Supplemental Material

Engineering of the hyperthermophilic archaeon *Thermococcus kodakarensis* for chitin-dependent hydrogen production

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Supplementary Table S1. Specific growth rates of engineered strains of *T. kodakarensis* in ASW-YT-Pyr medium

Strains	Specific growth rate (h^{-1})
KOD1	0.47 ± 0.03
KU216	0.45 ± 0.04
KC01	0.39 ± 0.04
KC04	0.40 ± 0.05
KU216Δt	0.37 ± 0.02
KC04Δt	0.34 ± 0.03
KC04ΔtM1	0.42 ± 0.05

The data represents the average of four independent cultures and are shown with standard deviations.

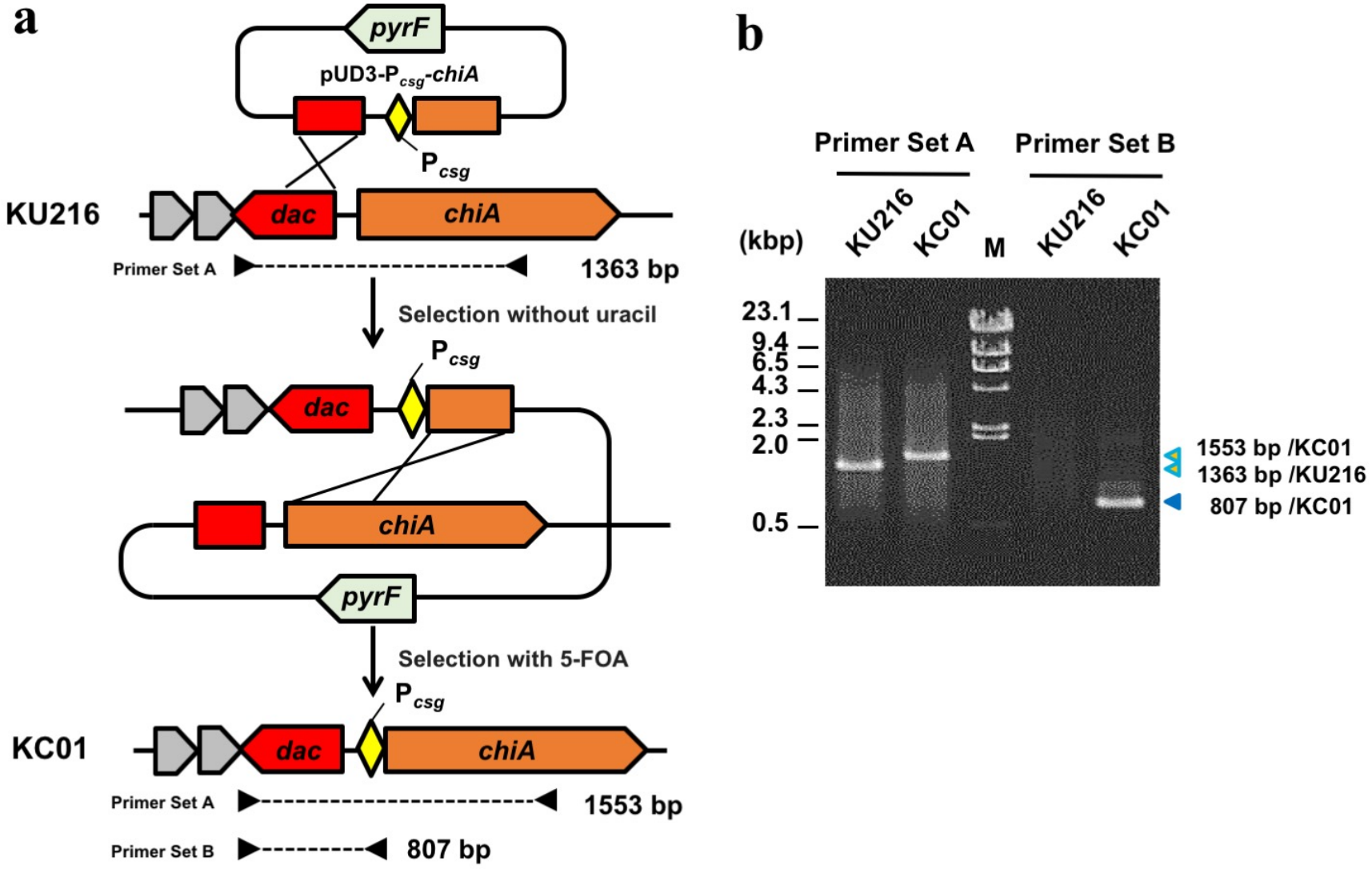

Fig. S1 Construction of a ChiA overexpression strain of *T. kodakarensis*. (a) A general strategy to construct strain KC01 *via* single-crossover insertion/pop-out recombination. A strong promoter (P_{csg}) was placed just before *chiA* ORF (b) Insertion of P_{csg} was confirmed by PCR using Primer Set A (attached in *dac* or *chiA*) and Primer Set B (attached in P_{csg} promoter or *dac*). Analysis by Primer Set A shows an amplified product (1363bp) in KU216, while a longer product (of 1553bp) was amplified in in KC01 as a result of P_{csg} insertion. Analysis by Primer Set B does not give any product in the case of KU216, while a product (of 807bp) is amplified for KC01, indicating the presence of P_{csg} next to *dac*.

