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Methylotrophic bacterium-based molecular sensor for the detection of low concentrations of methanol

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Methylotrophic bacterium *Methylorubrum extorquens* is a promising microorganism for the production of value-added compounds from methanol. This study focused on the development of a single-cell level biosensor system that detects methanol by using the intrinsic regulatory machinery which responds to the presence of methanol in this bacterium. A green fluorescent protein (GFP) gene located downstream of the promoter region of the serine glyoxylate aminotransferase gene (P_{sga}) or the methanol dehydrogenase subunit 1 precursor gene (P_{mxaF}) was inserted into the chromosome of *M. extorquens* wild-type strain AM1. The expression of GFP upon methanol exposure was measured by spectrofluorometer and fluorescence-activated cell sorting (FACS). The strain harboring P_{sga}-*gfp* emitted fluorescence only when methanol was supplied to the culture medium, while the other strain harboring P_{mxaF}-*gfp* showed high basal fluorescence even in the absence of methanol. The fluorescence intensity of the P_{sga}-*gfp* strain depended on a methanol concentration higher than 25 μM, and the sensitivity and dose-dependency of this strain were much higher than previous systems using *Escherichia coli*. The methanol-sensing properties of the engineered *M. extorquens* strain were comparable to those of a methylotrophic yeast-based biosensor, suggesting the usefulness of methylotrophic microorganisms as platforms for single-cell sensing of C_1 compounds. The constructed methanol sensor strain, coupled with flow cytometry techniques, provides a high-throughput and highly sensitive screening method for the selection of functional methanol-producing enzymes.

[**Key words:** Methanol; Methane; Sensor cell; *Methylorubrum extorquens*; Methylotrophic bacterium]

Methanol is ubiquitously present in the global carbon cycle as an intermediate between methane and CO_2, two major greenhouse gases. It is not only a versatile feedstock in the chemical industry but also an important substrate in the industrial biotechnology field. The facultative methylotrophic bacterium *Methylobacterium extorquens* (recently reclassified as *Methylorubrum extorquens* (1)) has been extensively investigated as a useful bacterium for the methanol-based bioindustry. In this bacterium, methanol is dehydrogenized to formaldehyde by pyrroloquinoline-quinone (PQQ)-dependent methanol dehydrogenase, condensed with tetrahydromethanopterin, and subsequently oxidized to formate. Formate reacts with tetrahydrofolate to form methylenetetrahydrofolate and then enters the serine cycle, one of the C_1-assimilation pathways existing in nature. The serine cycle is associated with the ethylmalonyl-CoA pathway to regenerate glyoxylate in order to maintain the cycle. Noteworthy, several intermediate metabolites of the serine cycle and ethylmalonyl-CoA pathway can act as precursors to various value-added compounds such as amino acids, polyhydroxyalkanoates (PHAs), and fine chemicals (2).

Recently, cellular regulatory components, capable of binding to metabolites, that regulate the expression of actuators such as selection markers or fluorescent reporters have attracted much attention as sensors for detecting small molecules generated within the cells. The combination of such single cell-biosensors with flow cytometry techniques allows high-throughput screening for the detection of functional enzymes from mutant libraries (3). *M. extorquens* is thought to be suitable for the development of a highly sensitive single-cell sensor of C_1 compounds because this methylotroph possesses intrinsic regulatory machinery that responds to the presence of C_1 compounds (4). One of the most interesting and important C_1 compound-converting enzymes is methane monooxygenase (MMO). At present, methanol is industrially produced from methane in abundant natural gas or waste gases via syngas (CO/H_2) by chemical gas-to-liquid conversion requiring high temperatures and pressures as well as technical complexity. The biological conversion of methane to methanol catalyzed by MMOs offers several advantages over the conventional chemical processes, such as mild reaction conditions, high selectivity, and consequent less energy consumption (5,6). Functional expression of *mmo* in methanol-utilizing microorganisms such as *M. extorquens* is expected to enable the production of valuable substances from methane, contributing to the reduction of greenhouse gas emissions. However, a fully active form of MMO has not been obtained in heterologous hosts so far, although many efforts have been made for heterologous expression of engineered soluble

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and particulate MMOs aiming at the elucidation and application of enzymatic methane oxidation (7–12).

