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BIOTECHNOLOGY

Engineering of the Hyperthermophilic Archaeon *Thermococcus kodakarensis* for Chitin-Dependent Hydrogen Production

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ABSTRACT *Thermococcus kodakarensis* is a hyperthermophilic archaeon that harbors a complete set of genes for chitin degradation to fructose 6-phosphate. However, wild-type *T. kodakarensis* KOD1 does not display growth on chitin. In this study, we developed a *T. kodakarensis* strain that can grow on chitin via genetic and adaptive engineering. First, a chitinase overproduction strain (KC01) was constructed by replacing the chitinase gene promoter with a strong promoter from the cell surface glycoprotein gene, resulting in increased degradation of swollen chitin and accumulation of *N*-,*N*′-diacetylchitobiose in the medium. To enhance *N*-,*N*′-diacetylchitobiose assimilation in KC01, genes encoding diacetylchitobiose deacetylase, exo-β-D-glucosaminidase, and glucosamine-6-phosphate deaminase were also overexpressed to obtain strain KC04. To strengthen the glycolytic flux of KC04, the gene encoding Tgr (transcriptional repressor of glycolytic genes) was disrupted to obtain strain KC04Δt. In both KC04 and KC04Δt strains, degradation of swollen chitin was further enhanced. In the culture broth of these strains, the accumulation of glucosamine was observed. KC04Δt was repeatedly inoculated in a swollen-chitin-containing medium for 13 cultures. This adaptive engineering strategy resulted in the isolation of a strain (KC04ΔtM1) that showed almost complete degradation of 0.4% (wt/vol) swollen chitin after 90 h. The strain produced high levels of acetate and ammonium in the culture medium, and, moreover, molecular hydrogen was generated. This strongly suggests that strain KC04ΔtM1 has acquired the ability to convert chitin to fructose 6-phosphate via deacetylation and deamination and further convert fructose 6-phosphate to acetate via glycolysis coupled to hydrogen generation.

IMPORTANCE Chitin is a linear homopolymer of β-1,4-linked *N*-acetylglucosamine and is the second most abundant biomass next to cellulose. Compared to the wealth of research focused on the microbial degradation and conversion of cellulose, studies addressing microbial chitin utilization are still limited. In this study, using the hyperthermophilic archaeon *Thermococcus kodakarensis* as a host, we have constructed a strain that displays chitin-dependent hydrogen generation. The apparent hydrogen yield per unit of sugar consumed was slightly higher with swollen chitin than with starch. As gene manipulation in *T. kodakarensis* is relatively simple, the strain constructed in this study can also be used as a parent strain for the development and expansion of chitin-dependent biorefinery, in addition to its capacity to produce hydrogen.

KEYWORDS chitin, chitinase, hyperthermophile, archaea, *Thermococcus kodakarensis*, *Archaea*, *Thermococcus*, chitinases, hyperthermophiles

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Chitin is a linear homopolymer of β-1,4-linked *N*-acetylglucosamine (GlcNAc) and is largely present in the exoskeleton of crustaceans and insects and in the cell walls of fungi. The annual steady-state amount of chitin formation has been estimated to be 10^{10} to 10^{11} tons (1), making it next in abundance to cellulose. However, the majority of chitin is unused by mankind and accumulates as part of the surplus biomass. Therefore, effective methods are desired to enhance the utilization of this unused biomass.

Microbial conversion of biomass into useful products provides an environmentally friendly alternative to the conventional manufacturing processes dependent on fossil fuel. In particular, the use of (hyper)thermophiles for this purpose has several advantages over use of their mesophilic counterparts as they are able to hydrolyze biomass at elevated temperatures, which promotes higher substrate solubility and catalytic rates, as well as decreasing the risk of contamination (2–6).

Thermococcus kodakarensis strain KOD1 is a hyperthermophilic archaeon, isolated near the coast of Kodakara Island, Kagoshima, Japan (7, 8). *T. kodakarensis* KOD1 can grow on a variety of carbon sources, such as amino acids, starch (maltodextrin), and pyruvate. The strain is also known to produce molecular hydrogen at a relatively high rate (9–12). The entire genome sequence of *T. kodakarensis* KOD1 has been determined (13), and gene manipulation systems based on homologous recombination and shuttle vectors have been developed (14–18). These have frequently been used to study gene function *in vivo* as well as to engineer *T. kodakarensis* for biotechnological purposes (10, 19).

T. kodakarensis harbors a gene cluster for chitin degradation/assimilation on its genome (20). Our group has previously characterized the chitinase (ChiA; encoded by the TK1765 gene) (21–23), the diacetylchitobiose deacetylase (Dac; encoded by the TK1764 gene) (24), the exo-β-D-glucosaminidase (GlmA; encoded by the TK1754 gene) (24, 25), and the glucosamine-6-phosphate deaminase (GlmD; encoded by the TK1755 gene) (20). The chitin degradation pathway of *T. kodakarensis* is unique (Fig. 1). Chitin is first degraded into the disaccharide *N*-,*N*′-diacetylchitobiose [$(GlcNAc)_2$] by ChiA, which possesses two catalytic domains, one exhibiting endo-type activity and the other exhibiting exo-type activity (21–23). The $(GlcNAc)_2$ is then presumed to be taken up by an ABC transport system, whose genes (*dppABCDF*) are located adjacent to *glmDA*. The acetyl group of the nonreducing side of $(GlcNAc)_2$ is first deacetylated by Dac, and the partially deacetylated disaccharide is hydrolyzed into glucosamine (GlcN) and GlcNAc by GlmA, followed by further deacetylation of GlcNAc to GlcN by Dac (24, 25). The gene cluster lacks a gene for the phosphorylation of GlcN to generate glucosamine 6-phosphate (GlcN6P). However, it is thought to be mediated by an ADP-dependent glucokinase (GK) encoded at a different locus (the TK1110 gene), as the orthologs from closely related archaea, *Pyrococcus furiosus* and *Thermococcus litoralis*, are capable of phosphorylating GlcN to GlcN6P *in vitro* (26). GlcN6P is further deaminated and converted to fructose 6-phosphate (F6P) by GlmD (20) flowing into the glycolytic pathway.

As described above, *T. kodakarensis* KOD1 harbors a complete set of genes for chitin degradation and assimilation. In addition, the transcription levels of the genes in the cluster are induced in the presence of $(GlcNAc)_2$ (20, 24, 25). Intriguingly however, *T. kodakarensis* does not show robust growth on medium containing crystal/swollen chitin as the major carbon source. In the present study, we have engineered *T. kodakarensis* via repeated genetic manipulations followed by an adaptive engineering approach. As a result, a *T. kodakarensis* strain that can degrade and assimilate chitin was obtained that can efficiently produce molecular hydrogen dependent on the degradation of swollen chitin.

RESULTS

Construction of *chiA* overexpression strain KC01 and its characterization. In order to enhance the chitin degradation capability of *T. kodakarensis*, a chitinase gene (*chiA*) overexpression strain (KC01) was constructed by replacing its original promoter

