary to proceed with an experimental approach which can be used in a high-throughput fashion to survey global gene regulation networks in methanotrophs. The *Mc. capsulatus* LacZ reporter strain developed in this study can in future be used as a host for transposon mutagenesis for

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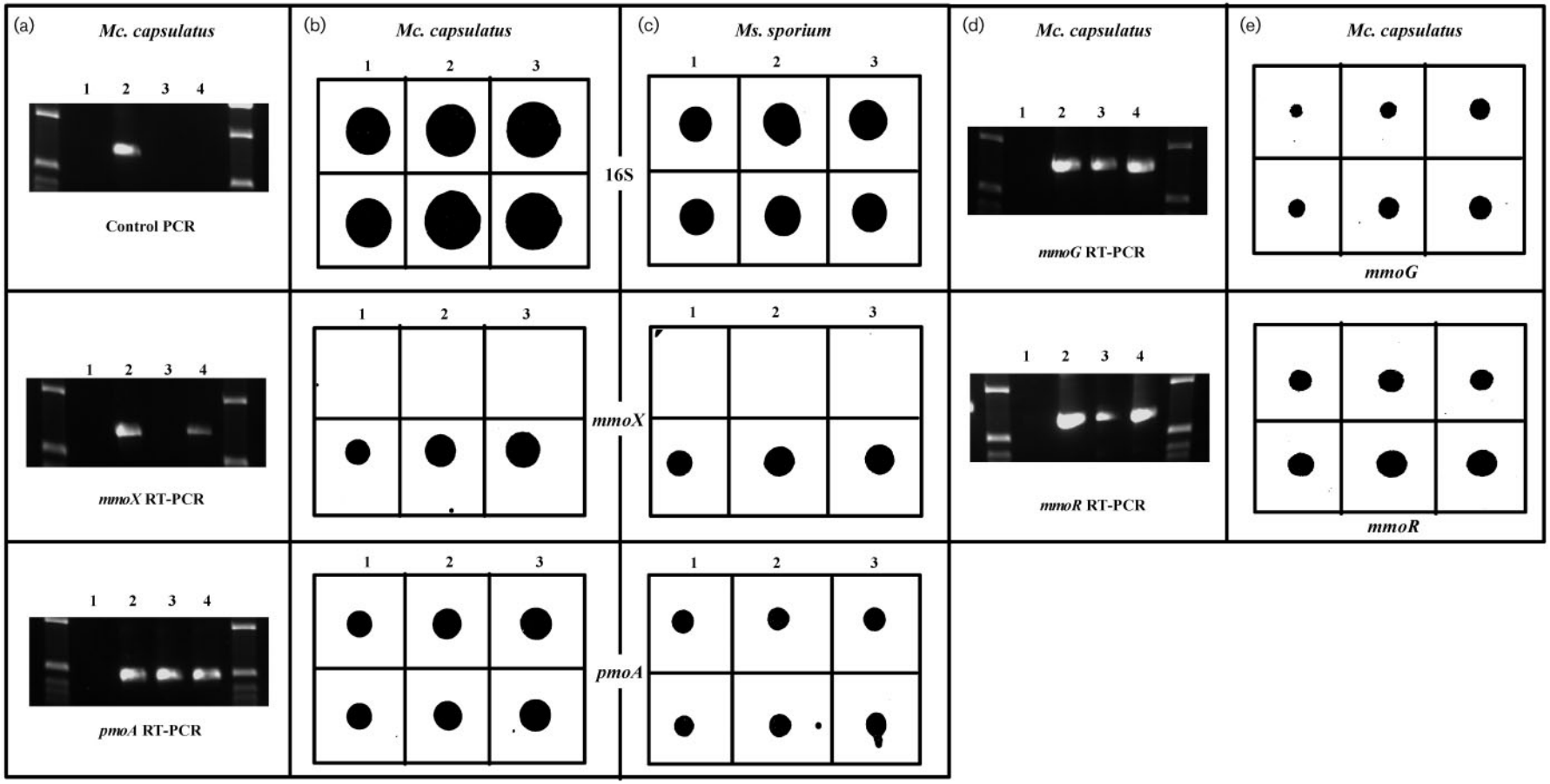

Fig. 4. Transcriptional analysis of *mmoX*, *pmoA* and the sMMO regulatory genes *mmoG* and *mmoR* by RT-PCR and RNA dot-blot analysis. (a, d) RT-PCR. A control PCR on DNase I treated RNA was done using *mmoX* primers to ensure the removal of all traces of DNA from RNA preparations. Lanes: 1, negative control (no template); 2, positive control (*Mc. capsulatus* genomic DNA); 3, RNA extracted from cells expressing pMMO (high-copper growth conditions); 4, RNA extracted from cells expressing sMMO (low-copper growth conditions). (b, c, e) RNA dot-blots. Each RNA dot-blot contains RNA extracted from *Mc. capsulatus* or *Ms. sporium* cultures expressing pMMO (top row) or sMMO (bottom row), which was hybridized with ^{32}P-labelled specific gene probe as indicated. Columns 1, 2 and 3 contain approximately 0.5, 1.5 and 3.0 μg total RNA, respectively. A control dot-blot loaded with equivalent amounts of RNA was hybridized with a specific gene probe of 16S rRNA to ensure that consistent amounts of RNA were loaded for the two copper conditions in each blot.

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identifying genes that are essential for sMMO transcription from the σ^{54} promoter. The MUG assay developed here will provide a powerful phenotypic screen for detecting sMMO-minus mutants and to investigate further the complex regulatory networks involved in MMO regulation.

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