Highly sensitive single-cell methanol biosensors are, therefore, expected to be useful for the selection of active forms of recombinant MMOs *in vivo*. There were several reports of C_1 compounds biosensor strains built with non-methylotrophic *Escherichia coli* based on chimeric two-component systems (13–15) or combination of transcription factors and C_1 compounds-converting enzymes (16). Takeya et al. (17) utilized methylotrophic yeast *Pichia pastoris* (*Komagataella phaffii*) as a host for the development of methanol-sensing cells. They constructed recombinant yeast strains expressing a fluorescent protein under the control of methanol-inducible promoters (18,19), and observed actual change in fluorescence in response to intracellular methanol formed by pectin methylesterase, demonstrating successful evaluation of *in vivo* activity levels.

This study focused on the development of methanol-sensing strains of *M. extorquens*. Two promoters were chosen based on previously observed changes in gene expression and enzymatic activity in response to growth substrates. (4,20). Skovran et al. (4) reported that expression of serine cycle genes and enzymatic activity of serine-glyoxylate aminotransferase (Sga) considerably increased after the transition from succinate growth to methanol growth. Similarly, methanol oxidation activity during methanol growth was much higher than that during succinate growth (4,20), although the gene expression of the methanol dehydrogenase (MDH) subunit gene *mxaF* temporarily decreased in the initial phase of the post-methanol transition (4). In this study, the promoter regions of *sga* and *mxaF*, fused to *gfp*, were inserted into the chromosome of *M. extorquens* and the methanol sensing properties of the resulting strains were evaluated. Fluorescence-activated cell sorting (FACS) analysis demonstrated the high methanol-sensing ability of the *sga* promoter-based strain.

MATERIALS AND METHODS

Bacterial strains and plasmids *E. coli* strains DH5α and JM109 were used as host strains for general genetic engineering. *M. extorquens* wild-type strain AM1 and the derivative recombinant strains were routinely cultivated at 30°C in nutrient rich (NR) medium containing 1% (w/w) meat extract, 1% (w/v) polypeptone, 0.2% (w/w) yeast extract dissolved in tap water, or hypho minimal medium (21) supplemented with the appropriate carbon source. Kanamycin (100 μg/mL) or ampicillin (100 μg/mL) was added into the medium when necessary.

Construction of plasmids and strains The primer sequences used in this study are listed in Table S1. The promoter region for *sga* (P_{sga}), a 662-bp intergenic region between *sga* (Mex_1p1726) and a hypothetical protein gene (Mex_1p1725), was amplified by PCR using the genomic DNA of *M. extorquens* AM1 as template and primer pair Psga-5/Psga-3Sal. Inverse PCR using primers depC-Inv1/depC-Inv2 was performed on pK18-Δ*depC* (22) in order to amplify the plasmid backbone along with flanking regions (approximately 1000 bp each) of *depC* (Mex_1p4148). The P_{sga} region and the inverse PCR product were ligated, forming plasmid pK18-P*sga*Δ*depC*. A *gfp-rrnB* terminator (T_{rrnB}) region was amplified by PCR using pBLac-gfp (23) as template with primer pair gfp-5Sal/rrnB-3. A linear fragment of pK18-P*sga*Δ*depC*, cut downstream of P*sga*, was prepared by inverse PCR using primer pair Psga-3Sal/depC-Inv2. The amplified fragments were digested by SalI and ligated to each other. The resulting plasmid was designated pK18-P*sga*gfpTrrnB.

pK18-P*mxaF*gfpTrrnB was constructed by replacing P_{sga} in pK18-P*sga*gfpTrrnB with the promoter region of *mxaF* (P_{mxaF}, a 406 bp-intergenic region between *mxaF* (Mex_1p4538) and *mxaJ* (Mex_1p4537)). The P_{mxaF} region was amplified using the genomic DNA of *M. extorquens* AM1 as template and primers PmxaF-5/PmxaF-3Sal. The pK18-P*sga*gfpTrrnB backbone lacking P_{sga} was prepared by inverse PCR using pK18-P*sga*gfpTrrnB as template and primer pair depC-Inv1/gfp-5Sal. These fragments were subsequently digested by SalI and then ligated to each other to obtain pK18-P*mxaF*gfpTrrnB.