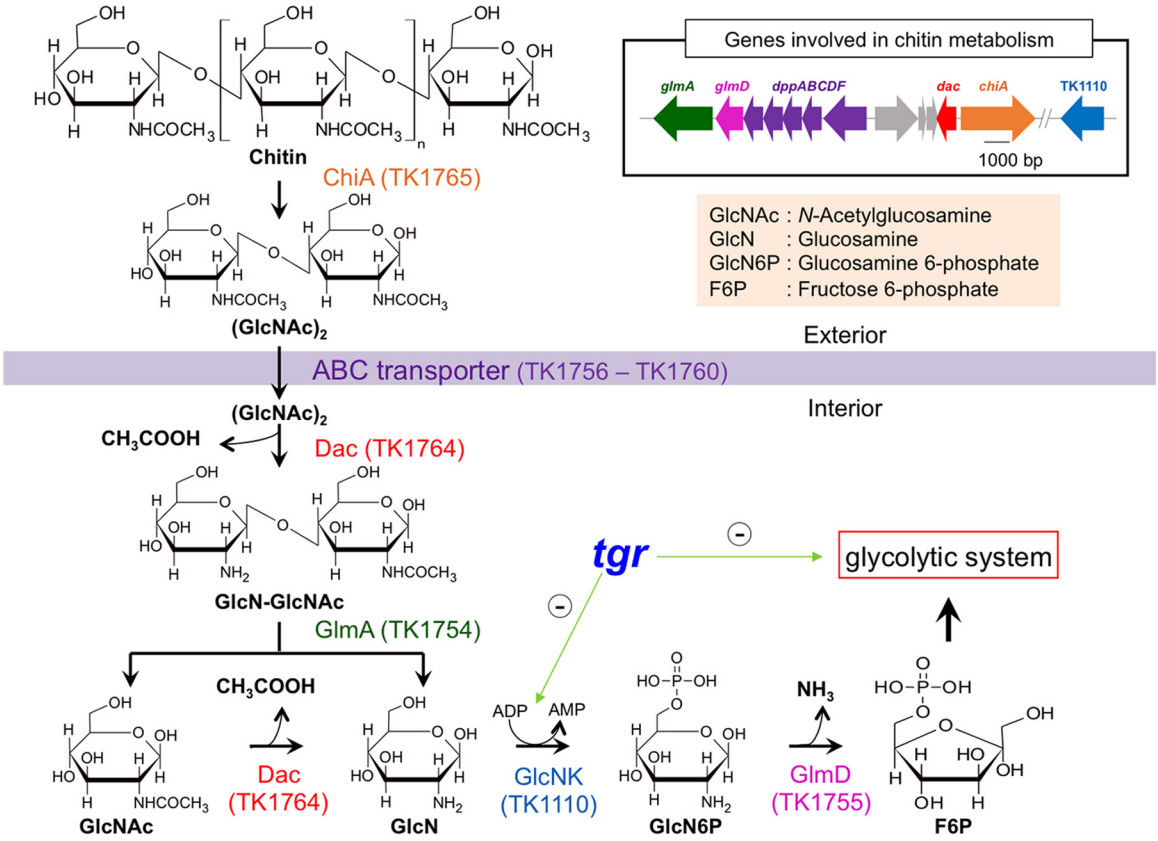

FIG 1 Chitin metabolic pathway of *T. kodakarensis*. Chitin is converted into *N*-,*N*′-diacetylchitobiose, $(GlcNAc)_2$, by extracellular chitinase (ChiA) encoded by the TK1765 gene. An ABC transporter (DppABCDF) encoded by the genes at locus TK1756 to TK1760 may allow $(GlcNAc)_2$ to enter into the cell, where deacetylation of the nonreducing end occurs by diacetylchitobiose deacetylase (Dac) encoded by the TK1764 gene. Exo-β-D-glucosaminidase (GlmA) encoded by the TK1754 gene hydrolyzes the product (GlcN-GlcNAc) to glucosamine (GlcN) and *N*-acetylglucosamine (GlcNAc). Dac further converts GlcNAc to GlcN with release of one more acetate. In *T. kodakarensis*, there is no homolog for GlcN kinase (GlcNK) that produces glucosamine 6-phosphate (GlcN6P), but this reaction is thought to be performed by an ADP-dependent glucokinase encoded by the TK1110 gene. GlcN6P is further deaminated, leaving ammonium, by GlcN6P deaminase (GlmD) encoded by the TK1755 gene. The fructose 6-phosphate (F6P) formed in this reaction further enters into the glycolytic pathway. Tgr is a transcriptional repressor for glycolytic genes including TK1110 (green arrows with minus signs represent effects of Tgr on transcriptional repressions of glycolytic genes).

with a strong promoter from the TK0895 gene (Fig. 2). TK0895 encodes a cell surface glycoprotein (Csg) that is strongly expressed in *T. kodakarensis*, and its promoter has previously been used to overexpress ChiAΔ4 (the C-terminal domain of *T. kodakarensis* ChiA) (19), endogenous pantoate kinase (27), and α-1,4-glucan phosphorylase from *Sulfolobus solfataricus* (28) in *T. kodakarensis*. The *csg* promoter was placed upstream of *chiA* (TK1765) in plasmid pUD3 (27) harboring *pyrF* used for transformant selection (see Fig. S1 in the supplemental material). The plasmid constructed (pUD3-P_{csg}-chiA) (Fig. S2) was used to transform *T. kodakarensis* KU216 (host strain) to obtain strain KC01.

Protein expression levels of ChiA were analyzed using KU216 and KC01 cells cultivated in nutrient-rich medium containing chitin oligosaccharides (ASW-VMT-Mdx-CO medium, consisting of artificial salt water [ASW], vitamin mixture with trace minerals [VMT], maltodextrin [Mdx], and chitin oligomers [CO]) (Table 1). Culture supernatants and cell extracts were prepared and subjected to SDS-PAGE. Western blot analysis using anti-ChiA antibodies revealed that ChiA was overexpressed and secreted efficiently to the supernatant of KC01 cultures (Fig. 3). A chitinase assay of the culture supernatants using *p*-nitrophenyl-$(GlcNAc)_2$ revealed the presence of chitinase activity only for KC01 (data not shown), indicating that ChiA secreted in the culture supernatant was active. In this strain, increased expression levels of GlmD, GlmA, and Dac were also observed. This is most likely caused by the generation of $(GlcNAc)_2$, which is the product of the ChiA reaction, in the culture medium. The expression of GlmD, Dac,

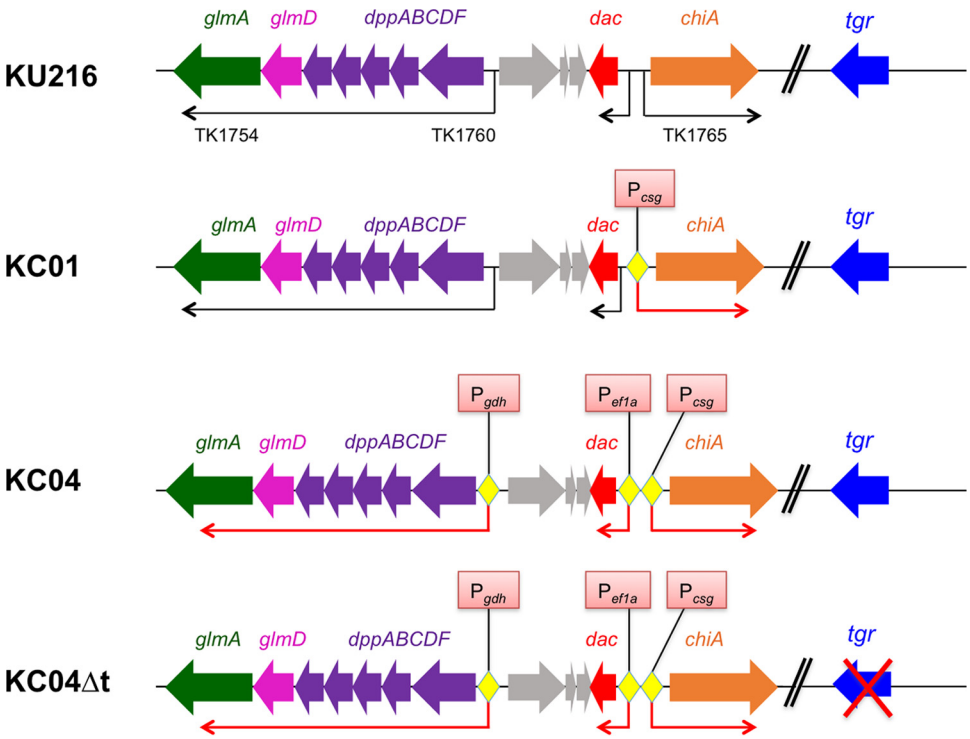

FIG 2 Engineering of *T. kodakarensis* strains. KU216 is a *T. kodakarensis* host strain expressing GlmA, GlmD, ABC transporter, Dac, and ChiA with their native promoters. In strain KC01, the native promoter of ChiA was replaced with a strong promoter, P_{csg}. KC01 was further modified to produce strain KC04 by replacing promoters of an operon (*dppABCDF-glmDA*) and the *dac* gene with strong promoters of P_{gdh} and of the EF-1α gene, respectively. Strain KC04 was further modified to produce strain KC04Δt by deleting *tgr* that is involved in transcriptional repression of glycolytic enzymes.

and GlmA has previously been shown to be induced in the presence of $(GlcNAc)_2$ (20, 24, 25).

Chitin degradation abilities of KC01 were examined at 85°C using a medium containing 0.4% (wt/vol) swollen chitin (ASW-VMT-SC medium, where SC is swollen chitin) (Table 1). After 48 h of cultivation, a clear decrease in swollen chitin was detected for KC01, and almost half disappeared after 90 h, with a slight color change (yellowish) of the culture broth (Fig. 4). On the other hand, no sign of chitin degradation was observed for the host strain, KU216, even after 90 h.

