Biohydrogen production of obligate anaerobic archaeon *Thermococcus onnurineus* NA1 under oxic conditions via overexpression of *frhAGB*-encoding hydrogenase genes

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**Abstract**

**Background:** The production of biohydrogen (H₂) as a promising future fuel in anaerobic hyperthermophiles has attracted great attention because H₂ formation is more thermodynamically feasible at elevated temperatures and fewer undesired side products are produced. However, these microbes require anoxic culture conditions for growth and H₂ production, thereby necessitating costly and time-consuming physical or chemical methods to remove molecular oxygen (O₂). Therefore, the development of an O₂-tolerant strain would be useful for industrial applications.

**Results:** In this study, we found that the overexpression of *frhAGB*-encoding hydrogenase genes in *Thermococcus onnurineus* NA1, an obligate anaerobic archaeon and robust H₂ producer, enhanced O₂ tolerance. When the recombinant FO strain was exposed to levels of O₂ up to 20% in the headspace of a sealed bottle, it showed significant growth. Whole transcriptome analysis of the FO strain revealed that several genes involved in the stress response such as chaperonin β subunit, universal stress protein, peroxiredoxin, and alkyl hydroperoxide reductase subunit C, were significantly up-regulated. The O₂ tolerance of the FO strain enabled it to grow on formate and produce H₂ under oxic conditions, where prior O₂-removing steps were omitted, such as the addition of reducing agent Na₂S, autoclaving, and inert gas purging.

**Conclusions:** Via the overexpression of *frhAGB* genes, the obligate anaerobic archaeon *T. onnurineus* NA1 gained the ability to overcome the inhibitory effect of O₂. This O₂-tolerant property of the strain may provide another advantage to this hyperthermophilic archaeon as a platform for biofuel H₂ production.

**Keywords:** *frhAGB*-encoding hydrogenase, Obligate anaerobe, *Thermococcus onnurineus* NA1, O₂ tolerance, Biohydrogen
the reduction of O₂ tension. Chemical reducing agents containing sulfur, such as cysteine hydrochloride (E₀’ = −210 mV, redox potential defined at pH 7 and 298 K), sodium thioglycollate (E₀’ = −140 mV), or Na₂S (E₀’ = −571 mV), are very effective at maintaining the low redox potential of the medium [4, 5]. Even though various physical or chemical methods are effective at keeping the culture medium anaerobic, these strategies are costly and time consuming.

Hyperthermophiles have advantages in biotechnological applications because of reduced burden on reactor cooling processes, low chance of contamination, and increased reaction rates [6]. Biohydrogen (H₂) production using hyperthermophiles is furthermore advantageous because, at elevated temperatures, H₂ formation is more thermodynamically feasible and fewer undesired side products are produced [7, 8]. Thermococcus onnurineus NA1 is a hyperthermophilic obligate anaerobic archaeon that is capable of producing H₂ using starch, formate, or carbon monoxide (CO). It has been recently reported that T. onnurineus NA1 possesses high H₂ production rates during growth on formate, comparable to those of various bacteria and archaea with a formate dehydrogenase and a hydrogenase in the form of formate hydrogen lyase (FHL) or hydrogen-dependent CO₂ reductase (HDCR), or separately [9–17]. In particular, H₂ production by T. onnurineus NA1 using steel-mill waste gas was successfully demonstrated, indicating that environmentally friendly H₂ production is feasible [18, 19]. Over the years, H₂ production by this strain has been improved by employing various strategies of genetic engineering [18, 20, 21], adaptive laboratory evolution [22, 23], and fermentation process engineering [24]. Even though the strain has great potential for practical applications as an H₂ producer, it must be carefully handled and cultivated to prevent exposure to O₂ in all the steps. In addition to inhibition of cell growth, H₂ production is also inhibited by O₂ since membrane-bound [NiFe] hydrogenases, involved in H₂ evolution, are O₂ sensitive to some degree [25, 26].

In this study, we present a recombinant strain of T. onnurineus NA1 that can grow and produce H₂ under oxic conditions, where any physical or chemical methods were not applied to remove O₂ from the medium and the bioreactor headspace, which is a condition under which the wild-type strain cannot grow at all. The FO strain exhibited a very similar cell yield and only a 10% reduction in the H₂ production rate compared to the strain grown under anoxic conditions. This study may enhance the prospects of exploiting this obligate anaerobe as a robust tool for biotechnology.

