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Disruption of a Sugar Transporter Gene Cluster in a Hyperthermophilic Archaeon Using a Host-Marker System Based on Antibiotic Resistance[▽]

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We have developed a gene disruption system in the hyperthermophilic archaeon *Thermococcus kodakaraensis* using the antibiotic simvastatin and a fusion gene designed to overexpress the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase gene (hmg_{Tk}) with the glutamate dehydrogenase promoter. With this system, we disrupted the *T. kodakaraensis* amylopullulanase gene (apu_{Tk}) or a gene cluster which includes apu_{Tk} and genes encoding components of a putative sugar transporter. Disruption plasmids were introduced into wild-type *T. kodakaraensis* KOD1 cells, and transformants exhibiting resistance to 4 μM simvastatin were isolated. The transformants exhibited growth in the presence of 20 μM simvastatin, and we observed a 30-fold increase in intracellular HMG-CoA reductase activity. The expected gene disruption via double-crossover recombination occurred at the target locus, but we also observed recombination events at the hmg_{Tk} locus when the endogenous hmg_{Tk} gene was used. This could be avoided by using the corresponding gene from *Pyrococcus furiosus* (hmg_{Pf}) or by linearizing the plasmid prior to transformation. While both gene disruption strains displayed normal growth on amino acids or pyruvate, cells without the sugar transporter genes could not grow on maltooligosaccharides or polysaccharides, indicating that the gene cluster encodes the only sugar transporter involved in the uptake of these compounds. The Δapu_{Tk} strain could not grow on pullulan and displayed only low levels of growth on amylose, suggesting that Apu_{Tk} is a major polysaccharide-degrading enzyme in *T. kodakaraensis*.

Hyperthermophiles are organisms that exhibit optimal growth at temperatures above 80°C (57). The organisms have attracted much attention from an evolutionary viewpoint as they occupy the deepest lineages within the phylogenies of both *Archaea* and *Bacteria* based on rRNA sequences (56, 57). Hyperthermophiles are also focused upon as a source of (thermo)stable enzymes that have the potential for application in a broad range of technologies (3, 21). There are now 14 complete genome sequences and many more in progress, providing a wealth of primary structural data from which we can estimate the presence or absence of various metabolic and regulatory mechanisms. However, although biochemical and structural analyses of hyperthermophile proteins are proceeding at a rapid pace, genetic studies to examine gene function in vivo are still limited in number.

In contrast to the hyperthermophilic archaea, a wealth of gene disruption and shuttle vector systems has been developed for the mesophilic archaea. In the halophilic archaea, procedures allowing the uptake of bacteriophage (15), plasmid (14), and genomic DNA (17) have been established with *Halobacterium halobium* and *Halobacterium* (now *Haloferax*) *volcanii*. Stable shuttle vectors have been developed (29, 37, 45), and homologous recombination has been demonstrated (34, 43). Selection methods include changes in phenotype from auxotrophy to prototrophy (17) and resistance against a variety of antibiotics such as mevinolin (34, 37, 45, 63), novobiocin (29), thiostrepton, and anisomycin (43). Genetic systems have also been developed for *Haloarcula* strains (16) and other *Halobacterium* strains including *Halobacterium* sp. strain NRC-1 (8, 47, 62). In the methanogenic archaea, shuttle vectors and gene disruption systems have been developed in *Methanococcus maripaludis* based on puromycin and the puromycin *N*-acetyltransferase gene (*pac*) (25, 48, 61) or neomycin and aminoglycoside phosphotransferase genes (2). The puromycin-*pac* system has also been applied for genetic manipulation in *Methanococcus voltae* (6, 7, 60), as well as in various *Methanosarcina* species (19, 44). Genetic transformation has also been observed in the thermophilic methanogen *Methanobacterium thermoautotrophicum* (now *Methanothermobacter thermautotrophicus*) (64).

