

# CO-Dependent H<sub>2</sub> Production by Genetically Engineered *Thermococcus onnurineus* NA1

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Hydrogenogenic CO oxidation ( $\text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + \text{H}_2$ ) has the potential for H<sub>2</sub> production as a clean renewable fuel. *Thermococcus onnurineus* NA1, which grows on CO and produces H<sub>2</sub>, has a unique gene cluster encoding the carbon monoxide dehydrogenase (CODH) and the hydrogenase. The gene cluster was identified as essential for carboxydrotrophic hydrogenogenic metabolism by gene disruption and transcriptional analysis. To develop a strain producing high levels of H<sub>2</sub>, the gene cluster was placed under the control of a strong promoter. The resulting mutant, MC01, showed 30-fold-higher transcription of the mRNA encoding CODH, hydrogenase, and Na<sup>+</sup>/H<sup>+</sup> antiporter and a 1.8-fold-higher specific activity for CO-dependent H<sub>2</sub> production than did the wild-type strain. The H<sub>2</sub> production potential of the MC01 mutant in a bioreactor culture was 3.8-fold higher than that of the wild-type strain. The H<sub>2</sub> production rate of the engineered strain was severalfold higher than those of any other CO-dependent H<sub>2</sub>-producing prokaryotes studied to date. The engineered strain also possessed high activity for the bioconversion of industrial waste gases created as a by-product during steel production. This work represents the first demonstration of H<sub>2</sub> production from steel mill waste gas using a carboxydrotrophic hydrogenogenic microbe.

Carbon monoxide (CO) is highly toxic to most living creatures, but it can be utilized by microorganisms as an energy and carbon source for the production of fuels and chemicals, such as acetate, butyrate, ethanol, butanol, and H<sub>2</sub>. Among those carboxydrotrophic microbes, CO-dependent H<sub>2</sub> production has been observed in three distinct groups, i.e., mesophilic bacteria, thermophilic bacteria, and hyperthermophilic archaea (1–3). Generally, growth rates of the mesophilic hydrogenogenic bacteria on CO are low, and high levels of CO are inhibitory. Predominant within this group are nonsulfur purple bacteria, including *Rubrivivax gelatinosus* and *Rhodospirillum rubrum*, which require light for optimal cell growth. Although *Rhodopseudomonas palustris* P4 is capable of hydrogenogenic CO conversion in the dark, it does not grow under this condition (4). Nonphototrophic *Citrobacter* strain Y19 also converts CO to H<sub>2</sub>, but it only grows slowly under anaerobic conditions and an aerobic growth phase is required to generate sufficient biomass before the anaerobic CO conversion phase (5). The second group includes thermophilic, hydrogenogenic bacteria isolated from freshwater and marine environments with temperatures ranging from 40 to 85°C. *Carboxydotherrmus hydrogeniformans*, *Carboxydocella thermautotrophica*, *Thermosinus carboxydivorans*, and *Caldanaerobacter subterraneus* subsp. *pacificus* are capable of chemolithotrophic growth on high concentrations of CO. The third group includes two hydrogenogenic CO-converting archaea, *Thermococcus* sp. strain AM4 and *Thermococcus onnurineus* NA1 (6, 7). Both strains are hyperthermophiles isolated from deep-sea hydrothermal vents and can grow on 100% CO (8).

Anaerobic carboxydrotrophic hydrogenogenic prokaryotes conserve energy by performing the following well-known water-gas shift reaction:  $\text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + \text{H}_2$  ( $\Delta G^{\circ\prime} = -20 \text{ kJ/mol}$ ) (33). In this metabolism, CO is oxidized by a carbon monoxide dehydrogenase (CODH) and electrons are transferred to an energy-converting hydrogenase that reduces protons to H<sub>2</sub>. These enzymatic systems have been studied most extensively in *R. rubrum*

and *C. hydrogeniformans* (9–11). In these organisms, the enzymes are encoded by a gene cluster comprised of a CODH gene (*cooS*), a ferredoxin-like protein gene (*cooF*), genes for a six-subunit hydrogenase, and genes encoding accessory proteins. *Thermococcus barophilus* MP is also likely to possess the capacity for hydrogenogenic carboxydrotrophy due to the presence of a gene cluster with close similarity to those from two *Thermococcus* strains mentioned above (12). The three gene clusters in *Thermococcus* strains are distinguished from those in mesophilic or thermophilic bacteria by the presence of genes encoding Na<sup>+</sup>/H<sup>+</sup> antiporter (see Fig. S1 in the supplemental material).