### Results

**Construction and phenotype of a recombinant strain (FO)**

In our previous report, the frhAGB-encoding hydrogenase from T. onnurineus NA1, homologous to the F₄₂₀-reducing hydrogenases, a key enzyme in methanogenesis, was characterized [27]. To obtain a higher yield of the enzyme complex for biochemical studies, the frhAGB operon was overexpressed in the native strain using a strong constitutive promoter, resulting in the FO strain (Additional file 1: Fig. S1) [27]. The production of frhAGB in the FO strain was measured by Western blot, and the Frh α subunit encoded by the frhA gene was observed to be markedly increased (Fig. 1). The FO strain grew well using formate as an energy source and showed 3.4- and 1.4-fold higher cell densities at 6 and 12 h of culture, respectively, than those of the wild-type strain (Fig. 2a). The filh2–mfh2–mnh2 gene cluster was previously determined to be essential for the oxidation of formate and subsequent production of H₂ coupled with ATP synthesis [10]; therefore, the concentration of H₂ produced as a product of formate oxidation was measured. The FO strain produced 2.8- and 1.4-fold higher amounts of H₂ at the two measured time points than those of the wild-type strain, respectively, which correspond to the increasing levels of cell density (Fig. 2b). These results indicated that the expression level of membrane-bound formate-dependent hydrogenase (Mfh2) was not changed, and the protein level of a large subunit of Mfh2 hydrogenase was confirmed to be similar to that of the wild-type strain (Additional file 1: Fig. S2).

![Fig. 1](https://example.com/figure1.png) **Expression of the frhA gene in T. onnurineus NA1.** Western blot analysis of the expression of frhA-encoding protein in the FO strain (upper panel). The position of the target protein is denoted by an arrow. SDS-PAGE gel was stained with Coomassie Brilliant Blue (lower panel). WT wild-type strain, FO a recombinant strain overexpressing frhAGB; the numbers denote molecular masses in kilodaltons.
Transcriptome analysis of the FO strain
To explore the physiological changes that occurred through the overexpression of frhAGB genes, the expression changes were investigated at the whole transcriptome level. We performed microarray-based transcriptome analysis using samples of the wild-type and FO strains that were cultured in formate-supplemented medium under the anoxic condition. The results showed that 24 and 33 genes were identified as being up- and down-regulated with fold changes of ≥ 2 or ≤ 0.5 in expression compared with the wild-type strain, respectively (Additional file 1: Tables S1, S2). Among the down-regulated group, a gene cluster was identified to be related to the biosynthesis of disaccharides, oligosaccharides and polysaccharides (locus tag TON_1857 ~ 1862), including galactosyltransferases and glycosyltransferases. Some of the genes encoding proteins such as sulfate adenylyltransferase (TON_1707), transcriptional regulatory protein (TON_0836 and TON_1663), and ATP-dependent helicase (TON_1042 and TON_1380) were down-regulated. Among the up-regulated group, three gene clusters were identified, whose functions are predicted as transport (TON_0014 ~ 0015 and TON_0656 ~ 0657) and thiamine biosynthesis (TON_0853 ~ 0854). Two genes related to the stress response, chaperonin β subunit (TON_1877) and universal stress protein (TON_1493), were also up-regulated. Interestingly, two genes encoding peroxiredoxin (TON_0786) and alkyl hydroperoxide reductase subunit c (TON_0847), which are known to protect cells against oxidative stress by reducing hydrogen peroxide and/or alkyl hydroperoxides, were up-regulated.

O₂ tolerance test
Based on the transcriptomic data, we speculated that the changes at the transcriptional level of several stress response genes caused by frhAGB overexpression might affect O₂ tolerance of the strain. Therefore, we compared O₂ sensitivity between the wild-type and FO strains. Each strain was cultured in sealed vials containing 0, 10, 20, 30, and 40% of atmospheric air (21% O₂, v/v) in the headspace. With increasing percentages of atmospheric air, the wild-type strain showed gradual decreases in cell density, ranging from 17.9 to 56.7% of the control (0% air) (Fig. 3a). On the other hand, the growth of the FO strain remained constant, with changes in cell density ranging from only 7.7 to 11.9%, indicating a significant enhancement in O₂ tolerance.