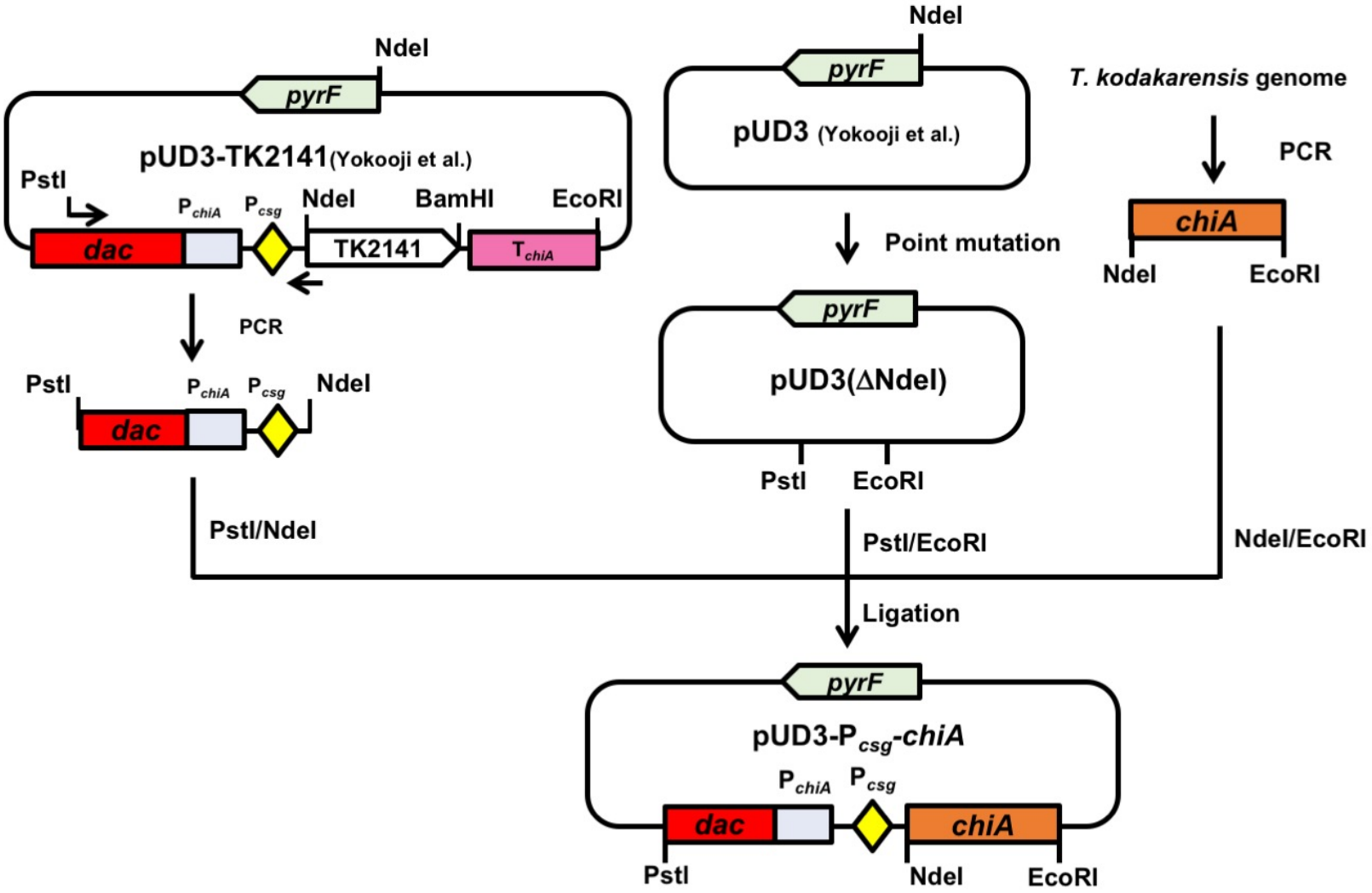

Fig. S2 Construction of the *chiA* overexpression vector pUD3-P_{csg}-*chiA*.