The hydrogenogenic CO-converting reaction has a potential application in the production of H<sub>2</sub> gas as a clean renewable fuel from waste gases or synthesis gas (syngas). Syngas is a mixture of mainly H<sub>2</sub>, CO, CO<sub>2</sub>, N<sub>2</sub>, and CH<sub>4</sub>, produced by partial oxidation or autothermal reforming of hydrocarbon-rich fossil fuels, domestic and agricultural wastes, and other biomass. Biological processes, while slower than chemical reactions, have a number of advantages such as higher yields, specificity, requirement of minimum energy due to lower operating temperatures and pressures, and lower cost (13). Bioconversion of syngas to H<sub>2</sub> was investigated in *R. rubrum* (14–16). Producer syngas had no negative effect on growth rates, biomass production, H<sub>2</sub> production, or CO

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consumption (17). Industrial waste gases created as a by-product during steel production could be an attractive source of CO and have been targeted for the production of both ethanol and 2,3-butanediol using acetogenic bacteria (18, 19). So far, however, this application has not been reported using carboxydrotrophic hydrogenogenic microbes.

Over the years, numerous studies have been performed to improve the H<sub>2</sub> productivity from CO with regard to selecting strains resistant to CO toxicity and stimulating CO gas-liquid mass transfer rates (16, 20, 21). In the present study, a different approach to increase the H<sub>2</sub> productivity was examined. The expression of the CODH gene cluster was upregulated. This experiment took advantage of the recently developed genetic system in *T. onnurineus* NA1 (22). Here, we identify the CODH gene cluster responsible for hydrogenogenic CO-converting activity. The strain constructed to overproduce the CODH-hydrogenase enzyme complex possessed higher H<sub>2</sub> productivity than did the wild-type strain. Using the engineered strain, the potential of H<sub>2</sub> production from steel mill waste gas was investigated for the first time.

## MATERIALS AND METHODS

**Strain, media, and culture conditions.** *T. onnurineus* NA1 (KCTC 10859) was isolated from a deep-sea hydrothermal vent area in the Papua New Guinea-Australia-Canada-Manus (PACMANUS) field (23). This strain was routinely cultured in YPS (yeast extract-peptone-sulfur) medium as previously reported (23).

The modified medium 1 (MM1) (7, 22) was prepared with 1 g liter<sup>-1</sup> yeast extract, 35 g liter<sup>-1</sup> NaCl, 0.7 g liter<sup>-1</sup> KCl, 3.9 g liter<sup>-1</sup> MgSO<sub>4</sub>, 0.4 g liter<sup>-1</sup> CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.3 g liter<sup>-1</sup> NH<sub>4</sub>Cl, 0.15 g liter<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, 0.03 g liter<sup>-1</sup> NaSiO<sub>3</sub>, 0.5 g liter<sup>-1</sup> NaHCO<sub>3</sub>, 0.5 g liter<sup>-1</sup> cysteine-HCl, and 0.001 g liter<sup>-1</sup> resazurin. One milliliter liter<sup>-1</sup> of Holden's trace element-Fe-EDTA solution (24) and 1 ml liter<sup>-1</sup> of Balch's vitamin solution (25) were added as a supplement to the medium. After autoclaving, the medium was kept in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) filled with an anoxic gas mixture (N<sub>2</sub>:H<sub>2</sub>:CO<sub>2</sub>, 90:5:5) to equilibrate, and the final pH of the medium was adjusted to 6.5 with 2 N HCl.

For the cultures in serum bottles, the media were reduced with 0.005% Na<sub>2</sub>S·9H<sub>2</sub>O, and the headspaces were filled with 100% CO (MM1-CO). The serum bottles were sealed with bromobutyl rubber stoppers and aluminum crimp caps.

For the pH-stat batch culture, *T. onnurineus* strain NA1 was serially cultured in a 100-ml serum bottle and 3-liter bioreactors (Fermentec, Cheongwon, South Korea), the working volumes of which were 50 ml and 2 liters, respectively, at 80°C. For the cultures in bioreactors, MM1 was supplemented with 10 g liter<sup>-1</sup> yeast extract and 10 times more Holden's trace element-Fe-EDTA solution. The bioreactors were sparged with pure argon gas (99.999%) through a microsparger. The agitation speed was 300 rpm, and the pH was controlled at 6.1 to 6.2 using 0.2 M NaOH in 3.5% NaCl. The inlet gas of 100% CO was supplied by using a mass flow controller (MKPrecision, Seoul, South Korea) at feeding rates in the range of 40 to 400 ml min<sup>-1</sup>. The steel mill waste gas (composition, 57.4% CO, 25.9% N<sub>2</sub>, 13.9% CO<sub>2</sub>, and 2.8% H<sub>2</sub>; collected from a Linz-Donawitz gas [LDG] furnace in a Hyundai Steel plant in Dangjin, South Korea) was used for bioreactor cultures at rates of 60 or 180 ml min<sup>-1</sup>. The gas outlet was open to let the H<sub>2</sub> and CO<sub>2</sub> gases escape and maintain the total pressure at 10<sup>5</sup> Pa.