TABLE 1 Compositions of culture media used in this study

Medium type and name	Composition	Reference or source
Base media		
ASW-YT	0.8× ASW, 5.0 g/liter liter yeast extract, and 5.0 g $liter^{-1}$ tryptone	14
ASW-AA	0.8× ASW, a mixture of 20 amino acids, a vitamin mixture, and a modified Wolfe's trace minerals mixture	14
ASW-VMT	0.8× ASW, 5.0 g $liter^{-1}$ tryptone, a vitamin mixture, and a modified Wolfe's trace minerals mixture (including 3μM $NiCl_2$ and 10μM Na_2WO_4)	This study
Culture media		
ASW-YT-S^0	ASW-YT plus 2.0 g/liter sulfur powder	27
ASW-YT-Pyr	ASW-YT plus 5.0 g/liter sodium pyruvate	27
ASW-AA-S^0	ASW-AA plus 2.0 g/liter sulfur powder	14
ASW-VMT-SC	ASW-VMT plus 4.0 g/liter swollen chitin	This study
ASW-VMT-CO	ASW-VMT plus 1.0 g/liter chitin oligomers	This study
ASW-VMT-Mdx-CO	ASW-VMT plus 5.0 g/liter maltodextrin and 1.0 g/liter chitin oligomers	This study
ASW-VMT-Pyr	ASW-VMT plus 5.0 g/liter sodium pyruvate	This study

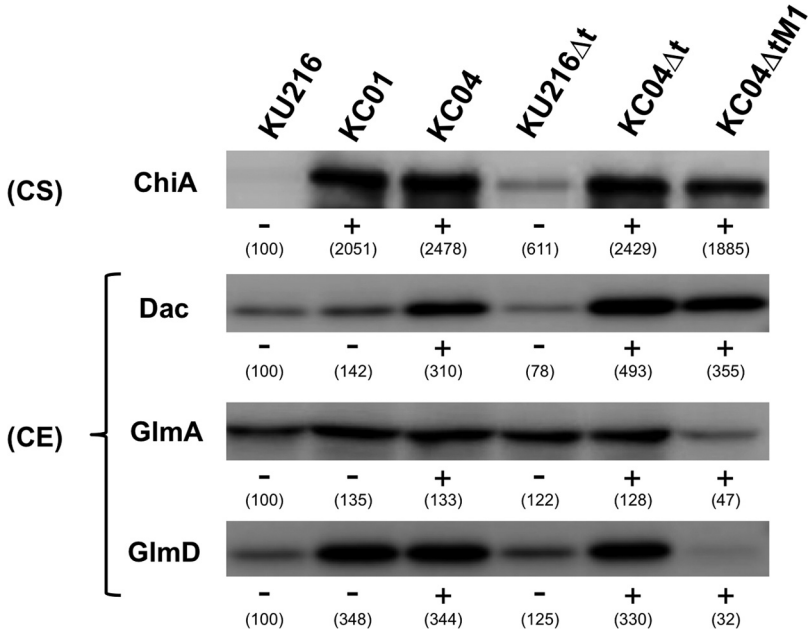

FIG 3 Expression levels of ChiA, Dac, GlmA, and GlmD in engineered *T. kodakarensis* strains. All strains were grown in ASW-VMT-Mdx-CO medium. Western blot analysis of ChiA was performed using culture supernatants (CS). Culture supernatant corresponding to a volume of 150 μl was loaded on each lane. Western blot analyses of Dac, GlmA, and GlmD were performed using cell extracts (CE). An equal amount (10 μg) of protein was loaded on each lane. A plus sign indicates overexpression of the enzyme performed by promoter replacement, and a minus sign indicates that there is no promoter replacement. Numbers in parentheses indicate band intensities (percent) relative to those of KU216 cultivated under the same medium condition (which is defined as 100%).

Degradation products of swollen chitin in the culture supernatants of KC01 were analyzed by thin-layer chromatography (TLC). Cells were cultivated at 85°C in ASW-VMT-SC medium, and culture supernatants were collected at different cultivation periods (until 90 h). In the culture supernatants of KC01, accumulation of $(GlcNAc)_2$ as well as of GlcNAc was detected. The abundances of these products increased with cultivation time and reached their maxima at around 72 to 90 h (Fig. 5). On the other hand, no such degradation was detected with KU216 cells.

Construction and characterization of strains KC04 and KC04Δt. As we observed accumulation of $(GlcNAc)_2$ in the culture supernatant of KC01, we next set out to enhance the production of enzymes involved in $(GlcNAc)_2$ assimilation. Strain KC04 was constructed in which strong promoters, the *csg* promoter, the promoter of the EF-1α gene, and *gdh* promoter, were introduced upstream of *chiA*, *dac* (TK1764), and the *dppABCDF-glmDA* operon (TK1754-TK1760), respectively (Fig. 2).

Based on KC04, strain KC04Δt was further constructed by disrupting the TK1769 gene (Fig. 2). TK1769 encodes a transcriptional regulator, Tgr (TrmBL1), which controls the transcription levels of genes involved in glycolysis and gluconeogenesis in *T. kodakarensis* (29). In the case of glycolytic genes, Tgr binds to a sequence motif (TGM; *Thermococcales* glycolytic motif) that is located downstream of the BRE/TATA sequence (30). Binding of Tgr to the motif blocks transcription through the inhibition of RNA polymerase recruitment to the promoter. Binding of maltotrioses to Tgr triggers their release from the TGM sites, resulting in the derepression of glycolytic genes. Gene disruption of Tgr thus results in an increase in the transcript levels of the glycolytic genes even in the absence of α-glucans (29). If GK, encoded by the TK1110 gene, is also responsible for glucosamine kinase activity, the Tgr gene disruption should lead to an increase in glucokinase/glucosamine-kinase activity in the cells. Moreover, *tgr* disruption should also increase the total glycolytic flux of the cells, particularly from F6P to pyruvate. Therefore, we expected that KC04Δt would satisfy all the requirements for the breakdown and assimilation of chitin.

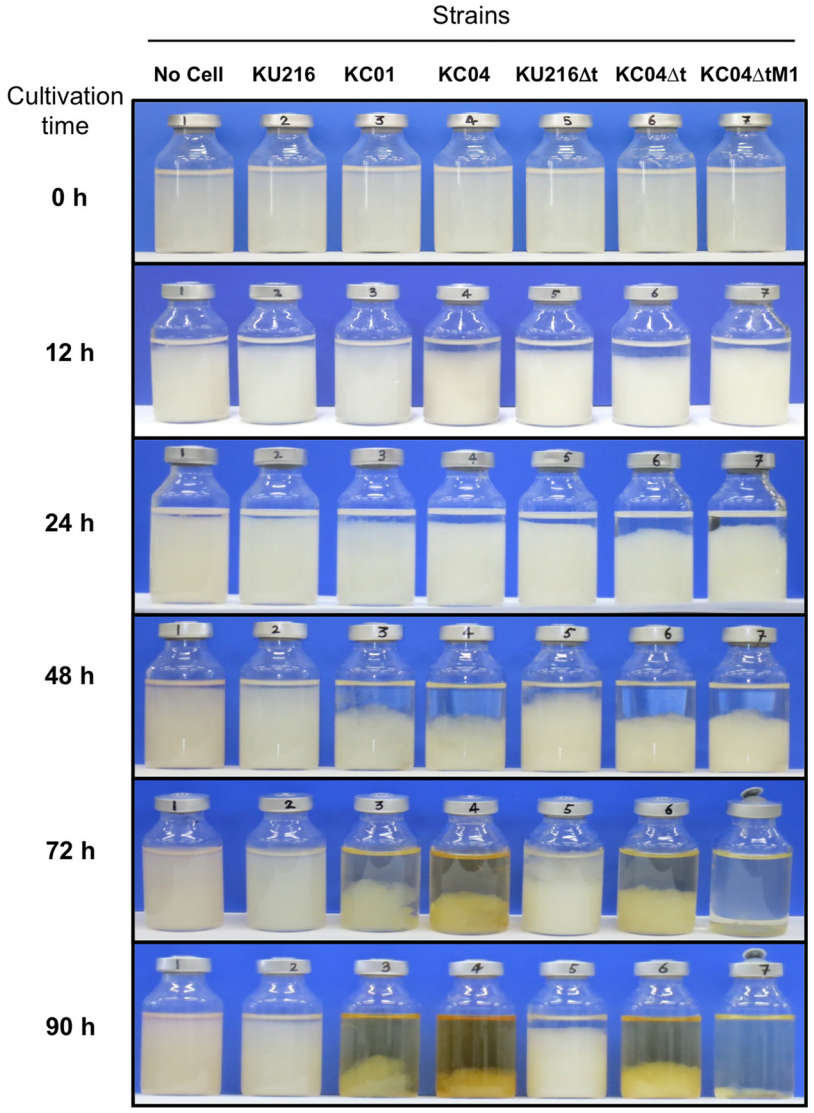

FIG 4 Degradation of swollen chitin by engineered strains of *T. kodakarensis*. Cultivations were performed in ASW-VMT-SC medium at 85°C, and degradation of swollen chitin was observed at different time points until 90 h. An increased capacity to degrade swollen chitin was observed in strain KC04ΔtM1.