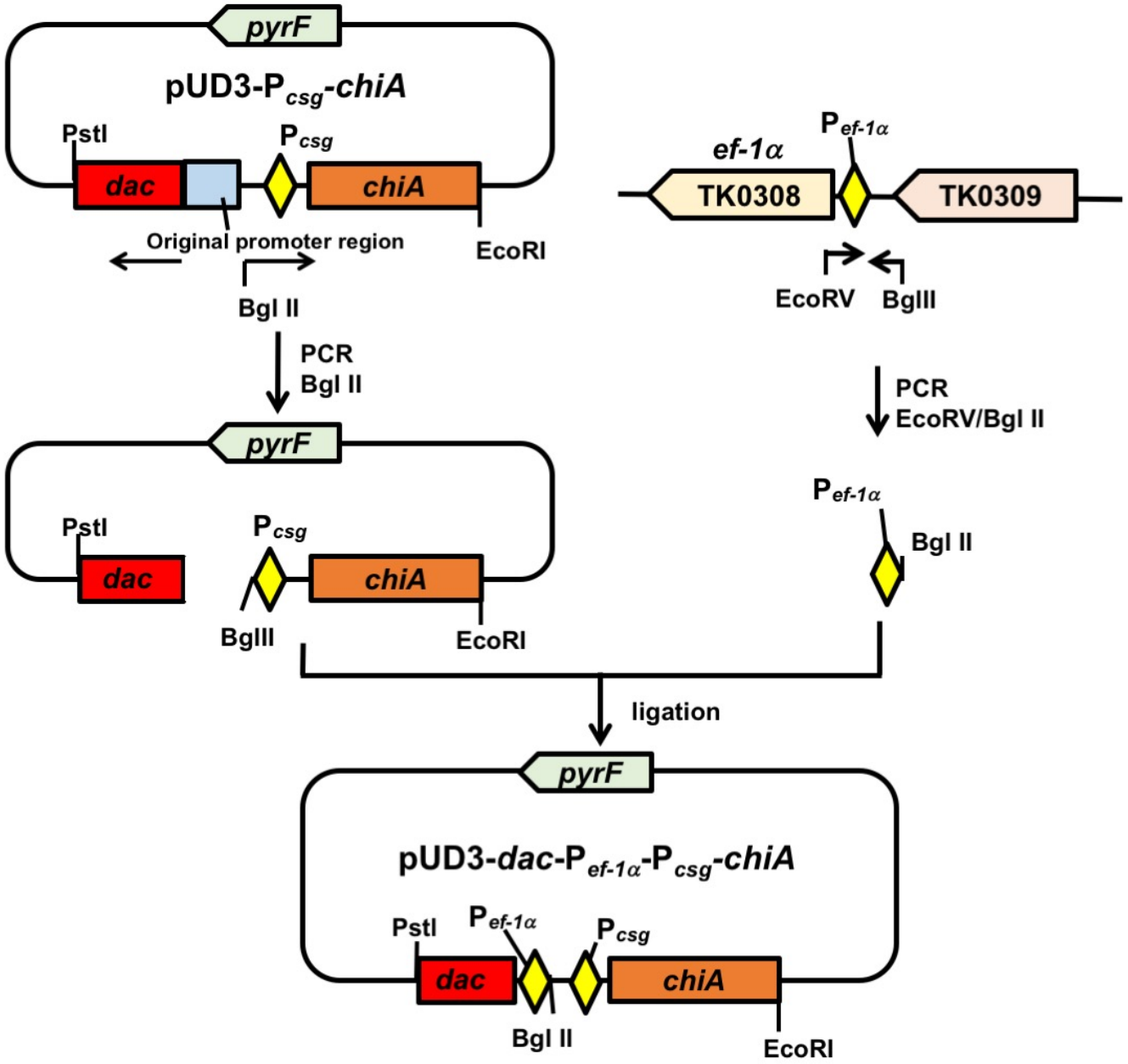

Fig. S3 Construction of the *dac* and *chiA* overexpression vector pUD3-*dac*-$P_{ef\text{-}1\alpha}$-P_{csg}-*chiA*.