**Analytical methods.** Cell growth was monitored by measuring optical density at 600 nm (OD<sub>600</sub>) with a biophotometer plus a UV-visible spectrophotometer (Eppendorf, Hamburg, Germany). Biomass was determined by measuring the amount of cellular proteins of cell lysates using the DC protein assay kit (Bio-Rad, Hercules, CA) based on the assumption that protein comprises approximately 50% of dry cell weight (DCW) (26). The unit value of OD<sub>600</sub> corresponded to 0.361 g (DCW) liter<sup>-1</sup>. The H<sub>2</sub> production rate was calculated on the basis of H<sub>2</sub> content in the gases

produced from a bioreactor, and the gas flow rate was measured with a wet gas meter (Shinagawa, Tokyo, Japan).

The amounts of CO, H<sub>2</sub>, and CO<sub>2</sub> were measured by using a YL6100 gas chromatograph (GC) (YL Instrument Co., Anyang, South Korea) equipped with a Molsieve 5A column (Supelco, Bellefonte, PA), a Porapak N column (Supelco), a thermal conductivity detector, and a flame ionization detector. Argon was used as the carrier gas at a flow rate of 30 ml min<sup>-1</sup>.

**DNA microarray analysis.** A custom microarray was manufactured by Roche NimbleGen (Basel, Switzerland). Six unique 60-mer oligonucleotides for all of the predicted 1,986 open reading frames (ORFs) in the annotated genome of *T. onnurineus* NA1 were designed and synthesized. Isolation of mRNA, labeling, and hybridization were performed as described previously (22). After hybridization, arrays were scanned with a GenePix 4000B scanner (Molecular Devices), and the data were extracted using NimbleScan 2.4 software (Roche NimbleGen, Basel, Switzerland). Data analysis was performed using GeneSpring GX 7.3.1 (Agilent Technologies, Palo Alto, CA).

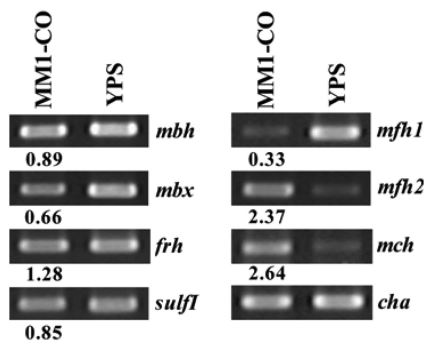
**Reverse transcription-PCR (RT-PCR).** cDNAs were synthesized from 350 ng of total RNA using SuperScript II reverse transcriptase, and PCRs were performed with gene-specific primers using rTaq DNA polymerase (TaKaRa, Otsu, Japan) (see Table S1 in the supplemental material). Expression levels were calculated using GellPro32 software (Media Cybernetics, Bethesda, MD) using a chaperonin-encoding gene (*cha*, TON\_1276) as a control to normalize expression levels.

**Quantitative reverse transcription-PCR (RT-qPCR).** RNA was prepared with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions with some modifications. Genomic DNA was eliminated by DNase I (Thermo Scientific Fermentas, St. Leon-Rot, Germany) treatment. One microgram of RNA was incubated with 1 unit of DNase I at 37°C for 30 min and purified through chloroform extraction and ethanol precipitation. RNA was quantified with a spectrophotometer, and cDNA was created using Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Thermo Scientific Fermentas, St. Leon-Rot, Germany). One microgram of RNA was incubated with 40 units of reverse transcriptase, 5 μM random hexamers, and 1 mM deoxynucleoside triphosphate (dNTP) at 37°C for 1 h in reverse transcription buffer (1×, as supplied by the enzyme manufacturer). The reaction products were serially diluted to find the adequate concentration for real-time PCR analysis, and the samples were amplified with SYBR green real-time PCR master mix (Toyobo, Osaka, Japan). Amplified signals were detected using the StepOnePlus system (Applied Biosystems, Foster City, CA), and all primers used were as listed in Table S1 in the supplemental material. The relative amount of each gene was calculated from cycle threshold (C<sub>T</sub>) values using a relative standard curve after normalization with corresponding 16S rRNA (TON\_1979) quantity.