Electroporation of the constructed vectors into the *M. extorquens* wild-type strain AM1, and selection of transformants formed via pop-in/pop-out recombination were done as described previously (24,25). The genotype of the resulting *M. extorquens* strains AM1Δ*depC*::P*sga*gfp and AM1Δ*depC*::P*mxaF*gfp were confirmed by PCR with the appropriate primer pairs.

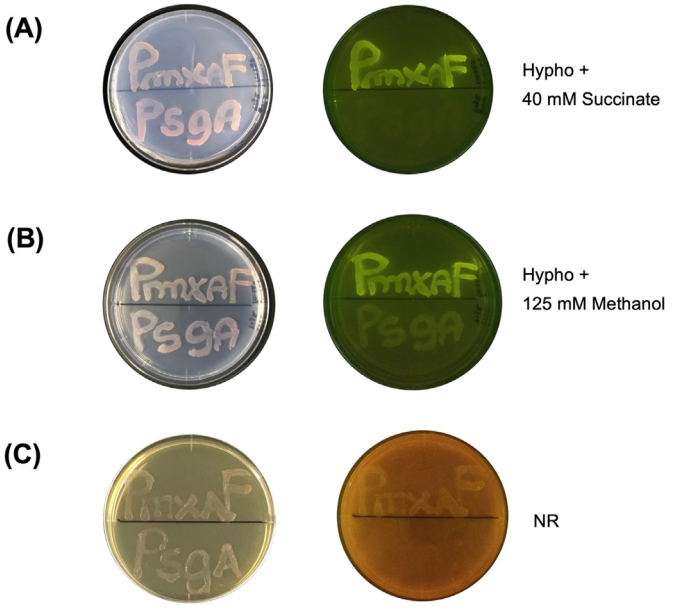

FIG. 1. GFP fluorescence of *M. extorquens* strains AM1Δ*depC*::P*mxaF*gfp and AM1Δ*depC*::P*sga*gfp grown on agar medium plate. (A, B) Hypho minimal medium plate with the addition of succinate 0.5% (v/v) (A) or methanol 0.5% (v/v) (B) as a sole carbon source. Cells were pre-cultivated in hypho minimal medium with succinate 0.5% (v/v) at 30°C for 48 h. (C) NR medium plate. Cells were pre-cultivated in NR medium at 30°C for 72 h. Plate cultivation was done at 30°C for several days. The same culture plate is shown under normal light (left) and blue LED light of 470 nm (right).

Spectrofluorometry and fluorescence microscopy *M. extorquens* strains were pre-cultivated in 50 mL hypho minimal medium supplemented with 0.5% (w/v) succinate as a carbon source at 30°C for 48 h with reciprocal shaking (120 strokes/min). The cells were harvested, resuspended with fresh hypho minimal medium, and then inoculated into 100 mL hypho minimal medium containing various concentrations of methanol and/or 40 mM succinate with an initial OD_{600} of 0.1. The cultivation was done at 30°C with shaking.

Ten mL of the culture broth were taken at appropriate intervals, and the cells were collected by centrifugation at 10,000 *g*, 4°C for 5 min, and resuspended in the same volume of 50 mM Tris–HCl buffer (pH 7.5). Fluorescent emission at 510 nm (488 nm excitation) by the green fluorescent protein (GFP) expressed in the whole cell was recorded with a fluorescence spectrophotometer F-7000 (Hitachi, Tokyo, Japan). Fluorescence unit per OD_{600} of the suspension was used for evaluation of the sensor cells.

Fluorescence microscopy was performed using a motorized inverted microscope (IX81; Olympus, Tokyo, Japan) equipped with an Uplan-Apochromat 100×/1.35 NA oil iris objective lens. Images were captured with a charge-coupled device camera DP30BW (Olympus) at a fixed exposure time of 200 ms, and the data were acquired and analyzed by using the MetaMorph software version 7.0 (Molecular Devices, San Jose, CA, USA).