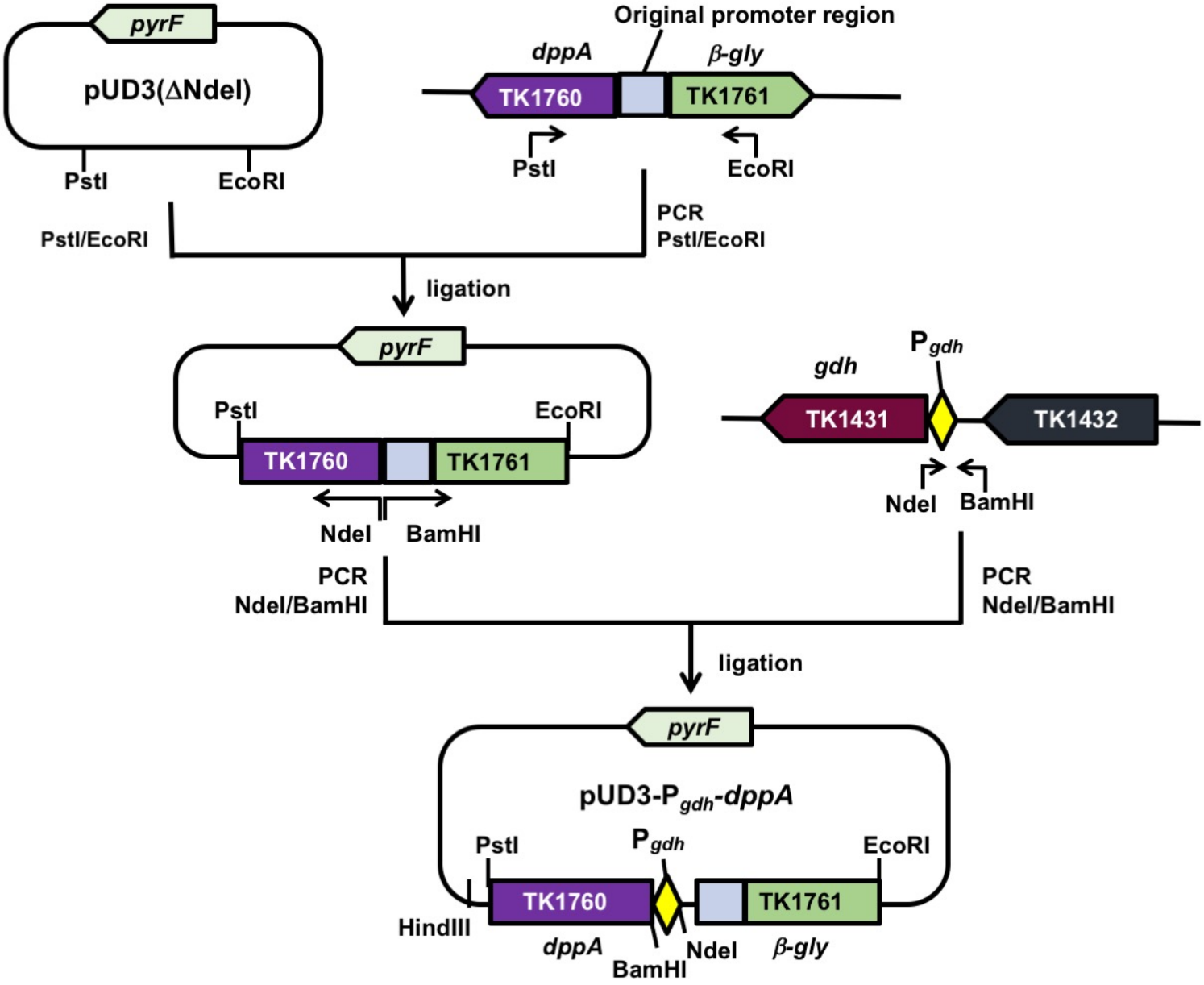

Fig. S4 Construction of the plasmid pUD3-P_{gdh}-*dppA* for overexpression of ABC transporter genes, *glmD* and *glmA*.

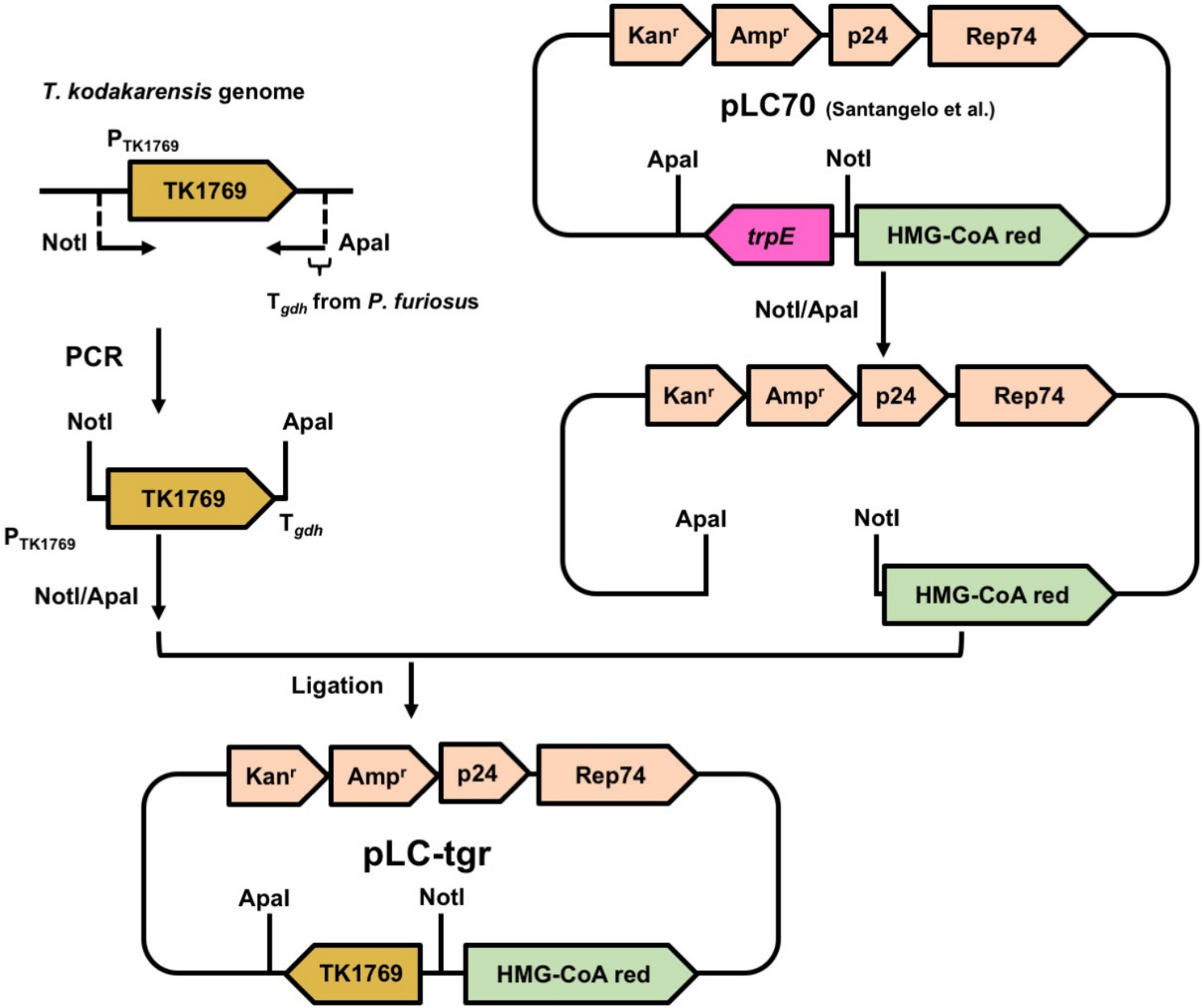

Fig. S5 Construction of the plasmid pLC-tgr for complementation of *tgr*. The region containing *trpE* in pLC70 was replaced by TK1769 (*tgr*). HMG-CoA reductase was used as a selectable marker for transformation.