**Gene disruption.** Mutants of each large subunit of *mfh2* (TON\_1569) and *mch* (TON\_1023) hydrogenases and the MC01 (TON\_1016) mutant were constructed by applying the gene disruption system used for a hyperthermophilic archaeon, *Thermococcus kodakarensis* KOD1 (27). Briefly, target genes were replaced with the P<sub>gdh</sub>-*hmgP<sub>flu</sub>* cassette through homologous recombination. *T. onnurineus* NA1 cells were transformed and incubated in the presence of 10 μM simvastatin as a selection marker. The sequences of the primers used for gene disruption and construct verification are given in Table S1 in the supplemental material.

**Western blotting.** Western blots were prepared and analyzed using a chemiluminescent dye with the Immun-Star horseradish peroxidase (HRP) chemiluminescent kit (Bio-Rad, Hercules, CA). Antibodies were generated against each protein encoded by TON\_1018 and TON\_1023, which were overexpressed in *Escherichia coli* Rosetta(DE3)pLysS cells (Stratagene, La Jolla, CA) and purified through His-Bind resin (Novagen, Madison, WI).

**Measurement of H<sub>2</sub> production activity.** Cell lysates of the wild-type strain and the MC01 mutant were prepared by sonication of exponentially growing cells (0.097 g, wet weight) in 2 ml of 50 mM Tris-HCl buffer (pH



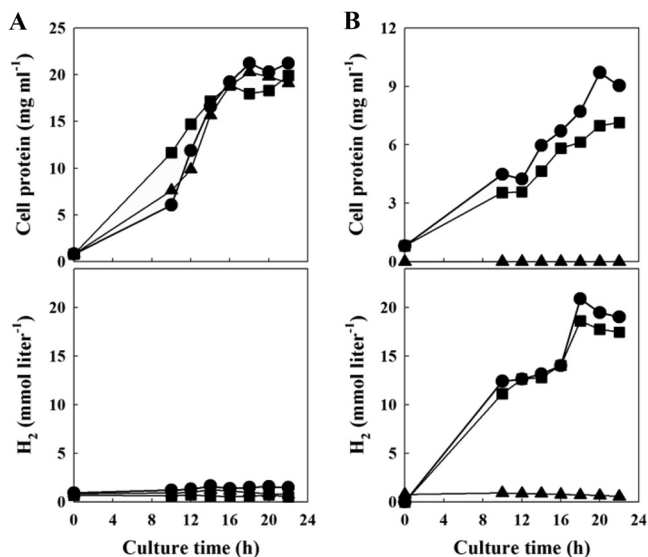
**FIG 1** Expression analysis of *T. onnurineus* NA1 hydrogenase gene clusters by RT-PCR on MM1-CO. RT-PCR analysis of each large subunit of *mbh* (TON\_1593), *mbx* (TON\_0489), *frh* (TON\_1560), *sulfI* (TON\_0534), *mch* (TON\_1023), *mfh2* (TON\_1569), and *mfh1* (TON\_0276) hydrogenases was performed with samples grown on MM1-CO with 0.5 g liter<sup>-1</sup> of yeast extract and YPS medium. *cha*, a chaperonin-encoding gene, was used as a control to normalize the expression level. The numbers under PCR bands represent band intensities relative to those on YPS medium.

7.5) containing 5 mM MgSO<sub>4</sub>, 10% glycerol, 2 mM dithiothreitol, and a protease inhibitor cocktail tablet (Roche Applied Science, Madison, WI) and then centrifugation at 14,000 × g for 20 min to remove cell debris. Samples were resuspended in 40 mM imidazole buffer (pH 6.5) containing 600 mM NaCl, 30 mM MgCl<sub>2</sub>, 10 mM KCl, and 2 mM dithiothreitol at a concentration of 0.1 mg ml<sup>-1</sup>. The assay was carried out in glass vials, which were filled with 100% CO at 10<sup>5</sup> Pa and sealed with bromobutyl rubber stoppers. The amounts of CO, H<sub>2</sub>, and CO<sub>2</sub> were measured by using a gas chromatograph (GC).