To examine the expression levels of ChiA, Dac, GlmD, and GlmA in *T. kodakarensis* KC04 and KC04Δt, Western blot analyses were performed. The two strains were grown in ASW-VMT-Mdx-CO medium. As a result, levels of ChiA were much higher in the culture supernatants of KC04 and KC04Δt than in the supernatant of KU216, and levels of Dac, GlmD, and GlmA were also higher in the cell extracts of KC04 and KC04Δt than in the cell extract of KU216 (Fig. 3).

Chitin degradation capabilities of strains KC04 and KC04Δt were examined using ASW-VMT-SC medium. In KC04, the degree of swollen chitin degradation was slightly higher than that in KC01, while the color of the culture broth looked darker (yellowish) after 90 h (Fig. 4). As for the KC04Δt strain, swollen chitin degradation was comparable to that of KC04, while the color of the culture broth was relatively light.

When degradation products of chitin in the culture supernatants were analyzed, we noticed the accumulation of GlcN in addition to $(GlcNAc)_2$ in both strains (Fig. 5). This suggests that $(GlcNAc)_2$ conversion to GlcN was enhanced, while a bottleneck reaction was present after GlcN generation.

The chitin degradation capacities of KC04 and KC04Δt were also estimated by examining acetate and ammonium concentrations in the culture supernatants after

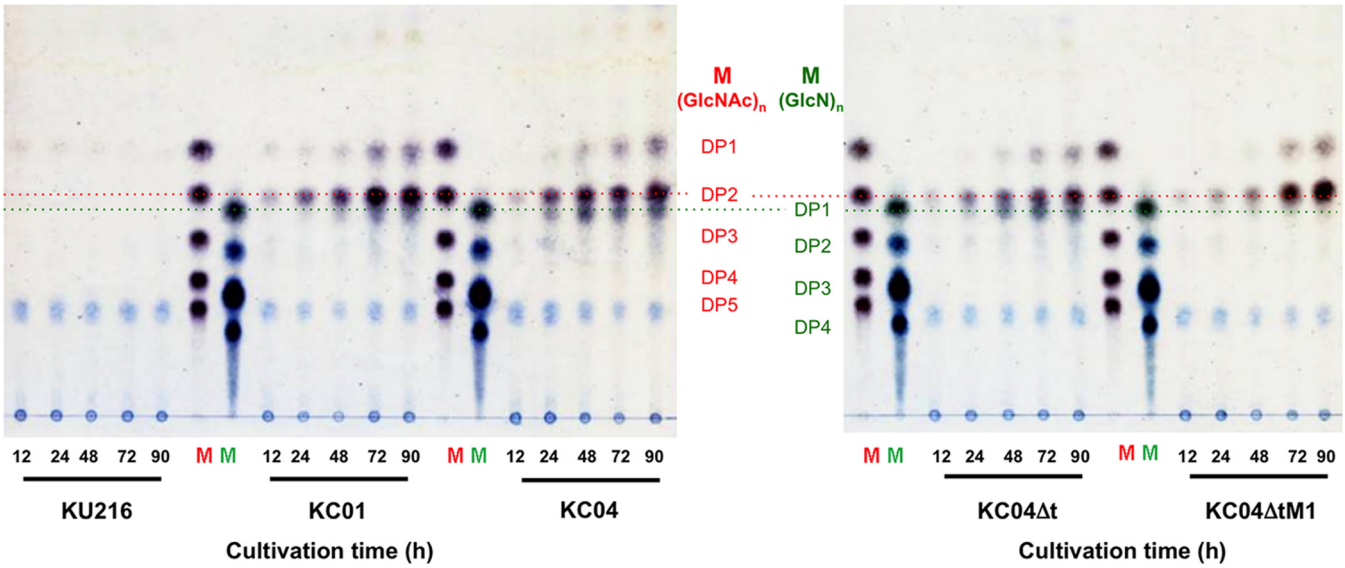

FIG 5 TLC analysis of supernatants after cultivation of engineered *T. kodakarensis* strains in ASW-VMT-SC medium. Chitin degradation products in culture supernatant were analyzed at different time points until 90 h. Lane M, standard GlcNAc oligomers ranging from DP1 to DP5, or standard GlcN oligomers ranging from DP1 to DP4. DP indicates the degree of polymerization. Culture supernatants of KC04ΔtM1 cells displayed increased accumulation of $(GlcNAc)_2$ with very little accumulation of GlcN.

growth in ASW-VMT-SC medium for 72 h. Acetate levels in the cultures of KC04 and KC04Δt were similar to each other and were higher than those in the cultures of KC01 and KU216 (Fig. 6A). On the other hand, ammonium levels of KC04 and KC04Δt were almost equal to the level of KC01 (Fig. 6B).

Overall, the studies on the metabolites indicated that the chitin degradation capacities of KC04 and KC04Δt were significantly increased by the genetic manipulations, but these strains still display GlcN accumulation in the culture broth. As GlcN contains an amino group and a reducing group, excess accumulation of GlcN at high temperatures should promote the Maillard reaction that leads to yellowish by-products, which agrees well with the yellowish appearance of the medium that we observed. The progression of the Maillard reaction should result in a substantial reduction in sugars that would otherwise be available and utilized for cell growth.

Adaptive engineering of *T. kodakarensis* on chitin. The previous analyses suggested that minimizing GlcN accumulation might lead to a more efficient degradation and assimilation of chitin. As it seemed difficult to rationally optimize the balance between GlcN generation and utilization, we next employed an adaptive engineering approach. The KC04Δt strain was cultivated in ASW-VMT-SC medium up to eight serial cultivations. Although chitin degradation was observed for at least 90 h (Fig. 4), aliquots of the cultures were inoculated to the next medium after 24 h in order to enrich cells that displayed relatively faster growth on chitin. With the progression of the serial cultures, the time required to completely degrade the swollen chitin became shorter. After the eighth culture, an initial isolation was carried out on ASW-VMT-SC solid medium, and three isolates were subjected to a further five serial cultivations in ASW-VMT-SC medium. Isolation was carried out from the medium that displayed the most rapid degradation rate and resulted in the isolation of strain KC04ΔtM1. When cultures were performed with KC04ΔtM1 in ASW-VMT-SC medium, almost all of the medium became transparent by 72 h, with little color change (Fig. 4). Western blot analyses indicated that protein levels of GlmA and GlmD were unexpectedly much lower in KC04ΔtM1, while protein levels of ChiA and Dac were comparable to those in KC04Δt (Fig. 3). TLC analysis of chitin degradation products indicated that KC04ΔtM1 exhibited increased accumulation of $(GlcNAc)_2$ but almost no accumulation of GlcN (Fig. 5). Moreover, accumulation of both acetate and ammonium was observed in the

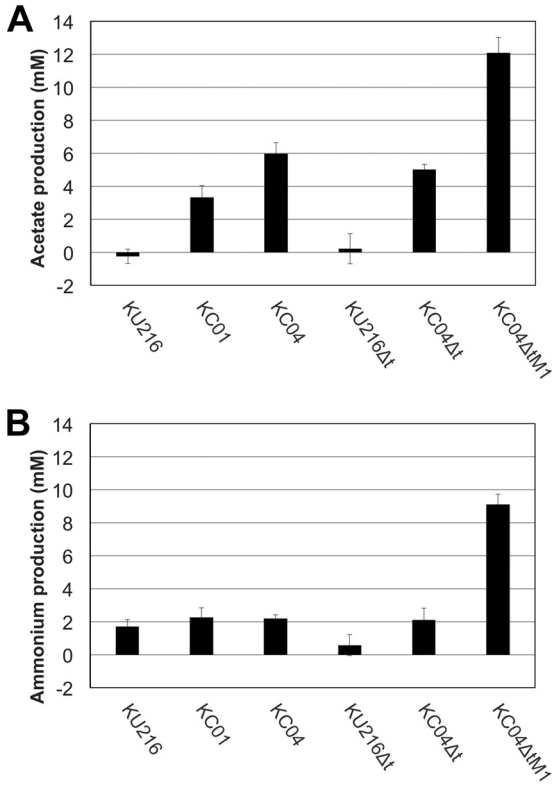

FIG 6 Quantification of acetate and ammonium concentrations in culture supernatants of engineered strains of *T. kodakarensis* growing on swollen chitin. Cultivations were performed in ASW-VMT-SC medium at 85 °C for 72 h, and culture supernatants prepared were analyzed. Results shown were determined by subtracting acetate or ammonium concentrations present in culture medium without inoculation. Error bars represent standard deviations of three independent measurements. KC04ΔtM1 showed increased production of both acetate and ammonium.

culture supernatant of KC04ΔtM1 (Fig. 6A and B), showing that both deacetylation and deamination processes were efficiently proceeding in this strain.