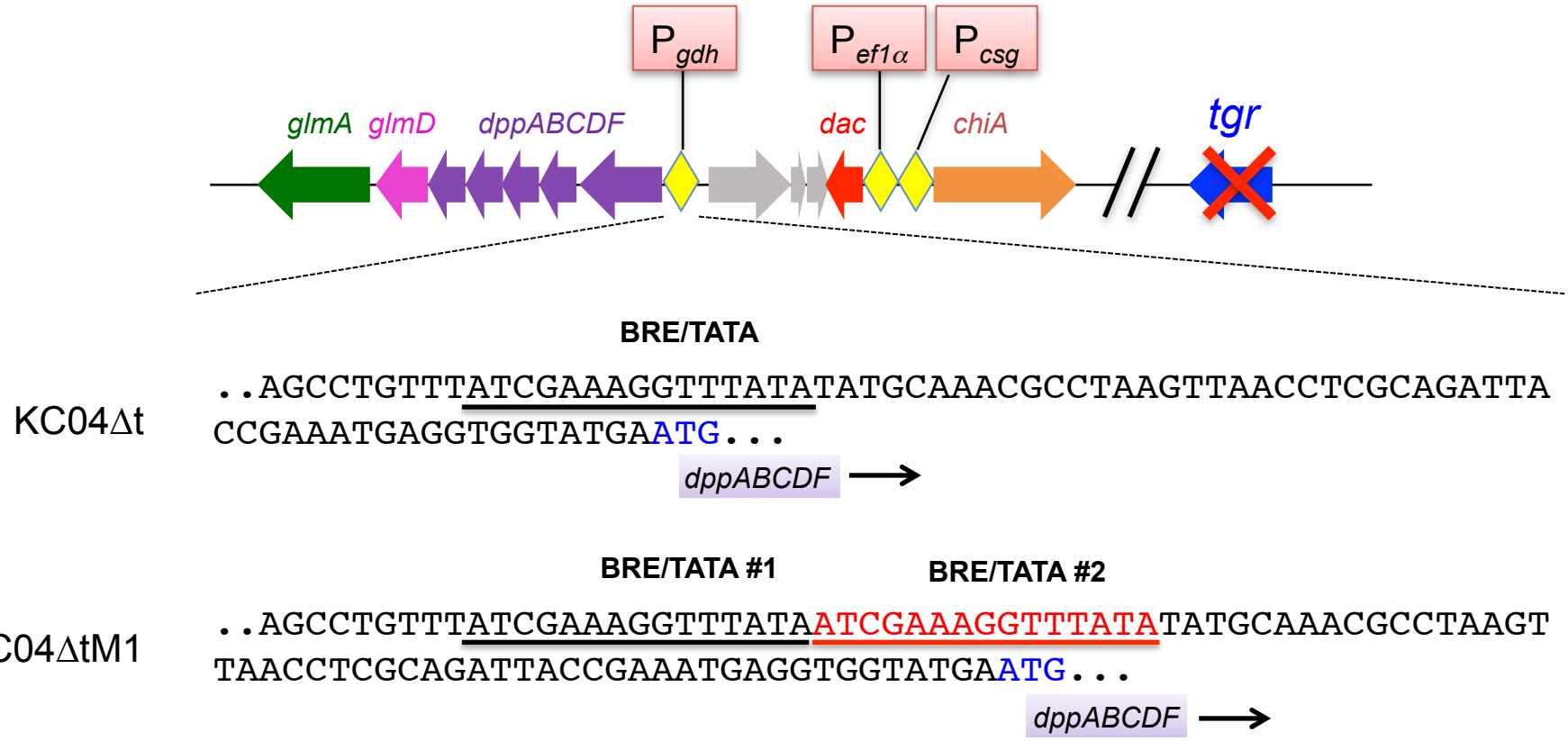

Fig. S6 Comparison in sequences in P_{gdh} regions of KC04Δt and KC04ΔtM1. A 15bp repeat sequence (underline) was found in BRE/TATA region of P_{gdh} in strain KC04ΔtM1 (shown as BRE/TATA #1 and BRE/TATA #2), which is not present in strain KC04Δt (the parental strain).