## RESULTS AND DISCUSSION

**CODH gene cluster responsible for carboxydrotrophic hydrogenogenic metabolism.** It was postulated that the CODH gene cluster would play a key role in the carboxydrotrophic hydrogenogenic metabolism in *T. onnurineus* NA1. To identify the hydrogenase gene cluster(s) for CO-dependent H<sub>2</sub> production, a DNA microarray was used to profile the gene expression of several distinct gene clusters encoding seven [NiFe]-hydrogenases (*mbh*, *sulfI*, *sulfII*, *mfh1*, *mfh2*, *mch*, and *frh*) and one homolog similar to Mbx (membrane-bound oxidoreductase, *mbx*). Relative expression ratios were derived by comparing mRNA abundance levels in cells grown in a medium containing CO (MM1-CO medium) to those in cells grown in a sulfur-containing complex medium (YPS medium). While transcript levels of most genes remained constant (fold change in expression of 0.5 to 2) or downregulated (fold change of <0.5), four to 10 out of 16 genes in the CODH gene cluster (TON\_1016–1031) were upregulated more than 2-fold, depending on the concentration of yeast extract (see Fig. S2 in the supplemental material). Interestingly, a few genes (two to four out of 18) in the *mfh2* gene cluster (TON\_1563–1580) were also upregulated. The expression pattern in the DNA microarray data was validated by reverse transcription-PCR (RT-PCR), showing upregulation (fold change of >2) of two genes encoding the large subunits of the *mch* and *mfh2* hydrogenases (TON\_1023 and TON\_1569) (Fig. 1). It is very intriguing that the expression level of the *mfh2* gene cluster was increased in the presence of CO, because the cluster had been identified previously as being responsible for growth on exogenous formate (22).

The relative importance of the *mch* gene cluster for growth on CO was assessed by gene disruption of the large subunits of the



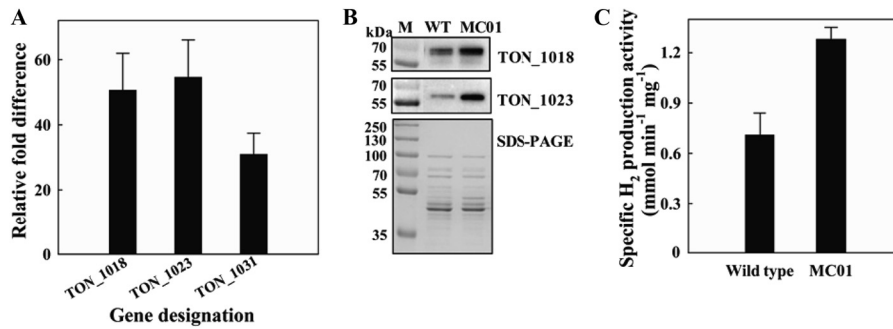
**FIG 2** Growth (top) and H<sub>2</sub> production (bottom) of wild-type (circles),  $\Delta mch$  (triangles), and  $\Delta mfh2$  (squares) strains on YPS medium (A) and MM1-CO with 0.5 g liter<sup>-1</sup> of yeast extract (B).

*mch* and *mfh2* hydrogenases (see Fig. S3 in the supplemental material). The growth of  $\Delta mch$  and  $\Delta mfh2$  mutants closely resembled the growth of the wild-type strain in YPS medium, so they seemed to be dispensable under this condition (Fig. 2A). However, in MM1-CO, the  $\Delta mch$  mutant showed a phenotype of severely impaired CO-dependent growth and H<sub>2</sub> production, which made it distinct from the  $\Delta mfh2$  mutant (Fig. 2B). This observation indicated that the *mch* gene was essential for carboxydrotrophic hydrogenogenic metabolism in *T. onnurineus* NA1.

**Overexpression of CODH gene cluster by promoter modulation.** Because the CODH gene cluster is intimately related to CO-dependent H<sub>2</sub> production, the gene cluster was overexpressed in an attempt to improve H<sub>2</sub> production from CO. In order to overexpress the CODH gene cluster, a strong constitutive promoter (*P<sub>gdh</sub>*), which drives expression of the gene encoding the glutamate dehydrogenase in *Thermococcus kodakarensis*, was inserted in front of the CODH gene cluster with a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase gene in *Pyrococcus furiosus* as a selection marker, substituting for the putative transcriptional regulator (TON\_1016) (see Fig. S3 in the supplemental material).

Quantitative reverse transcription-PCR (RT-qPCR) showed that the levels of transcripts for TON\_1018, TON\_1023, and TON\_1031 encoding subunits of CODH, hydrogenase, and Na<sup>+</sup>/H<sup>+</sup> antiporter, respectively, were 51.3-, 53.8-, and 31.5-fold higher in the engineered strain (MC01) than in the wild-type strain, in which transcription of the CODH gene cluster is controlled by the native promoter (Fig. 3A).