Flow cytometry Flow cytometry was performed with FACSAria IIIu (Becton Dickinson, San Jose, CA, USA) according to the previous report regarding methanol-sensing yeast (17), with slight modifications due to the smaller cell sizes of bacteria when compared to yeast. The cells were resuspended in ice-cold phosphate-buffered saline and kept on ice prior to the analysis. For flow cytometry, the forward scatter (FSC) and side scatter (SSC) were set at a photomultiplier tube (PMT) voltage of 250 and 300, respectively, with a threshold of 2000. GFP fluorescence was excited at 488 nm, and the emission at 530/30 nm was detected at a PMT voltage of 580. At least 30,000 cells were analyzed per sample. FACSDiva 8 software (Becton Dickinson) was used both for data acquisition and creation of scatter plots.

RESULTS

Construction of methanol sensor strains Two molecular sensor strains of *M. extorquens* for methanol detection were constructed by cloning a GFP gene downstream of the promoter of the gene encoding serine glyoxylate aminotransferase (Sga), an enzyme in the serine cycle, or downstream of the promoter of methanol dehydrogenase subunit 1 precursor (MxaF), involved in methanol oxidation. Each promoter-*gfp-rrnB* terminator cassette

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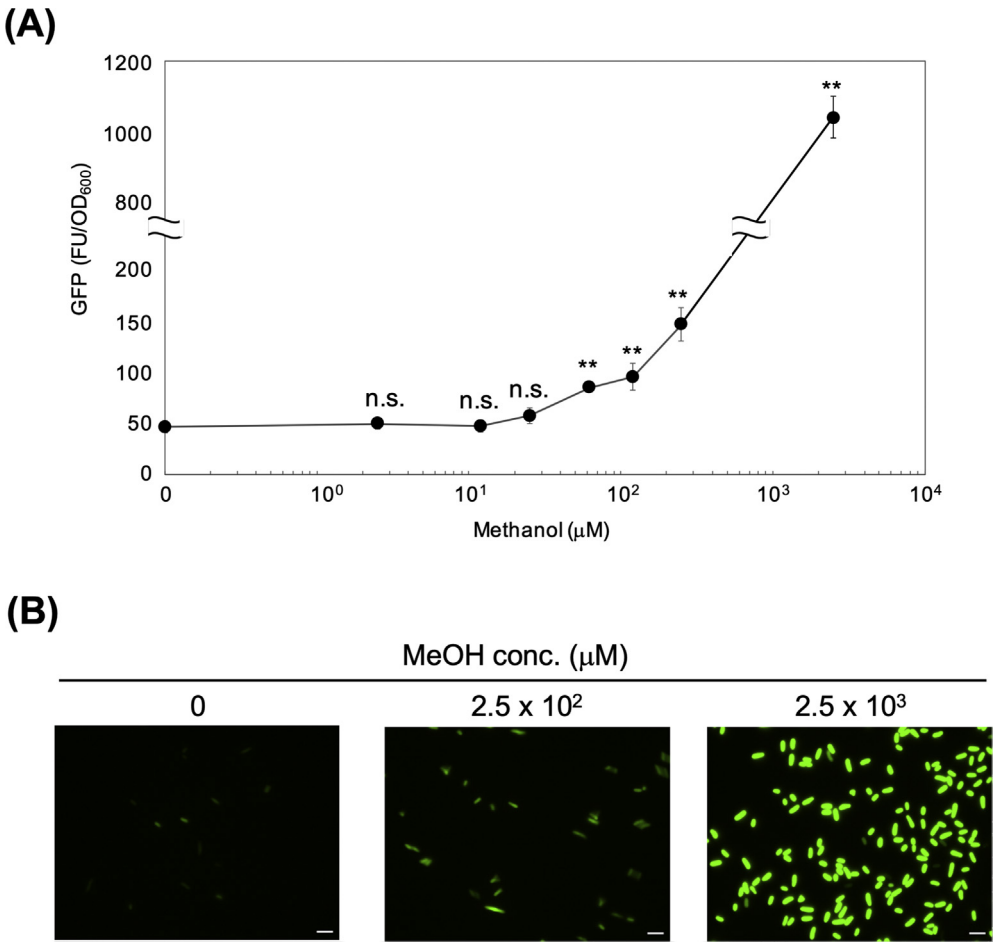