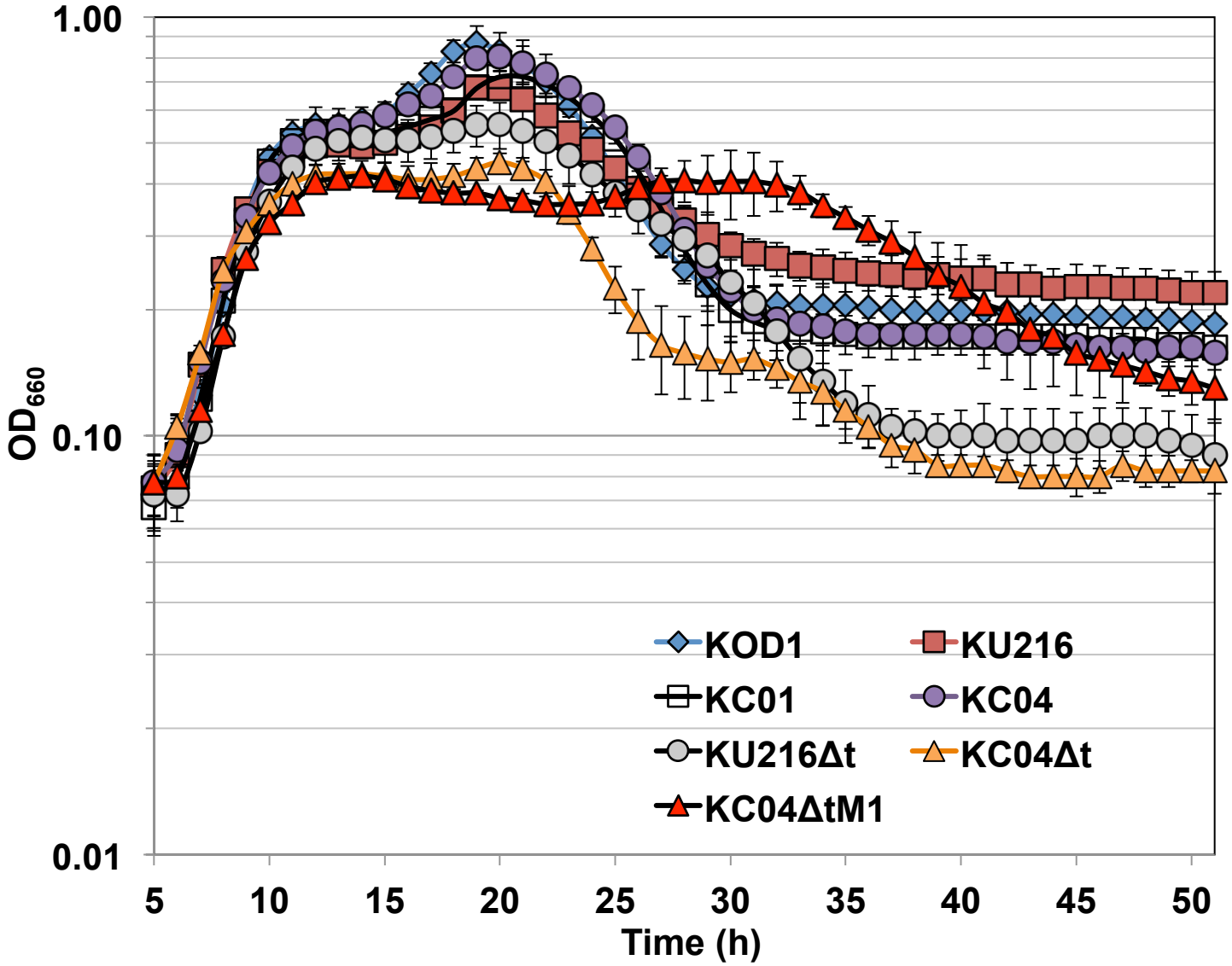

Fig. S7. Growth curve of *T. kodakarensis* strains in ASW-YT-Pyr medium. Batch cultivation of each strain was carried out at 85°C. Error bars represent the standard deviations of four independent growth experiments. We observed a tendency that strains with a higher number of overexpressed genes exhibited lower specific growth rates.