We next examined the possible cause of the lower expression levels of GlmD and GlmA in the KC04ΔtM1 strain. As genes for GlmD and GlmA are located in a single operon under the control of the *gdh* promoter, the promoter region was sequenced to examine whether it contains mutations. As a result, a 15-bp duplication (5′-ATCGAAA GGTTTATA-3′) was found that contains nearly a complete BRE/TATA sequence (Fig. S6). As the BRE/TATA sequence is involved in the process of transcriptional initiation, the duplication of BRE/TATA sequence may have caused a deleterious effect for transcription of this locus, resulting in decreased expression levels of both GlmA and GlmD.

Growth characteristics of the engineered *T. kodakarensis* strains. We have described the construction of multiple strains of *T. kodakarensis* with the aim of developing a strain with enhanced chitin degradation capacity. This required higher expression of a relatively large number of genes related to chitin degradation as well as glycolysis. We examined if the increased expression of a high number of genes affected growth of these strains on a conventional carbon source for *T. kodakarensis*. We grew each strain in a typical nutrient-rich medium supplemented with pyruvate (ASW-YT-Pyr, composed of ASW, yeast extract and tryptone [YT], and pyruvate [Pyr]) (Table 1). Overall, we found that all strains displayed growth in ASW-YT-Pyr medium (Fig. S7). Growth of *T. kodakarensis* KOD1 exhibited two peaks in this medium, one at around 10 h and the other at around 18 h. When we compared specific growth rates among these strains, we observed a tendency that strains with a higher number of overexpressed genes exhibited lower specific growth rates (Table S1). KC04Δt, which overexpresses all of the genes related to chitin degradation and those involved in

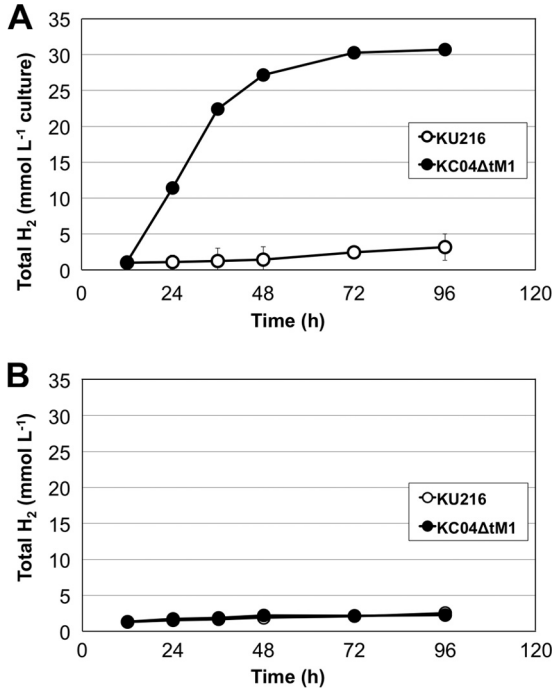

FIG 7 Comparison of chitin-dependent hydrogen (H_2) production in KU216 and KC04ΔtM1 in ASW-VMT-SC medium (A) and in ASW-VMT medium (B). Total hydrogen represents the amount of molecular hydrogen present in the headspace of a culture bottle per milliliter of culture volume (medium volume was 15 ml). Error bars represent standard deviations of three independent cultivations. The results show that strain KC04ΔtM1 exhibits hydrogen production dependent on swollen chitin.

glycolysis, displayed a 25% decrease in specific growth rate compared to that of KU216. In terms of cell yield, we observed that the KC04Δt and KC04ΔtM1 strains displayed the lowest values among the strains (Fig. S7). The differences we observed can be presumed to be due to the investment of starting material and energy directed toward the overexpression of multiple proteins.

Chitin-dependent hydrogen production by *T. kodakarensis* KC04ΔtM1. As *T. kodakarensis* is known to produce molecular hydrogen from maltooligosaccharides through primary metabolism (9, 12), we analyzed whether strain KC04ΔtM1 produced hydrogen from chitin. When *T. kodakarensis* KC04ΔtM1 and its host strain, KU216, were grown in ASW-VMT-SC medium, much higher levels (approximately 10-fold) of hydrogen were produced from KC04ΔtM1 than from KU216 (Fig. 7A). On the other hand, in a medium without swollen chitin (ASW-VMT medium) (Table 1), both strains produced only low levels of hydrogen (Fig. 7B), and this level was comparable to the hydrogen levels of KU216 grown in the presence of swollen chitin. This clearly indicates that most of the hydrogen produced from KC04ΔtM1 is dependent on the presence of chitin and the genetic modifications harbored in KC04ΔtM1.

We estimated the amounts of chitin consumed and compared these levels with those of the production of acetate, ammonium, and hydrogen. Although we introduced 60 mg of chitin into each culture (15 ml), the results of the TLC analysis shown in Fig. 5 indicated that residual $(GlcNAc)_2$ and GlcNAc were still present in the medium at the end of our cultures. By quantifying the residual sugars by high-performance liquid chromatography (HPLC), we found that 0.12 $\pm$ 0.01 mmol of chitin (expressed as GlcNAc units) was consumed during a 96-h culture. The concentration of hydrogen generated at this time was 30.1 $\pm$ 1.8 mmol liter^{-1} culture, corresponding to 0.46 $\pm$ 0.03 mmol hydrogen (Fig. 7A). We also examined the amounts of acetate and ammonium and found that 0.24 $\pm$ 0.01 mmol of acetate and 0.16 $\pm$ 0.00 mmol of ammonium were present in the medium. The maximum theoretical ratios of hydrogen/GlcNAc, acetate/GlcNAc, and ammonium/GlcNAc can be considered 4:1, 3:1, and 1:1, respec-

tively. The results of our cultures with KC04ΔtM1 cells gave ratios of 3.79:1, 2.01:1, and 1.29:1, respectively. In order to exclude the amounts deriving from tryptone metabolism, we subtracted the values obtained in cultures with KU216 cells, resulting in ratios of 3.40:1 (hydrogen/GlcNAc), 2.01:1 (acetate/GlcNAc), and 0.99:1 (ammonium/GlcNAc).

Effect of *tgr* knockout on hydrogen production of KC04ΔtM1. In order to analyze whether *tgr* disruption had effects on the hydrogen production of KC04ΔtM1, the *tgr* gene was reintroduced into KC04ΔtM1 using a pLC70-based plasmid vector (17). While introduction of pLC70M, a control vector without *tgr*, displayed virtually no effect on hydrogen production of KC04ΔtM1 in ASW-VMT-SC medium, reintroduction of the *tgr* gene resulted in a clear delay in the initiation of hydrogen generation (Fig. S8). However, hydrogen production was observed afterwards and reached levels comparable to those with KC04ΔtM1 and KC04ΔtM1/pLC70M cells. We confirmed that the reintroduction of the Tgr gene led to the production of Tgr protein in the cell (Fig. S9). These cells displayed levels of the glycolytic GK and phosphofructokinase (PFK) similar to those of strain KU216 when it was grown on pyruvate (ASW-VMT-Pyr medium), indicating that the Tgr protein in the reintroduced strain represses these two genes under gluconeogenic conditions, as expected (Fig. S10). However, when cells were grown in ASW-VMT-SC medium, we found that the effects of *tgr* reintroduction were less prominent, with the extents of GK and PFK repression much lower than those in cells grown on pyruvate (Fig. S9). Protein levels of Tgr were also lower than those in cells grown on pyruvate. This may be the reason why the *tgr*-complemented strain eventually displayed the ability to assimilate chitin and generate hydrogen.