FIG. 2. Effects of methanol concentration on GFP fluorescence of the *M. extorquens* AM1Δ*depC*::P*sga*gfp sensor strain. (A) GFP fluorescence measured by a fluorescence spectrophotometer. Statistical significance of the observed fluorescence levels against those obtained at 0 μM methanol was calculated by two-tailed paired *t* test (nonsignificant, n.s., $p > 0.05$; *$p < 0.05$; **$p < 0.01$). Measurements were performed with three independent cultivations and the fluorescence unit values were normalized to cell concentration (OD_{600}). (B) Fluorescent microscopy images of *M. extorquens* AM1Δ*depC*::P*sga*gfp sensor cells grown in various methanol concentrations indicated in the panels. Scale bars in the images are all 5 μm.

was inserted into the poly (3-hydroxybutyrate) depolymerase C gene (*depC*) locus in the chromosome of the wild type strain AM1. It has been reported that disruption of *depC* has no influence on both the methylotrophic growth and the PHA production of *M. extorquens* (22). The occurrence of the desired homologous recombination was confirmed by PCR using primer pairs bound to *gfp* and to regions adjacent to the homologous regions used for the recombination (data not shown).

In the constructed strains, the GFP expression in response to methanol was roughly evaluated on plate media, as shown in Fig. 1. The cells pre-grown on succinate were inoculated onto a hypho minimal medium plate containing 40 mM succinate or 125 mM methanol, and onto a NR medium plate. The strain harboring the P_{mxaF} promoter cassette (AM1Δ*depC*::P*mxaF*gfp) showed very high fluorescence both on succinate and on methanol, and *gfp* expression could also be observed on the NR medium. Conversely, the other strain harboring the P_{sga} promoter cassette (AM1Δ*depC*::P*sga*) exhibited GFP fluorescence only when methanol was available. Although the fluorescence was lower than that of the strain harboring the P_{mxaF} promoter cassette, the AM1Δ*depC*::P*sga*gfp cells did not emit fluorescence on the succinate-containing and on the NR media plates. The weaker promoter activity of P_{sga} on methanol is consistent with a previous study reporting that GFP expression regulated by P_{sga} was about 5% of that controlled by P_{mxaF} in *M. extorquens* (26). Considering the faint background fluorescence in the absence of methanol, the P_{sga}-based strain showed potential as a highly specific sensor toward methanol.

Sensitivity to methanol of *M. extorquens* AM1Δ*depC*::P*sga*gfp The effects of methanol concentration on the cellular fluorescence intensity of the AM1Δ*depC*::P*sga*gfp sensor strain was investigated using a spectrofluorometer to evaluate the response and sensitivity to methanol. The cells pre-cultured on succinate were incubated in hypho minimal media containing various concentrations of methanol as sole carbon source (0 μM–2.5×10^3 μM) for about 72 h. The fluorescence intensity in the cells increased as the methanol concentration in the medium increased (Fig. 2A). The lowest detectable concentration of methanol by this procedure was 62 μM ($p < 0.05$ by two-tailed test comparing with cells incubated in media not containing a carbon source). The dose-dependent increase in GFP fluorescence was also observed in fluorescence microscopy (Fig. 2B).

The fluorescence intensity of the AM1Δ*depC*::P*sga*gfp strain was further evaluated by FACS at the single-cell level. The cells grown on succinate were transferred into media containing methanol with concentrations ranging from 0 μM to 2.5×10^2 μM, and subjected to FACS analysis after a 4-h incubation. Single cells were analyzed after excluding dividing/multiple cells as well as other particles distinguished by areas of forward (FSC-A) and side (SSC-A) scatters, respectively. As shown in Fig. 3A, higher methanol concentrations led to larger GFP-A values owing to a dose-dependent response of *gfp* expression. The positive correlation between GFP-A values and methanol concentration demonstrated the biological sensing of low concentrations of methanol using bacterial cells. A

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statistically significant increase of the GFP-A value could be observed at 25 μM, where about half of the cells showed a higher GFP-A value than the maximum fluorescence level shown by the cells grown in the absence of methanol (Fig. 3A and B). Single-cell level analysis combining whole-cell biosensors and flow cytometry proved to be a highly sensitive procedure for methanol detection.