In our previous report, the frhA-deletion mutant, ΔfrhA, showed very limited changes in cell density and H₂ production compared with the wild-type strain [28]. To correlate the intracellular level of the frhAGB-encoding hydrogenase with O₂ tolerance, we tested how the O₂ sensitivities of the wild-type strain, the frhA-deletion mutant strain, and the FO strain were affected at higher concentrations of O₂ in the headspace of culture vials. Instead of air, pure O₂ was added to a concentration of 20%, equivalent to the atmospheric concentration of O₂. Once again, the FO strain grew much better than the wild-type strain (Fig. 3b). On the other hand, the ΔfrhA mutant strain was more sensitive to O₂ than the wild-type strain.

Growth of the FO strain under oxic conditions
Since the FO strain showed strong performance in the presence of O₂, we tested whether enhanced O₂ tolerance
could enable the FO strain to grow under oxic conditions. Therefore, several pretreatment methods that are typically applied to remove O₂ from a culture vessel were omitted, namely the addition of reducing agent Na₂S, autoclaving, and inert gas purging. In the bioreactor, the growth of the wild-type strain was severely impaired under oxic conditions, as expected (Fig. 4a). In contrast, after a prolonged lag phase, the FO strain started to grow and reached a cell density similar to that obtained under anoxic conditions (Fig. 4a). Maximum specific growth rates were quite similar in the two conditions (Table 1). This result is quite surprising, considering that the experimental strain used in this study, *T. onnurineus* NA1, is an obligate anaerobe. To our knowledge, it has never been reported that the overexpression of only one gene caused an obligate anaerobe to grow under oxic conditions. Then we sought to examine what would happen to H₂ production under oxic conditions. Although H₂ production was
antioxidant-related genes, such as thioredoxin peroxidase (TON_0862), ruberythrin (TON_0864), ruberythrin-related protein (TON_0873), NAD(P)H rubredoxin oxidoreductase (TON_0865), and thioredoxin reductase (TON_1603), which were identified to be strongly up-regulated in the presence of O2 (manuscript forthcoming). We speculated that the FO strain, with two antioxidant-related genes up-regulated, might exhibit changes in O2 tolerance. To verify this hypothesis, the O2 sensitivities of the FO strain, wild-type strain, and ΔfrhA mutant were compared. The expression level of the frhAGB gene cluster had a strong influence on oxidative stress defense. In the ΔfrhA mutant, however, the transcript levels of genes encoding peroxiredoxin and alkyl hydroperoxide reductase subunit c were similar to those in the wild-type strain [28]. This result implies that there are more genes that contribute to oxidative stress defense in the FO strain.

O2 tolerance in obligate anaerobes by means of hydrogenases has been reported previously. In obligate anaerobic bacteria Desulfovibrio vulgaris strains, H2-consuming periplasmic soluble hydrogenase and c-type cytochrome couples were identified to play a role in O2 reduction, thereby protecting against oxidative stress [29, 30]. It has been reported that the hydrogenase was up-regulated when D. vulgaris Hildenborough was exposed to O2 [30]. In another obligate anaerobic bacteria Geobacter sulfurreducens, a periplasmic hydrogenase Hya was identified to be necessary for growth after exposure to oxidative stress. While the Hya-deficient strain was more sensitive to the presence of superoxide or hydrogen peroxide, overexpression of Hya enabled the strain to endure oxidative stress better than the wild-type strain. However, the mechanism by which this hydrogenase contributed to the defense against oxidative stress relative to the promotion of antioxidant enzyme activity was not elucidated [31].