DISCUSSION

In this study, we have constructed *T. kodakarensis* strains that exhibit improved abilities to degrade/assimilate chitin. In the case of strain KC04, chitin degradation was enhanced, but accumulation of GlcN in the culture supernatant as well as browning of the medium was observed, suggesting that the excess GlcN was subjected to the Maillard reaction (31, 32). Gene disruption of *tgr* (TK1769) was previously shown to increase activity levels of ADP-dependent GK (the TK1110 protein) in the cell extract of *T. kodakarensis* (29). If ADP-dependent GK also exhibits glucosamine kinase activity, activity levels of glucosamine kinase (as well as the activities of glycolytic enzymes) should also be higher in the KC04Δt strain. This might be the reason why the yellowish appearance of the medium was decreased in KC04Δt compared to that in KC04 (Fig. 4). However, the ammonium levels in the culture supernatants of KC04Δt and KC04 were similar, indicating that the flux of the following deamination reaction did not increase significantly and that chitin degradation was still incomplete (Fig. 6B).

KC04ΔtM1 exhibited the highest capacity of chitin degradation, and accumulation of GlcN was not observed (Fig. 4 and 5). Increases in both acetate and ammonium levels indicated that most of the chitin was converted through the chitin metabolic pathway (Fig. 6). As we observed only very little color change in the medium, GlcN accumulation and the progression of the Maillard reaction can be presumed to be minimal in this strain. Metabolites from chitin were efficiently assimilated and resulted in cell growth, as high levels of hydrogen production, which occur only in growing cells (12, 33), were observed. Western blot analyses revealed that expression levels of GlmD and GlmA were significantly decreased in KC04ΔtM1 compared to the level in the direct parent strain KC04Δt (Fig. 3). This was an unexpected result but can be interpreted as follows. The decrease in the levels of GlmA (exo-β-D-glucosaminidase), which generates GlcN, reduces its production rate. This can be expected to reduce the accumulation of GlcN, which is actually the case, as shown in Fig. 5. As GlcN accumulation readily causes progression of the Maillard reaction under high-temperature environments, a decrease in accumulation may prevent the loss of carbon and energy provided by GlcN. The prevention of the Maillard reaction can clearly be observed when the colors of the cultures of KC04Δt and KC04ΔtM1 at 90 h shown in Fig. 4 are compared. In addition to the loss of carbon and energy, the products of the Maillard reaction have a strong

inhibitory effect on cell growth of the aerobic hyperthermophilic archaeon *Aeropyrum pernix* (34), and this may also be the case for *T. kodakarensis*.

To analyze the effect of *tgr* disruption on chitin assimilation of KC04ΔtM1, the *tgr* gene was reintroduced to the strain. This resulted in a delay in the initiation of hydrogen generation, but the strain still generated hydrogen in ASW-VMT-SC medium to a level similar to that observed with KC04ΔtM1 (see Fig. S8 in the supplemental material). The weak effect of *tgr* reintroduction in cells cultivated in ASW-VMT-SC medium agrees well with the fact that GK and PFK are only slightly repressed (~30% decrease) (Fig. S9). By Western blotting, we observed that levels of Tgr protein in a *tgr*-complemented strain grown in ASW-VMT-SC medium was almost one-third of that grown in ASW-VMT-Pyr medium, in which levels of the repression of PFK and GK were much more significant (50 to 90% decrease) (Fig. S9). This is intriguing and may be due to the fact that the *tgr* gene is located on a plasmid and not on the genome, but further analysis will be necessary to elucidate the weakened repression by Tgr in chitin-grown cells.

We find it intriguing that *T. kodakarensis*, with a complete set of chitin-degrading genes, does not display robust growth on chitin. When we look for genes involved in chitin degradation in other hyperthermophilic archaea, we find that the distribution is very limited. Genomes that harbor homologs of the chitin-degrading enzymes of *T. kodakarensis* are found only in *P. furiosus*, *Thermococcus chitonophagus*, and *Thermococcus nautili*. In *P. furiosus*, two consecutive chitinase genes (*Pf-chiA* and *Pf-chiB*) are separated by a single base insertion, and the removal of this base leads to a fusion gene whose product is structurally similar to that of *T. kodakarensis chiA* (*Tk-chiA*) harboring two different catalytic domains (endo- and exo-type) (35, 36). The growth of *P. furiosus* on chitin seems to vary depending on the conditions of the medium or on how the cells are adapted to the presence of chitin (35–38). Recently, genetic engineering of *P. furiosus* was reported that conducted a deletion of the single base insertion located between *Pf-chiA* and *Pf-chiB* (39). The engineered strain with a single chitinase gene reached a maximum cell density of 1×10^8 ml^{-1} on colloidal chitin-containing medium, which is a 10-fold higher cell concentration than that of the wild-type strain. It is interesting that *P. furiosus*, that had lost its intact chitinase gene, could easily restore growth on chitin, whereas *T. kodakarensis*, with an intact chitinase gene, does not display growth on chitin.

T. chitonophagus is known to possess multiple chitinases (40–42). Recently, two groups of investigators have conducted genome analyses of this archaeon, and both predicted the presence of three chitinase genes (43, 44). Reclassification of the organism to the genus *Pyrococcus* has also been proposed (species name *Pyrococcus chitonophagus*) (43). Biochemical characterization of one of the *T. chitonophagus* chitinases, ChiD, has revealed that the enzyme is a structurally novel chitinase exhibiting exo-type activities and mainly releases $(GlcNAc)_2$ units from the reducing ends of chitin chains (44). As *T. kodakarensis* ChiA contains an endo-chitinase domain and an exo-chitinase domain that recognizes only the nonreducing ends of chitin chains, introduction of the ChiD gene into *T. kodakarensis* KC04ΔtM1 may further improve its chitin degradation capabilities.

Using the engineered *T. kodakarensis* KC04ΔtM1 strain, a system for molecular hydrogen production from chitin has been established. According to our search, there is only one report on hydrogen production from chitin or chitin-containing materials. Evvyernie et al. has reported hydrogen production from a mesophilic bacterium, *Clostridium paraputrificum* M-21, using *N*-acetylglucosamine, chitin, and chitin-containing wastes (45, 46). From 1 g of raw chitinous wastes, the evolved hydrogen was reported as 5.2 to 7.6 mmol. *T. kodakarensis* KC04ΔtM1 exhibited a higher hydrogen conversion (18.7 $\pm$ 1.1 mmol of H_2 per gram of swollen chitin [consumed]), but we should note that our study employed pure chitin.

The results of our cultures with KC04ΔtM1 cells gave ratios of 3.40:1 (hydrogen/GlcNAc), 2.01:1 (acetate/GlcNAc), and 0.99:1 (ammonium/GlcNAc). It has been shown that in the metabolism of *T. kodakarensis*, the pyruvate formed from GlcNAc can be

directed to either acetate (with hydrogen production) or alanine (no hydrogen production) (9, 12). As the ratios of hydrogen/GlcNAc and acetate/GlcNAc, particularly the latter, are lower than the maximum values, we can expect that a portion of the pyruvate formed from GlcNAc is directed toward alanine formation. When soluble starch is used as the sugar source, the theoretical maximum ratios of hydrogen/glucose and acetate/glucose are 4:1 and 2:1, respectively. In a previous study, we observed hydrogen/glucose and acetate/glucose ratios of 3.33:1 and 1.12:1, respectively, indicating that the conversion of chitin to hydrogen is comparable to that of starch to hydrogen (9).

Our results indicate that hydrogen production ceased prior to the total consumption of $(GlcNAc)_2$ in the medium. A possible reason for the cessation of hydrogen production and cell growth might be the accumulation of hydrogen itself. Another reason might be the drop in pH of the culture medium. We observed a decrease in pH from 6.4 to 5.4 after 72 h of cultivation in ASW-VMT-SC medium, which might be due to accumulation of acetate in the culture medium. Cultivation methods that reduce hydrogen and/or acetate accumulation (e.g., increase in the volume of the gas phase or cultivation using dialysis membrane) should contribute to further increasing the hydrogen conversion rate from chitin.