Time-dependent response of GFP fluorescence to methanol Succinate-grown cells of the AM1Δ*depC*::P*sgag*fp strain were inoculated into hypho minimal media containing 2.5 mM or 125 mM methanol, and the GFP fluorescence in the cells was measured at 4 h, 12 h, 24 h, 36 h, and 48 h using a spectrofluorometer (Fig. 4). It has been reported that, except for enolase, expression of serine cycle genes and activities of the corresponding enzymes increased immediately after the transition of carbon source from succinate to methanol (4). Concordantly, we observed fluorescence levels that were higher than basal levels in the sensor cells at approximately 4 h after the transfer of the cells to the methanol medium. The GFP expression increased in a time-dependent manner until 36 h on 125 mM methanol, while fluorescence levels

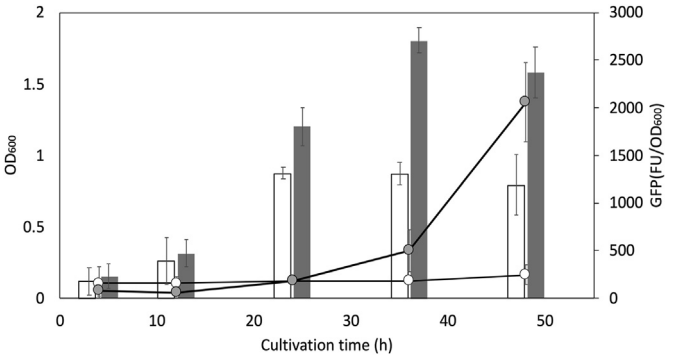
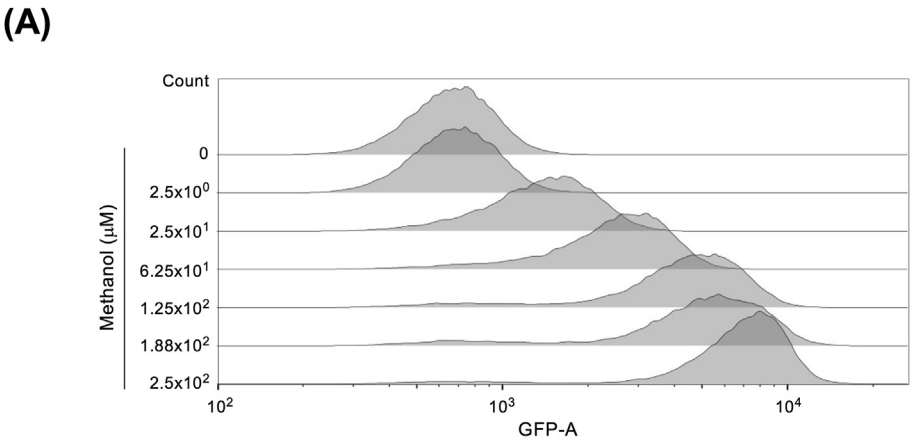


FIG. 4. Growth (lines) and time-dependent response of GFP fluorescence to methanol (bars) of *M. extorquens* AM1Δ*depC*::P*sgag*fp in hypho minimal medium with the addition of 2.5 mM (open bars) or 125 mM methanol (closed bars). GFP fluorescence was determined by spectrofluorometry at different cultivation time points.