In the hyperthermophilic archaea, exchange and recombination of chromosomal markers, as well as homologous recombination of exogenous DNA, have been reported for *Sulfolobus acidocaldarius* (27, 28, 35, 50). A plasmid based on pNOB8 has been demonstrated to transform *Sulfolobus solfataricus* (22). An autonomously replicating vector harboring a mutant hygromycin phosphotransferase gene for selection has also been developed for this species (12, 13, 18). In the *Euryarchaeota*, a shuttle vector has been developed for use in *Pyrococcus abyssi* (41). In terms of gene disruption, however, only two systems have been reported so far: one for *Thermococcus kodakaraensis* (52, 53) from the *Euryarchaeota* and the other for *S. solfataricus* from the *Crenarchaeota* (65). Both systems rely on homologous recombination. The former system utilizes various host strains with amino acid/nucleotide auxotrophy and corre-

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sponding marker genes that complement the auxotrophy. The latter utilizes a *lacS*-deficient host strain and a modified but active *lacS* marker gene with selection based on lactose-dependent growth. The two systems have proved to be powerful tools in examining gene function in the respective strains (5, 30, 54, 55) and can be expected to provide further genetic evidence that will help in understanding the physiological roles of genes in these and closely related organisms.

In this study, we aimed to develop a gene disruption system in hyperthermophiles using antibiotics and a marker gene that would confer resistance to transformant cells. This would relieve the necessity to prepare auxotrophic host cells and also allow selection of transformants in a nutrient-rich medium. Thus, the methodology should not only provide a convenient alternative for gene disruption in *T. kodakaraensis* but also be helpful in establishing gene disruption systems in other hyperthermophilic archaea. We examined the possibilities of utilizing the mevinolin system established in the halophilic archaea. Mevinolin, along with its analog simvastatin, is a specific inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, an enzyme essential for archaeal membrane lipid biosynthesis (11, 36). HMG-CoA reductases have been extensively examined from a number of archaeal species (9, 10, 31). An overexpression construct of the HMG-CoA reductase gene can be expected to be applicable as a marker gene. Additionally, as the gene is originally present in the hyperthermophile, there is no need for concern about the thermostability of the marker gene product. As all archaeal strains are presumed to require the function of HMG-CoA reductase for lipid and membrane generation, the system described in this study has the potential for application in all hyperthermophilic archaea.

MATERIALS AND METHODS

Strains, media, and plasmids. *T. kodakaraensis* KOD1 (4) and the mutant strains were cultivated under anaerobic conditions at 85°C in a nutrient-rich medium, ASW-YT (53), supplemented with various organic substrates or elemental sulfur when appropriate. ASW-YT medium was composed of 0.8× artificial seawater, 5.0 g liter^{-1} of yeast extract, and 5.0 g liter^{-1} of tryptone. Resazurin was added at a concentration of 0.8 mg liter^{-1}, and prior to inoculation, Na_2S was added to the medium until it became transparent. In the case of plate culture, instead of elemental sulfur and Na_2S $9H_2O$, 2 ml of a polysulfide solution (10 g of Na_2S $9H_2O$ and 3 g of sulfur flowers in 15 ml of H_2O) per liter and Gelrite (10 g liter^{-1}) were added to solidify the medium. All components were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan). When simvastatin was added to the medium, simvastatin was dissolved in ethanol, and the amount of the solution added was adjusted so that the ethanol concentration in the medium was constant at 0.1% (vol/vol).

DNA manipulation and sequence analysis. *Escherichia coli* strain DH5α and pUC18/pUC19 (Takara, Kyoto, Japan) were used for DNA manipulation and sequencing. *E. coli* strains were cultivated in Luria-Bertani medium at 37°C with ampicillin at a concentration of 100 μg ml^{-1}. Restriction and modification enzymes were purchased from Toyobo (Osaka, Japan) and Takara. Plasmid DNA was isolated with a plasmid mini kit from QIAGEN (Hilden, Germany). KOD Plus (Toyobo) was used as a polymerase for PCR, and a GFX PCR DNA and gel band purification kit (GE Life Sciences, Little Chalfont, United Kingdom) was used to recover DNA fragments from agarose gels after electrophoresis. DNA sequencing was performed using a BigDye terminator cycle sequencing kit, version 3.1, and a model 3100 capillary DNA sequencer (Applied Biosystems, Foster City, CA).