MATERIALS AND METHODS

Strains and media. *T. kodakarensis* strains were cultivated with basically the same methods as described elsewhere (14, 15). *T. kodakarensis* KU216 (15) and its derivative strains were grown under anaerobic conditions at 85°C in either a nutrient-rich ASW-YT medium, a synthetic ASW-AA medium or a semisynthetic medium supplemented with tryptone (ASW-VMT medium). The compositions of all media used in this study are shown in Table 1. ASW-VMT-SC medium, which is ASW-VMT medium supplemented with 4.0 g liter^{-1} swollen chitin, was the medium used for adaptive engineering cultures and to evaluate chitin degradation of various *T. kodakarensis* strains. ASW-AA medium (where AA is amino acids) was used in transformation procedures. ASW-YT medium was mainly used for growth examinations and precultivation. ASW-YT, ASW-AA, and ASW-VMT media were supplemented with 2.0 g liter^{-1} of S^0 (sulfur powder), sodium pyruvate (5.0 g liter^{-1}), maltodextrin (5.0 g liter^{-1}), or 1.0 g liter^{-1} chitin oligomers (NA-COS-Y; Yaizu Suisankagaku Industry, Yaizu, Japan) when necessary. The media used in individual experiments are indicated in the respective sections. For solid media, S^0 was replaced with 2 ml of a polysulfide solution (10 g of $Na_2S{\cdot}9H_2O$ and 3 g of sulfur powder in 15 ml of H_2O) per liter, and 10 g liter^{-1} of Gelrite was added to solidify the medium. When necessary, 4.0 g liter^{-1} swollen chitin was also added. All medium components, unless mentioned otherwise, were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan). Cell growth of *T. kodakarensis* strains grown in ASW-YT-Pyr medium at 85°C was continuously monitored at 660 nm in an incubator equipped with an optical density (OD) measurement apparatus (model OD-BR-43FH-VH; Taitec, Koshigaya, Japan) with water as a reference. *Escherichia coli* strain DH5α was cultivated in LB medium (47) at 37°C with 100 mg liter^{-1} ampicillin sodium salt. All strains and plasmids used in this study are listed in Table 2.

Preparation of swollen chitin. Chitin from crab shells (2.5 g; Nacalai Tesque) was mixed with 125 ml of phosphoric acid (85%, wt/vol, in water) and stirred at 4°C for 24 h. The suspension was poured into 1.25 liters of deionized water and centrifuged (15,300 $\times$ *g*, 10 min, 4°C). The resulting precipitate was washed with deionized water several times until the pH of the suspension became neutral. The phosphoric acid treatment disrupts the hydrogen bond networks of chitin chains (48). The networks are only partially restored after washes with water, resulting in swollen chitin. A similar phosphate treatment procedure conducted at room temperature produced chitin samples with average degrees of polymerization (DP) varying from 1,300 to 110 (49). The swollen chitin was finally suspended with 100 ml of deionized water. The concentration of chitin in the suspension was determined by drying an aliquot and measuring the dry weight. TLC/HPLC analyses indicated that GlcN and GlcNAc oligomers were not present at detectable levels in our swollen-chitin preparation.

Plasmid construction. Plasmids to construct overexpression strains for *chiA* (TK1765), *dac* (TK1764), and the *dppABCDF-glmDA* operon (TK1754-TK1760) were designed so that their native promoters were replaced with the following strong promoters: the cell surface glycoprotein gene (*csg*; TK0895) promoter (P_{csg}), the promoter of the archaeal elongation factor 1α subunit gene (TK0308), and the glutamate dehydrogenase gene (*gdh*; TK1431) promoter (P_{gdh}), respectively. Gene replacements were performed via a single-crossover insertion/pop-out recombination technique (see Fig. S1 in the supplemental material) previously reported in *T. kodakarensis* (27, 50). Nucleotide sequences of all plasmids were examined to confirm the absence of any unintended mutations. Sequences of primers used in this study are listed in Table 3.

(i) Construction of a plasmid for *chiA* overexpression. A *chiA* overexpression plasmid (pUD3-P_{csg}-*chiA*) was constructed as follows (Fig. S2). A DNA fragment containing part of *dac* and the promoter region of *chiA* (P_{chiA}) fused with P_{csg} was amplified from pUD3-TK2141 (27) with the primer set ChiA_UP1_PstI/P_{csg}R and digested with PstI/NdeI (fragment A). A part of the *chiA* coding region (containing the start codon) was amplified with the primer set OE-chiA-f-1/OE-chiA-r-1, and the fragment was digested with NdeI/EcoRI (fragment B). Fragment A and fragment B were inserted into pUD3(ΔNdeI)

TABLE 2 Strains and plasmids used in the study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli		
DH5α	λ^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	Stratagene (La Jolla, CA)
T. kodakarensis		
KOD1	Wild type	7, 8
KU216	KOD1 Δ*pyrF*	20
KC01	KU216 P_{csg}-*chiA*	This study
KC04	KU216 P_{csg}-*chiA* P_{gdh}-*dppABCDF-glmDA* EF-1α gene promoter-*dac*	This study
KU216Δt	KU216 Δ*tgr*	This study
KC04Δt	KC04 Δ*tgr*	This study
KC04ΔtM1	KC04Δt, after adaptive engineering	This study
Plasmids		
pUC118	General cloning vector, Amp^r	TaKaRa Bio (Otsu, Japan)
pUD3	pUC118 derivative; *pyrF marker* cassette	27
pUD3(ΔNdeI)	pUD3 derivative; NdeI site was deleted	This study
pUD3-P_{csg}-*chiA*	pUD3(ΔNdeI) derivative; P_{csg}-*chiA pyrF*	This study
pUD3-*dac*-$P_{ef\text{-}1\alpha}$-P_{csg}-*chiA*	pUD3(ΔNdeI) derivative; P_{csg}-*chiA* EF-1α gene promoter-*dac pyrF*	This study
pUD3-P_{gdh}-*dppA*	pUD3(ΔNdeI) derivative; P_{gdh}-*dppA pyrF*	This study
pUD3_1769D	pUD3 derivative; for *tgr* disruption	This study
pLC70	pLC64 derivative; *hmg trpE*	17
pLC70M	pLC70 derivative; *hmg*	This study
pLC-tgr	pLC70 derivative; *hmg tgr*	This study

digested with PstI/EcoRI, resulting in the *chiA* expression vector pUD3-P_{csg}-*chiA*. pUD3(ΔNdeI) is derived from pUD3 (27) with a point mutation within the NdeI site of pUD3 (CATATG to CACATG).

(ii) Construction of a plasmid for overexpression of both *chiA* and *dac*. The plasmid used to construct a *T. kodakarensis* strain that overexpresses both *chiA* and *dac* was constructed based on pUD3-P_{csg}-*chiA* (Fig. S3). Inverse PCR was performed with the primer set dPchiA-inverse-f/dPchiA-inverse-r, followed by digestion with BglII. A 234-bp region of the EF-1α gene promoter was amplified with the primer set PEF-1a-EcoRV-f/PEF-1a-r, followed by digestion with EcoRV/BglII. These digested products were ligated to obtain the plasmid pUD3-*dac*-$P_{ef\text{-}1\alpha}$-P_{csg}-*chiA*.

(iii) Construction of a plasmid for overexpression of an ABC transporter, *glmD*, and *glmA*. For overexpression of the *dppABCDF-glmDA* operon, a fragment containing part of the *dppA* (TK1760) coding region and part of the neighboring β-glycosidase gene (TK1761), together with the promoter region located in between these genes, was amplified with the primer set ABCtransporter-f/ABCtransporter-r, followed by digestion with PstI/EcoRI (Fig. S4). The fragment was ligated with pUD3(ΔNdeI) digested with PstI/EcoRI. Inverse PCR of the plasmid was performed to separate *dppA* with its promoter region with the

TABLE 3 Primers used in the study

Name	Sequence (5′–3′)[a]
ChiA_UP1_PstI	AAACTGCAGCCTTTCCTTTCTTATCACGG
P_{csg}R	GTGGTGGTGGTGGTGGTGCATATGACAACACCTCCTTGGGTT
OE-chiA-f-1	AAAAGTCGACCATATGAAGAAGATTTGGACTTCA
OE-chiA-r-1	AAAAGAATTCAGAGGATCAGCGTAGGTATC
dPchiA-inverse-f	AAAAAGATCTTATCGGCAAAAGGCGAATTATGTGT
dPchiA-inverse-r	ATGGTGTTTGAGGAGTTCAACAATT
PEF-1a-EcoRV-f	AAAAGATATCCAAACACCTCCATATTTTGGT
PEF-1a-r	AAAAAGATCTTGCGGGCTTTCTTCTTGTTCTCTC
ABCtransporter-f	AAAACTGCAGTATGTCGATGTAGTATGTACCGACG
ABCtransporter-r	AAAAGAATTCTGAGAAGGTTTTAACTGCCTCGTAG
ABCt-inverse-f	AAAAGGATCCCATATGAAGAAAGCTACCGCGGTTG
ABCt-inverse-r	AAAAGGATCCGGACACCACCACTTATAAGG
Pgdh-f	AAAAGTCGACCATATGTCATACCACCTCATTTCGGTAAT
Pgdh-r	AAAAGGATCCGCCCGTTGCCCGATGATTGGTTT
Δtk1769.1	AAAAAAGTCGACGGTGGAAAACGCCGTCGAGTAC
Δtk1769.2	TTTTTTGGATCCCGGTTATCACTTTCACGTTCTC
Δtk1769.3	CCCATCATTTTTAATTTCTAAACTT
Δtk1769.4	GGCTTAACCCCCAAAGACATTTAAG
TK1769compF1	GGGAATTCGCGGCCGCCACTCTGCCGTGGATGAAGT
TK1769compR1	GCATGGGCCCTAAAGCTATGCCCCAAAAATTCAGAAGAGAAATAG AAAAATGTAGAGGAATCACTCAAGGAGGATGAACT

[a]Restriction enzyme sites incorporated for genetic manipulation are underlined.

primer set ABCt-inverse-f/ABCt-inverse-r, and the amplified product was digested with NdeI/BamHI. The fragment was ligated with a 299-bp *gdh* promoter region that was amplified using genomic DNA of *T. kodakarensis* with the primer set Pgdh-f/Pgdh-r and digested with NdeI/BamHI. The plasmid was designated pUD3-P_{gdh}-*dppA*.