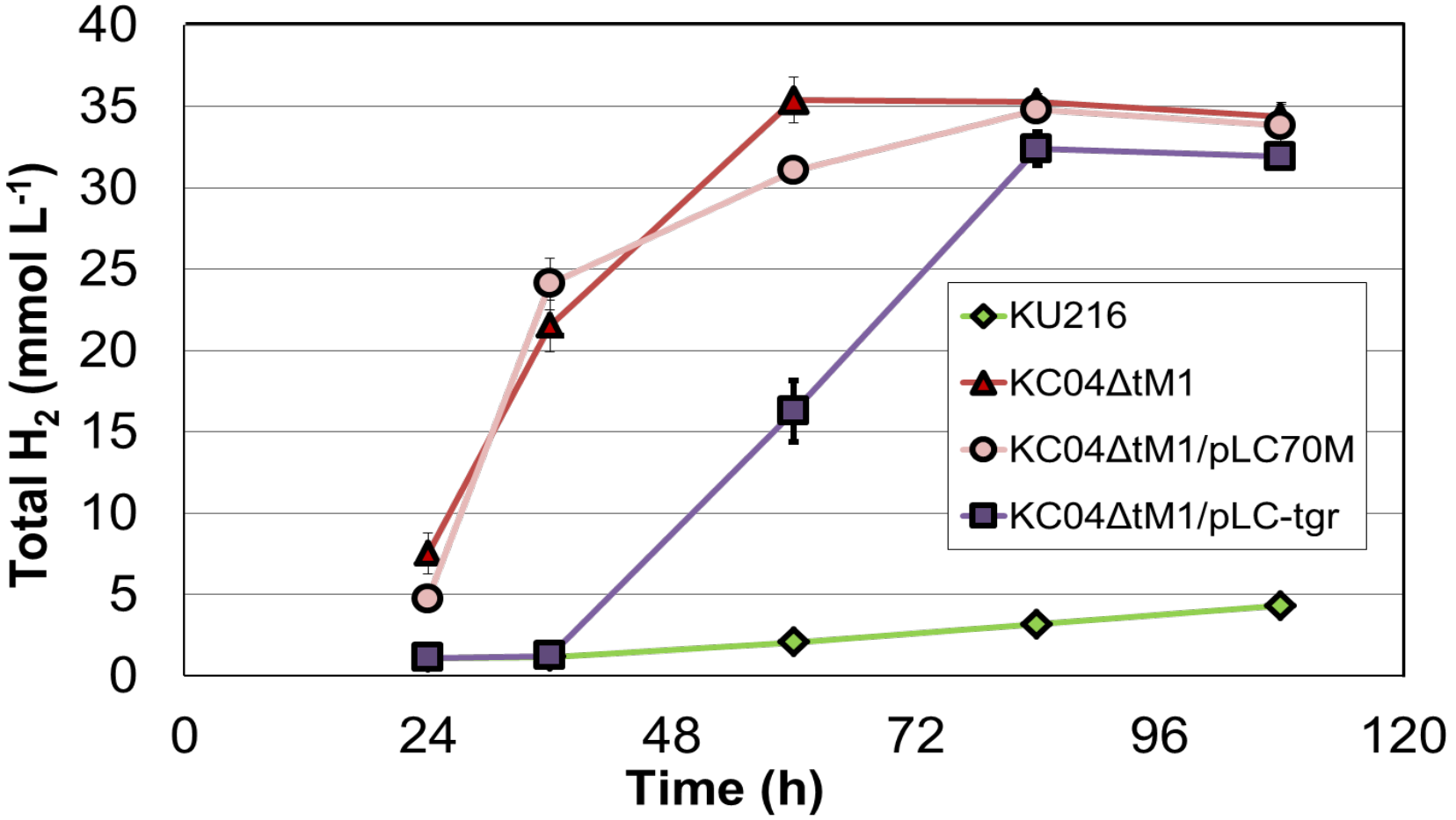

Fig. S8 Effect of *tgr* gene reintroduction on chitin-dependent hydrogen (H_2) production in KC04ΔtM1. KU216 is shown in *green* and *diamonds*, while KC04ΔtM1 is shown in *red* and *triangles*. KC04ΔtM1 complemented with pLC70M is shown in *pink* and *circles*, while KC04ΔtM1 complemented with pLC-tgr is shown in *purple* and *squares*. Cultivation was performed with ASW-VMT-SC medium at 85°C. Total H_2 (mmol L^{-1}) represents the amount of molecular hydrogen present in the headspace of a culture bottle per mL of culture volume (media volume was 15 mL). Error bars represent standard deviations of three independent cultivations. Reintroduction of Tgr gene to KC04ΔtM1 results in a delay in the H_2 production.