The expression level of proteins in the CODH gene cluster was monitored by Western blotting using polyclonal antibodies specific for the TON\_1018 and TON\_1023 proteins. The amounts of those proteins significantly increased in the MC01 mutant compared with the wild-type strain (Fig. 3B). In the MC01 mutant, the large subunit of *mch* hydrogenase (TON\_1023) was shown to be present exclusively in its mature form as the proteins migrated in a manner identical to that of proteins in the wild-type strain and



**FIG 3** Expression levels of genes in a *codh-mch-mnh3* gene cluster of the MC01 mutant on MM1-CO. (A) The mRNA quantity of three genes (TON\_1018, TON\_1023, and TON\_1031) of the wild-type strain and the MC01 mutant was measured by RT-qPCR and indicated as fold difference from the value of the wild-type strain, which is taken as 1. Error bars indicate the standard deviations from three independent experiments. (B) Western blot analysis of proteins encoded by TON\_1018 (67.7 kDa) and TON\_1023 (61.7 kDa). M, molecular mass marker; WT, wild-type strain. (C) H<sub>2</sub> production activity of cell lysates of the wild-type strain and MC01 mutant. Error bars indicate the standard deviations from three independent experiments.

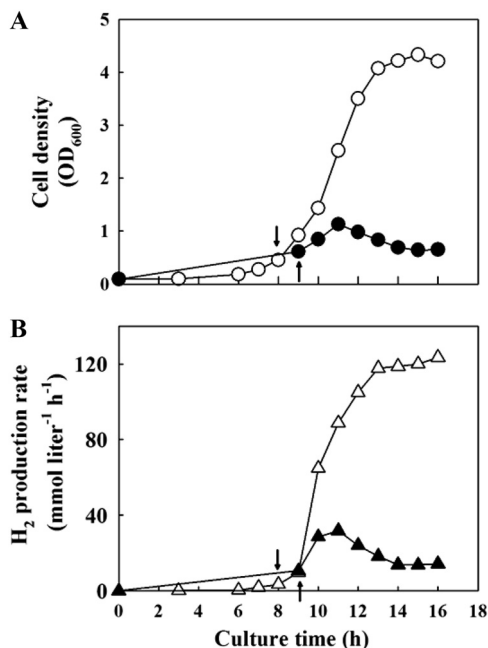
migrated faster than its precursor form (see Fig. S4 in the supplemental material).

The significantly increased CODH and Mch hydrogenase in the MC01 mutant might lead to an increased CO oxidation and hydrogenase activity. To corroborate this, cell lysates of the wild-type strain and the mutant were prepared, and the H<sub>2</sub> production activity was assayed based on the CO-dependent proton reduction activity. The specific H<sub>2</sub> production activity of the MC01 mutant was 1.8-fold higher than that of the wild-type strain (Fig. 3C).

**Measurement of growth and H<sub>2</sub> production.** Based on the increased enzymatic activity for CO-dependent H<sub>2</sub> formation of the MC01 mutant, the H<sub>2</sub> production potential of the strain was then tested in a bioreactor where 100% CO was continuously fed

with a flow rate of 240 ml min<sup>-1</sup>. The mutant might be expected to lead to an increased H<sub>2</sub> production and perhaps an increase in cell yield. When the growth rates of the two strains were compared, the MC01 mutant grew significantly better than the wild-type strain with a 3.8-fold increase in maximum biomass yield (Fig. 4A). Furthermore, the H<sub>2</sub> production rates of the MC01 mutant were higher than those of the wild-type strain, with a maximum rate of 123.5 mmol liter<sup>-1</sup> h<sup>-1</sup> for the mutant and 31.8 mmol liter<sup>-1</sup> h<sup>-1</sup> for the wild-type strain (Fig. 4B). Table 1 summarizes the kinetic parameters for the two strains. The maximum specific growth rate, biomass productivity, H<sub>2</sub> productivity, and H<sub>2</sub> yield coefficient with respect to biomass were also 2- to 4-fold higher for the mutant than for the wild-type strain. However, the molar ratios of H<sub>2</sub> and CO<sub>2</sub> produced with respect to CO consumed during cultures were constant at about 1:1:1 for both strains.