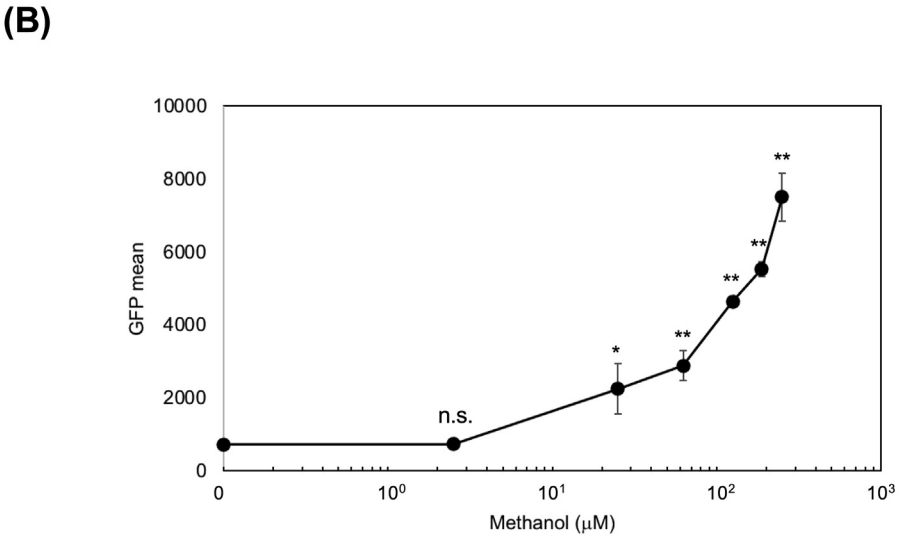


FIG. 3. Determination of GFP fluorescence in the *M. extorquens* AM1Δ*depC*::P*sgag*fp sensor strain by FACS analysis. (A) Distribution of GFP fluorescence (GFP-A) of the cells cultivated in different methanol concentrations. (B) Average of the GFP-A mean of triplicate samples at various concentrations of methanol. Statistical significance of the fluorescence levels against those obtained at 0 μM methanol were calculated by a two-tailed paired *t* test (n.s., $p > 0.05$; *$p < 0.05$; **$p < 0.01$).

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TABLE 1. GFP fluorescence intensity of the cells of *M. extorquens* AM1Δ*depC*::P*sga*gfp cultivated on methanol or multi-carbon sources.

Medium	Supplemented carbon source(s)	FU/OD_{600}	OD_{600}
Hypho	2.5 mM methanol	1401 ± 18	0.219 ± 0.010
	2 mM succinate	ND	0.433 ± 0.006
	2 mM succinate + 2.5 mM methanol	705 ± 50	0.495 ± 0.027
NR	–	ND	0.966 ± 0.049
	2.5 mM methanol	290 ± 15	1.253 ± 0.064

The cells cultivated in hypho minimal media containing 0.5% (w/v) succinate for 2 days were transferred into each medium and cultivated for 24 h. GFP fluorescence was determined by spectrofluorometry as described in materials and methods.

reached saturation after 24 h on 2.5 mM methanol, probably due to exhaustion of the carbon source.

Repression of *gfp* expression on mixed carbon sources The expression of serine cycle genes in *M. extorquens* is repressed when substrates other than methanol are available (27). Thus, we investigated the effects of multicarbon sources coexisting with methanol on the *gfp* expression of the AM1Δ*depC*::P*sga*gfp sensor strain (Table 1). The fluorescence in the hypho minimal medium containing 2.5 mM methanol and 2 mM succinate was 705 FU/OD_{600} after a 48-h cultivation, which was about half that of the cells cultivated solely on methanol (1401 FU/OD_{600}). Lower GFP expression was also observed in the NR medium, as the fluorescence was as low as 290 FU/OD_{600} even in the presence of 2.5 mM methanol.