(iv) Construction of a *tgr* disruption vector. The *tgr* gene (TK1769) encodes a transcriptional repressor of glycolytic enzymes (29). A disruption plasmid for the *tgr* gene (TK1769) (designated pUD3_1769D) was constructed as follows. The TK1769 gene together with its 5′- and 3′-flanking regions (~1 kbp) was amplified from the *T. kodakarensis* genomic DNA using the primer set Δtk1769.1/Δtk1769.2. The amplified fragment was digested with SalI/BamHI and inserted into the respective sites of pUD3. To remove the *tgr* coding region from the resulting plasmid, inverse PCR was performed using the primer set Δtk1769.3/Δtk1769.4. The PCR product was self-ligated to construct pUD3_1769D.

(v) Construction of a *tgr*-containing complementation vector. The *tgr* gene together with its promoter region was amplified from *T. kodakarensis* KOD1 genomic DNA using the primer set TK1769comp-F1/TK1769comp-R1. The primer TK1769comp-R1 contains the transcriptional terminator region (5′-TAAAGCTATGCCCCAAAAATTCAGAAGAGAAATAGAAAAATGTAGAGGAA-3′) of the *P. furiosus* glutamate dehydrogenase gene (PF1602). The amplified fragment contained the *tgr* gene flanked with its promoter region and the PF1602 terminator region and was digested with ApaI/NotI restriction enzymes. pLC70 is a shuttle vector which can autonomously replicate in both *E. coli* and *T. kodakarensis* (17). pLC70 was digested with ApaI/NotI and was ligated with the fragment containing the *tgr* gene, resulting in pLC-tgr (Fig. S5). A control plasmid was made to delete *trpE* by digestion of pLC70 using NotI/ApaI restriction enzymes, blunting, and self-ligation (pLC70M).

Transformation of *T. kodakarensis*. *T. kodakarensis* KU216 strain (15) was the parent strain for constructing recombinant strains that can degrade and assimilate chitin. Transformation methods were performed basically as described previously (14, 15). Plasmid for transformation (3 μg) was added to the cell suspension, and the mixture was kept on ice for a further 1 h, followed by heat shock at 85°C for 45 s. The cells were transferred to ASW-AA medium supplemented with S^0 (without uracil) and incubated at 85°C for 2 days. The cells were further cultivated for 2 days in the same medium to enrich the *pyrF*-containing transformants obtained via single-crossover recombination. Cells with *pyrF* deleted by pop-out recombination were selected in the presence of 5-fluoroorotic acid using ASW-AA or ASW-YT solid medium containing polysulfide. Genotypes of transformants were confirmed by PCR and direct sequencing. The shuttle plasmids pLC-tgr and pLC70M were introduced into *T. kodakarensis* KC04ΔtM1, and the transformants were selected by simvastatin resistance as described previously (16).

Western blot analysis of culture supernatants and cell extracts. *T. kodakarensis* strains were cultivated at 85°C in ASW-VMT-Mdx-CO medium and ASW-VMT-Pyr medium for 12 h and in ASW-VMT-SC medium for 90 h. Culture supernatants were prepared by centrifugation (5,000 × *g*, 15 min, 4°C) of cell cultures twice. The resulting culture supernatants were concentrated 15-fold with a Vivaspin ultracentrifugation device (30-kDa-cutoff membrane; Sartorius Stedim Biotech, Göttingen, Germany). Cell pellets after the first centrifugation were resuspended in 50 mM Tris-HCl (pH 7.5) buffer and disrupted by vortexing for 5 min. The resulting suspension was centrifuged (20,400 × *g*, 15 min, 4°C), and the supernatant obtained was used as cell extract. The culture supernatants and cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis ([SDS-PAGE] 12.5% or 15% acrylamide concentration), followed by blotting to a polyvinylidene difluoride membrane (Hybond-P; GE Healthcare Biosciences, Chicago, IL). For detection of ChiA, Dac, GlmD, GlmA, GK (encoded by the TK1110 gene), phosphofructokinase (PFK) (encoded by the TK0376 gene), or Tgr (encoded by the TK1769 gene), rabbit anti-ChiA, anti-Dac, anti-GlmD, anti-GlmA, anti-GK, anti-PFK, or anti-Tgr antiserum was used, respectively. Horseradish peroxidase-conjugated recombinant protein G (Zymed Laboratories, San Francisco, CA) was used as the secondary antibody. For signal detection, ECL Select Western blotting detection reagent (GE Healthcare Biosciences) and ImageQuant LAS 500 (GE Healthcare Biosciences) were used.

Analysis of chitin degradation capabilities. To examine the chitin degradation capabilities of recombinant strains, cells were precultivated in ASW-YT-S^0 medium at 85°C for 12 h. Cells were inoculated into ASW-VMT-SC medium (total volume, 20 ml) to obtain a theoretical initial OD at 660 nm (OD_{660}) of 0.0025 and cultivated at 85°C. The degrees of chitin degradation were examined at different time intervals (0, 12, 24, 48, 72, and 90 h).

Analysis of chitin metabolites in culture supernatants. Culture supernatants were collected at different time intervals (12, 24, 48, 72, and 90 h) to analyze metabolites of chitin degradation and assimilation. Aliquots of broth were centrifuged (20,400 × *g*, 15 min, 4°C), and the supernatants were spotted onto a silica gel plate (DC Kieselgel 60; Merck Co., Berlin, Germany). Products were developed with 1-butanol–methanol–25% ammonia solution–water (5:4:2:1 [vol/vol/vol/vol]) and separated. Products were identified by spraying the plate with aniline-diphenylamine reagent (4 ml of aniline, 4 g of diphenylamine, 200 ml of acetone, and 30 ml of 85% phosphoric acid), followed by baking at 180°C for 3 min. The amounts of chitin oligomers in culture supernatants after 96 h of cultivation in ASW-VMT-SC medium were determined by HPLC. A 10-μl aliquot of culture supernatant was applied to an Asahipak NH2P-50 4E column (Showa Denko, Kanagawa, Japan). As a mobile phase, 70% (vol/vol) acetonitrile was used at a flow rate of 1.0 ml min^{-1} at 40°C, and detection was performed with a UV detector at a wavelength of 210 nm.

Analysis of acetate and ammonium levels in culture supernatants. *T. kodakarensis* strains were cultivated in ASW-VMT-SC medium at 85°C, and culture supernatants were collected after 72 or 96 h. Concentrations of acetate and ammonium were determined enzymatically using F-kits for acetate and ammonium (Roche Diagnostics, Basel, Switzerland), respectively.

Analysis of hydrogen production. Chitin-dependent production of molecular hydrogen (H_2) was analyzed periodically (12, 24, 36, 48, 72, and 96 h) during cultivation in ASW-VMT-SC medium (total volume, 15 ml) at 85°C by a gas chromatograph (GC) equipped with thermal conductivity detectors (GC-17A, TCD-17 detector at 100°C, injector at 100°C [Shimadzu, Kyoto, Japan]). Hydrogen was measured by separation using a Sincarbon-T column (Shinwa Kako, Kyoto, Japan) at a temperature of 60°C using argon as the carrier gas with a flow rate of 30 ml min^{-1}.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00280-17.

SUPPLEMENTAL FILE 1, PDF file, 1.5 MB.

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We declare that there are no conflicts of interest.

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