In this study, the FO strain was distinct in that it was able to achieve similar growth yields under oxic conditions, where most anaerobes and the wild-type T. onnurinues NA1 strain are incapable of growth due to the high O2 level, compared with those of anoxic conditions. In addition, the maximum H2 production rates and maximum specific H2 production rates of the FO strain cultured under both oxic and anoxic conditions were quite similar to the high values for the WTF-156T strain, which had been engineered by adaptive evolution on formate-supplemented medium [23]. This feature can ameliorate the need to tightly maintain the anaerobic environment prior to cultivation in a batchwise or continuous system, where a certain level of O2 contamination is inevitable, by operating with the addition of carbon sources or other nutrients, or by maintaining a constant

Table 1 Kinetic analysis of the wild-type and FO strains under oxic or anoxic conditions

| Kinetic parameter | Wild type | FOb | Wild type | FOb |
|-------------------|-----------|-----|-----------|-----|
|                   | Anoxica   | Oxic| Anoxic     | Oxic|
| μmax (h−1)        | 0.3       | ND  | 0.60±0.08 | 0.60±0.02 |
| rmax (mmol L−1 h−1) | 31.7     | ND  | 118.5±3.9 | 101.7±16.6 |
| qmax (mmol g−1 h−1) | 198.2    | ND  | 364.8±8.1 | 323.3±54.5 |

a The data on the kinetic parameters of the wild-type strain under anoxic conditions were adapted from a previous study [23]
b The values for the FO strain show mean ± standard deviation for three independent cultures in this study

closely correlated with growth on formate under anoxic conditions [10], the maximum H2 production rate and maximum specific H2 production rate were approximately 90% of those under anoxic conditions (Fig. 4b and Table 1). In comparison with the wild-type strain cultured under anoxic conditions, the FO strain under oxic conditions showed 1.6- to 3.2-fold higher maximum specific growth rate, maximum H2 production rate, and maximum specific H2 production rate (Table 1).

Taken together, the overexpression of frhAGB genes endowed T. onnurinues NA1 with a substantial increase in tolerance to O2. This O2-tolerant property is a significant advantage in biotechnological applications because it is not necessary to strictly protect the growth and H2 production processes from O2. H2 production under micro-aerobic conditions would be a cost-effective and time-saving process.

Discussion

When the frhAGB gene cluster was overexpressed in T. onnurinues NA1, a recombinant strain (FO) showed better growth than the wild-type strain. The molecular mechanism underlying the enhanced cell growth is not yet understood. Instead, we attempted to exploit the potential of the FO strain using information from whole transcriptomic analysis. The expression of antioxidant-related genes encoding peroxideroxidin and alkyl hydroperoxide reductase subunit c was 2.5- and 4.1-fold up-regulated under the anaerobic conditions, respectively. The transcriptional up-regulation of antioxidant-related genes is usually associated with cellular responses to oxidative stress. However, the sources for oxidative stress are unidentified. Extracellular O2 is usually removed by the addition of a reducing agent, autoclaving the medium, and inert gas purging. The absence of O2 in our experiment was evidenced by minimal or no change in the transcriptional expression of other
pH using strong acid or bases. Therefore, the ability of the FO strain to tolerate O\textsubscript{2} would make it even more suitable for industrial applications. Our study implicates that other anaerobic H\textsubscript{2} producers might be relieved from the strict control of O\textsubscript{2} during growth through augmentation of the defense against oxidative stress. It would be helpful if we could get high amounts of whole cell biocatalysts and even purified enzymes from them easily by growing those strains under oxic conditions while the activity of enzymes, such as hydrogenases, FHLs, and HDCRs, essential for H\textsubscript{2} production, is untouched.

This study helps to understand the physiological role of the frhAGB-encoding hydrogenase, which is distinct from Frh hydrogenases of methanogens and is thus far poorly understood. In our previous report on the frhA-deletion mutant, it was shown that the hydrogenase might be associated with the regulation of gene expression in a non-methanogen [28]. For example, frhA gene deletion caused up-regulation of the cdh–mch–mnh3 gene cluster essential for CO-dependent H\textsubscript{2} production even in the absence of external CO and led to significant increases in cell growth (2.8-fold) and H\textsubscript{2} production (3.4-fold) [18]. In this study, overexpression of frhAGB genes also up-regulated the expression of antioxidant-related genes without exposing them to an oxidizing agent such as O\textsubscript{2}. Furthermore, the FO strain was superior to the wild-type strain with respect to growth under oxic conditions. Regulatory hydrogenases HupUV and HoxBC have been reported to participate in the transcriptional regulation of gene expression [25]. These hydrogenases function as H\textsubscript{2} sensors in two-component regulatory systems, which consist of a protein histidine kinase and a response regulator [32]. Two-component regulatory systems are distributed widely in bacteria, but only a few are found in archaea [33]. Therefore, if the soluble hydrogenase is playing a regulatory role, its mechanism in T. onnurineus NA1 awaits further studies. It is noteworthy that two genes encoding transcriptional regulatory proteins (TON_0836 and TON_1663) were down-regulated by overexpression of frhAGB genes. Further studies will be required to address whether these regulators are involved in the regulation of gene expression by the frhAGB-encoding hydrogenase. Contributions of other up- or down-regulated genes by overexpression of frhAGB genes cannot be ruled out; therefore, this issue requires further investigation.