DISCUSSION

In this study, the molecular machinery regulating the expression of genes involved in the methanol metabolism of the methylotrophic bacterium *M. extorquens* was applied for the establishment of methanol-sensing cells. Two different promoter regions, P_{mxaF} and P_{sga}, were selected to respond to methanol, and a promoter-*gfp*-terminator cassette was inserted into the *depC* locus in the chromosome of the wild-type strain AM1. P_{mxaF}, the native promoter region of the *mxa* operon involved in methanol oxidation, is the most widely used promoter for overexpression of target genes in *M. extorquens* under methanol growth conditions. In this study, we observed that the strain harboring the P_{mxaF}-*gfp* fusion emitted fluorescence when grown on succinate as well as when grown on methanol. This result disagreed with the drastic increase in methanol dehydrogenation activity, mainly mediated by MxaFI, after transition of the cells from succinate to methanol (4,20), while it was consistent with previous proteomic and transcriptomic analyses showing considerable expression of the *mxa* genes in succinate-grown cells (4,28). In *M. extorquens*, there are two distinct types of MDHs, PQQ-dependent MxaFI and lanthanide-dependent XoxF. It has been proposed that transcription of MDH genes is modulated by lanthanide availability (29,30). Although the lanthanide-dependent expression regulation has not been well understood to date, transcription by P_{mxaF} has been hypothesized to be activated by MxcQE and MxbDM two-component systems even on multicarbon substrates in the absence of lanthanides (29,30). As suggested previously (4), the low methanol dehydrogenation activity in succinate-grown cells of *M. extorquens* is likely due to post-translational modification of MxaFI. We here evaluated P_{mxaF} as a methanol-responding promoter taking into consideration past reports of successful protein expression in *M. extorquens* under methylotrophic growth conditions, however, the background transcription activity without methanol in the absence of lanthanides turned out to be higher than expected. We, therefore, concluded that P_{mxaF} was not suitable for methanol detection. In contrast, gene expression and enzymatic activity of serine glyoxylate aminotransferase (Sga) were tightly repressed on succinate, and induced by transition of the cells into the methanol medium (4,20). In support of the previous observation, insignificant levels of fluorescence were observed when the cells harboring the P_{sga}-*gfp* cassette were cultivated on succinate and NR medium plates, while the *gfp* expression was significantly stronger on the methanol medium plates. The strain harboring P_{sga}-*gfp*, AM1Δ*depC*::P*sga*gfp, was used for further analyses as the sensor strain successfully responding to methanol.

The sensor cells emitted fluorescence by sensing methanol in a dose-dependent manner. The fluorescence intensity was consistently increased until 36 h of cultivation under excessive concentrations of methanol (125 mM). Although the strain responded to very low concentrations of methanol (25 μM) when methanol was the only available carbon source, the fluorescence decreased in the medium containing multi-carbon compounds coexisting with methanol. It has been reported that intracellular activities of Sga and hydroxypyruvate reductase were much lower on mixed substrates of methanol and succinate than in methylotrophic growth conditions (31). Repression of the initial steps of the serine cycle during co-consumption of methanol and succinate was also suggested by metabolomic analyses (27). This repression is probably due to the weak binding of a Lys-R type regulator QscR (32–34), a transcriptional activator of the serine cycle genes, to the promoter regions in the presence of multi-carbon substrates. It is plausible to infer that the additional copy of P_{sga}, inserted into the *depC* locus, is also subject to the regulation mechanisms mediated by QscR. Optimization of cultivation conditions is, therefore, important for highly sensitive methanol detection by the whole-cell sensor based on the *M. extorquens* platform.

It has been reported that a methanol-sensing *E. coli* strain equipped with a chimeric two-component system using MxaY or FlhS derived from *Paracoccus denitrificans* (13,14), or chimeric MxcQZ/OmpR derived from *Methylobacterium organophilum* (15), detected concentrations as low as 0.001% (250 μM) methanol, whereas the responses did not depend on methanol concentration. Although a combination of a formaldehyde-responsive transcription factor derived from *E. coli* and methanol dehydrogenase derived from *Bacillus stearothermophilus* enabled *E. coli* to sense methanol with concentration dependency, the lowest detectable concentration was 25 mM (16). The methylotrophic bacterium-based sensor strain constructed in this study showed 10^3-10^4 times higher sensitivity to methanol, with dose-dependency, than the previously reported *E. coli* sensor cells. As mentioned above, Takeya et al. (17) developed a methylotrophic yeast-based methanol sensor strain and detected concentrations as low as 2.5 μM methanol by FACS under optimized analytical conditions. The sensitivity of the bacterial sensor was thought to be as high as that of the yeast system since a higher resolution between the fluorescence distribution emitted at 25 μM methanol and the basal-level fluorescence without methanol could be observed when comparing to the corresponding distributions by the yeast sensor strain. Taken together, whole-cell sensors using the intrinsic regulatory machineries of methylotrophic microorganisms appear to be suitable for highly sensitive detection of C_1 compounds. In the future, functional C_1-converting enzymes obtained through screening with methylotrophy-based sensor cells are expected to be useful in the C_1-bioindustry.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2021.05.002.

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