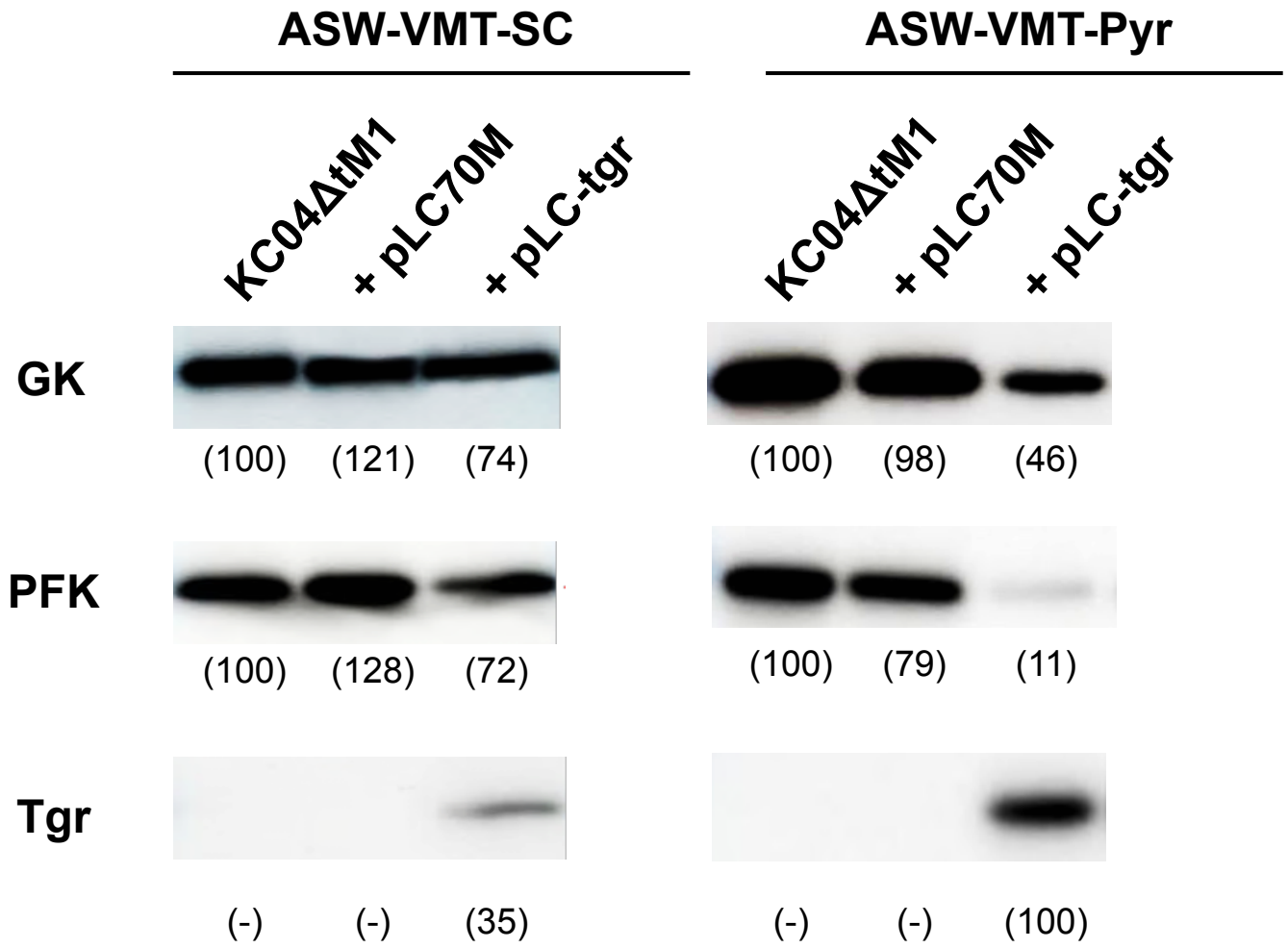

Fig. S9 Expression levels of GK, PFK and Tgr detected by Western blot analyses. Western blot analysis of GK, PFK and Tgr was performed with cell-free extracts from KC04ΔtM1, and KC04ΔtM1 strains complemented with pLC70M or pLC-tgr. Cells were cultivated at 85°C in ASW-VMT-SC or ASW-VMT-Pyr media. Numbers in parentheses indicate band intensities (%) relative to that of KC04ΔtM1 cultivated in the same medium condition (which is defined as 100%). In the case of quantification of Tgr, the amount of KC04ΔtM1 harboring pLC-tgr cultivated in ASW-VMT-Pyr medium was set as 100%. Tgr gene introduction to KC04ΔtM1 represses expressions of GK and PFK in ASW-VMT-Pyr medium, while the effects of repression are much lower in ASW-VMT-SC medium.

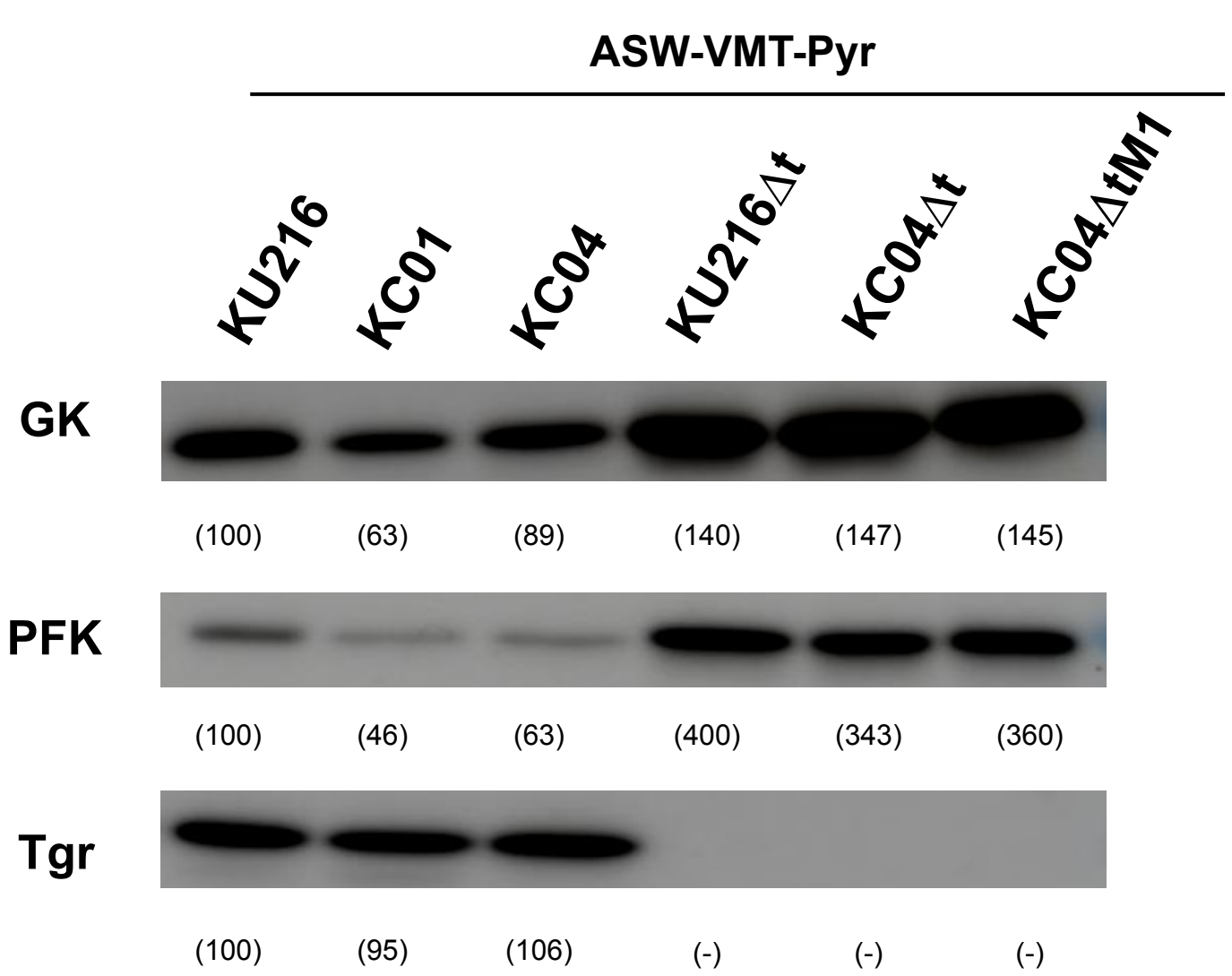

Fig. S10 Expression levels of GK, PFK and Tgr detected by Western blot analyses. Western blot analysis of GK, PFK and Tgr was performed with cell-free extracts from KU216, KC01, KC04, KU216Δt, KC04Δt and KC04ΔtM1 strains. Cells were cultivated at 85°C in ASW-VMT-Pyr media. Equal amount (3 μg) of protein was loaded on each lane for GK and PFK, while 10 μg of protein was loaded in the case of Tgr. Numbers in parentheses indicate band intensities (%) relative to that of KU216 (which is defined as 100%). Expression levels of GK and PFK are much higher in the Tgr gene knockout strains (KU216Δt, KC04Δt and KC04ΔtM1) compared to those of strains that harbor the Tgr gene.