Conclusions
In this study, we demonstrated that the overexpression of frhAGB-encoding hydrogenase genes significantly enhanced O\textsubscript{2} tolerance of obligate anaerobe T. onnurineus NA1. This engineered strain overcame the inhibitory effects of O\textsubscript{2} and showed growth and H\textsubscript{2} production under oxic condition. This study gives an insight into the development of biotechnologically useful anaerobic H\textsubscript{2} producer and identification of an unknown function of the hydrogenase in T. onnurineus NA1.

Methods
Strain, medium and culture conditions
The T. onnurineus NA1 wild-type (KCTC 10859), FO strain overexpressing frhAGB [27], and frhA-deletion mutant [28] strains were cultured at 80 °C in modified medium 1 (MM1) with 1% sodium formate (MM1-F) as previously described [10, 34]. In serum vial cultivation, the initial pH of the medium was adjusted to 6.5. In bioreactor cultivations, pH was maintained at 6.1–6.2, and MM1-F medium was modified as previously described [23].

Analytical methods
Cell growth was measured by the optical density value at 600 nm (OD\textsubscript{600}) using a UV–visible spectrophotometer (BioPhotometer Plus; Eppendorf, Hamburg, Germany). The dry cell weight (DCW) was deduced by the previous determination of the correlation between OD\textsubscript{600} and DCW values [18]. The H\textsubscript{2} concentration in the headspace was measured as previously described [10]. The H\textsubscript{2} production rate in the bioreactor experiment was determined as previously described [18].

SDS-PAGE and Western blot analysis
Cells were disrupted by sonication in 50 mM Tris–HCl buffer (pH 8.0) containing 100 mM NaCl, 19 mM KCl, 5% glycerol and a protease inhibitor cocktail tablet (Roche Applied Science, Madison, USA) and were analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Western blots were performed using primary polyclonal antibodies specific for the frhA-encoding protein or a large subunit of Mfh2 hydrogenase and were analyzed using a chemiluminescent dye with the Immob-Star horseradish peroxidase chemiluminescent kit (Bio-Rad, Hercules, USA).

O\textsubscript{2} sensitivity test
To test O\textsubscript{2} sensitivity of cells, atmospheric air (21% O\textsubscript{2}, v/v) or pure O\textsubscript{2} was injected into the headspace of culture vials after inoculation and the optical density of cells grown at 80 °C for 12 h was measured at 600 nm (OD\textsubscript{600}). To cultivate cells in a bioreactor under oxic condition (presence of O\textsubscript{2} in the headspace), reducing agent Na\textsubscript{2}S was omitted and autoclaving and inert gas purging were not performed.
Transcriptomic analysis
Transcriptomic profiling of the FO strain overexpressing frhAGB was carried out using mRNA samples prepared from two independent cultures. Manufacturing of the microarray slides, microarray processing and signal normalization were performed as previously described [10]. The microarray raw data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) with the accession code GSE88718.

Additional file

Additional file 1: Table S1. Genes identified as up-regulated by overexpression of frhAGB genes based on the transcriptomic analysis. Table S2. Genes identified as down-regulated by overexpression of frhAGB genes based on the transcriptomic analysis. Figure S1. Construction of the FO strain. Figure S2. Protein level of Mfh2 in the wild-type and FO strains.

Authors’ contributions
HSL and SGK designed research. SHL and M-SK performed research and analyzed data. SHL, SGK, and HSL wrote the paper with input from the coauthors. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

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