CO acts as both a substrate and an inhibitor in CO metabolism and at elevated concentrations can inhibit growth and catabolic activity due to its high affinity for metal-containing enzymes and hemes (28). To investigate the effect of CO content on the growth and H<sub>2</sub> production of the two strains, bioreactor cultures were supplied with CO at flow rates in the range of 40 to 400 ml min<sup>-1</sup>. Depending on the flow rates, the mutant consumed 27 to 37% of CO while the wild-type strain consumed 9.5 to 11% of CO during exponential growth phase (data not shown). The mutant showed 1.7- to 3.8-fold-higher values than the wild-type strain for maxi-



**FIG 4** Growth (A) and H<sub>2</sub> production (B) of wild-type strain (closed symbols) and MC01 mutant (open symbols) in a 3-liter bioreactor with CO supplied at 240 ml min<sup>-1</sup> of flow rate. Cell growth was monitored by measuring optical density at 600 nm (OD<sub>600</sub>). The initial CO flow rate of 20 ml min<sup>-1</sup> was raised to 240 ml min<sup>-1</sup> when the OD<sub>600</sub> reached about 0.3, as indicated by arrows.

**TABLE 1** Kinetic parameters between wild-type strain and MC01 mutant

Kinetic parameter <sup>a</sup>	Wild type	MC01 mutant	Fold difference
$\mu_{\max}$ (h <sup>-1</sup> )	0.31	0.72	2.3
$r_{\max}$ (mmol liter <sup>-1</sup> h <sup>-1</sup> )	31.8	123.5	3.9
$q_{\max}$ (mmol g <sup>-1</sup> h <sup>-1</sup> )	93.4	194.7	2.1
Biomass productivity (g liter <sup>-1</sup> h <sup>-1</sup> ) <sup>b</sup>	0.09	0.23	2.6
H <sub>2</sub> productivity (mmol liter <sup>-1</sup> h <sup>-1</sup> ) <sup>b</sup>	30.2	102.6	3.4
$Y_{p/x}$ (g H <sub>2</sub> g <sup>-1</sup> biomass)	0.32	0.9	2.8
CO:H <sub>2</sub> :CO <sub>2</sub> (mol:mol:mol)	1:1.04:1	1:1.07:1	1.0

<sup>a</sup> Kinetic parameters were calculated with data from graphs in Fig. 4.  $\mu_{\max}$ , maximum specific growth rate;  $r_{\max}$ , maximum H<sub>2</sub> production rate;  $q_{\max}$ , maximum specific H<sub>2</sub> production rate;  $Y_{p/x}$ , product yield coefficient with respect to biomass.

<sup>b</sup> Productivity was determined by dividing total yield by time difference from 9 to 11 h for the wild-type strain and from 8 to 14 h for the mutant.

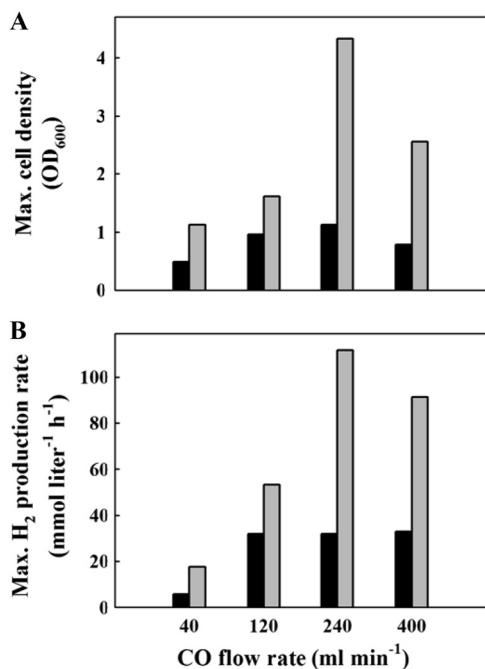


FIG 5 The effect of CO flow rate on the growth (A) and H<sub>2</sub> production (B) of the wild-type strain (black bars) and the MC01 mutant (gray bars) in a 3-liter bioreactor. Cell growth was monitored by measuring optical density at 600 nm (OD<sub>600</sub>).

imum H<sub>2</sub> production rate and maximum cell density (Fig. 5; see also Fig. S5 in the supplemental material). On the whole, as the CO flow rate was increased up to 240 ml min<sup>-1</sup>, the H<sub>2</sub> production and cell density increased for both strains. On the other hand, at a CO flow rate of 400 ml min<sup>-1</sup>, the cell density was decreased to about 60 to 70% of the values at 240 ml min<sup>-1</sup> for both strains, indicating that the growth was inhibited by CO. However, considering that the H<sub>2</sub> production rate was decreased only 20% for the mutant and maintained for the wild-type strain in comparison with the values at a CO flow rate of 240 ml min<sup>-1</sup>, CO metabolism does not seem to be inhibited by CO (Fig. 5).

TABLE 2 H<sub>2</sub> production rates of various carboxydrotrophic hydrogenogenic microbes

Organism	Cultivation method	H <sub>2</sub> production rate (mmol liter <sup>-1</sup> h <sup>-1</sup> ) <sup>a</sup>	Specific H <sub>2</sub> production rate (mmol g <sup>-1</sup> h <sup>-1</sup> ) <sup>a</sup>	Reference
<i>T. onnurineus</i> NA1 (MC01)	Batch	123.5 <sup>b</sup>	194.7 <sup>b</sup>	This study
<i>T. onnurineus</i> NA1 (wild type)	Batch	32.9 <sup>b</sup>	151.3 <sup>b</sup>	This study
<i>Rhodospseudomonas palustris</i> P4	Batch	41	41	29
<i>Citrobacter</i> sp. strain Y19	Batch	5.7	27.1	30
<i>Rhodospirillum rubrum</i>	Continuous	4.7	11	31
<i>Rubrivivax gelatinosus</i> CBS-2	Continuous	2.7	1.3–33	32

<sup>a</sup> Kinetic parameters were calculated with data from graphs in Fig. S5 in the supplemental material. The unit gram represents dry cell weight.

<sup>b</sup> The values were calculated from data obtained in a bioreactor where 100% CO was continuously fed with a flow rate of 240 ml min<sup>-1</sup> (MC01 strain) and 400 ml min<sup>-1</sup> (wild-type strain).

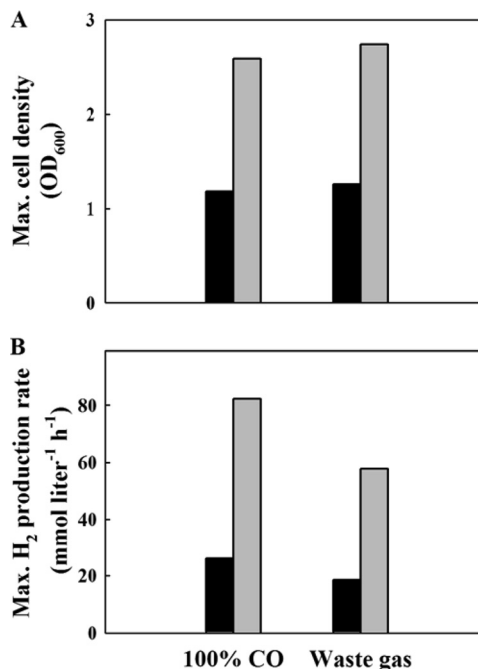


FIG 6 Comparison of the growth (A) and H<sub>2</sub> production (B) of the MC01 mutant cultured on steel mill waste gas supplied at flow rates of 60 (black bars) or 180 (gray bars) ml min<sup>-1</sup> in a 3-liter bioreactor. The maximum cell densities and maximum H<sub>2</sub> production rates of the MC01 mutant were estimated by regression of plots in Fig. 5.

It is worthy of note that the MC01 mutant showed the highest H<sub>2</sub> production rate and specific H<sub>2</sub> production rate among the CO-dependent H<sub>2</sub>-producing prokaryotes, to the best of our knowledge (Table 2).

**H<sub>2</sub> production from steel mill waste gas.** H<sub>2</sub> production by MC01 from waste gas, obtained as a by-product in the steel industry, was investigated. The increases of cell density and H<sub>2</sub> production rate were observed for both flow rates of waste gas, 60 and 180 ml min<sup>-1</sup> (see Fig. S6 in the supplemental material). The maximum H<sub>2</sub> production rate and the maximum cell densities (OD<sub>600</sub>) increased almost linearly with respect to the flow rate of waste gas. When 100% CO and waste gas were compared as CO suppliers at the same flow rates, the maximum cell densities were not much affected (Fig. 6A), but the maximum H<sub>2</sub> production rates were reduced about 30% by waste gas (Fig. 6B). The lower content of CO (57.4%) in waste gas might explain the result to some extent because the H<sub>2</sub> production rate was positively correlated with the content of CO (see Fig. S7 in the supplemental material).

In conclusion, the expression level of the CODH gene cluster involving CO-dependent H<sub>2</sub> production was modulated by insertion of a strong promoter and could achieve highly enhanced H<sub>2</sub> production from CO in *T. onnurineus* NA1. Waste gas from the steel industry is likely to be a potential substrate for H<sub>2</sub> production.

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