Construction of the gene disruption vectors. Two disruption vectors, pUDapu and pUDmal, were constructed for the targeted disruption of the *T. kodakaraensis* amylopullulanase gene *apu* (apu_{Tk}) and the sugar transporter gene cluster including apu_{Tk}, respectively, via double-crossover homologous recombination. Overexpression cassettes for the HMG-CoA reductase gene from *T. kodakaraensis* (hmg_{Tk}) were constructed by replacing the native promoter with a putative promoter region (−554 to −4) of the glutamate dehydrogenase gene (49). The region −3 to −1 was replaced by 5′-CAT-3′ in order to incorporate an NdeI site for fusion of the promoter to the coding region of hmg_{Tk}. Cassettes were designed so that one had SmaI sites at both ends, while another had an XbaI site upstream of the promoter and a BamHI site downstream of hmg_{Tk}. The two cassettes were inserted into pUC18 and sequenced. For construction of the apu_{Tk} disruption plasmid, a DNA fragment including apu_{Tk} along with its flanking regions (about 1,000 bp) was amplified from the genomic DNA of *T. kodakaraensis* KOD1 with the primer set APU-F1 and APU-TRANS-R1 (5′-AATT CAGAACGGCAAGCTCTACGTAACAGACGGCA-3′ and 5′-GCGTCGTA GATGTCCTCGGGCCTTATGCCGAAGAT-3′, respectively) and inserted into pUC18 at the HincII site. An inverse PCR was then carried out to amplify the flanking regions and pUC18, thereby removing the coding region of gene. The primers used were APU-R2 and APU-F2 (5′-CTTATCACCTCACTCTTTAA GGCCTCCAACAGTGA-3′ and 5′-AGAGGGTGGCGGAATCTGCGGCCC GGCGTTCCTCG-3′, respectively). The DNA fragment was ligated with the hmg_{Tk} overexpression cassette excised with SmaI and designated pUDapu. For disruption of the sugar transporter gene cluster, DNA fragments of the 5′ and 3′ flanking regions (about 1,000-bp) of the gene cluster were amplified with the primer pair TRANS-F1 and TRANS-R2 (5′-AGTTCTCAAATCGGACCTTC CGCCGATGGAAAAGT-3′ and 5′-TGTTTATCACCTAGTTATCTCGTTGC ATTTGAGTA-3′, respectively) and the pair TRANS-F2 and APU-TRANS-R1 (TRANS-F2, 5′-TCCCCAGGATCCGGCGGTGGTGAAGAGGGTGGCGG-3′). The 5′ flanking region was inserted into pUC19 at the HincII site, followed by insertion of the overexpression cassette in the XbaI and BamHI sites. The 3′ flanking region was then inserted in the BamHI and SmaI sites, resulting in the plasmid pUDmal.

Transformation of *T. kodakaraensis*. Transformation procedures were performed as described previously (52, 53), but the host strain used in this study was the wild-type *T. kodakaraensis* KOD1. After transformation, cells were cultivated in ASW-YT liquid medium supplemented with 0.2% (wt/vol) elemental sulfur (ASW-YT-S^0) in the presence of 4 μM simvastatin at 85°C for 12 h. The cells were further grown in ASW-YT-S^0 liquid medium with 8 μM simvastatin at 85°C and spread on ASW-YT (polysulfide) plate medium containing 4 μM simvastatin and incubated at 85°C. Genomic DNA was isolated from the transformants and analyzed by PCR and Southern blot analysis.

Southern blot analysis. A digoxigenin-DNA labeling and detection kit (Roche Diagnostics, Basel, Switzerland) was used according to the manufacturer's instructions. The probes within the coding regions of hmg_{Tk} and apu_{Tk} were amplified, respectively, with the primer pair HMG-F and HMG-R (5′-TGAGA ACATCGGGCACTACTCAATAGATCCCAACC-3′ and 5′-ACCAACGAGG TTCTTGCGGTAGTTCACCTCGGCTA-3′, respectively) and the pair APU-F and APU-R (5′-CTCAACGACAAGACCCTTGAAATCCTAGCGGAGAA-3′ and 5′-GGCTCATCTTATCTTTGTTTTCCATGAGGGCCTTT-3′, respectively). The probe within the coding region of the *malE* gene of *T. kodakaraensis* ($malE_{Tk}$) was amplified with the primers MalE-F and MalE-R (5′-CACTTCCCCGACCGA GACCACTACTACCTCACCCA-3′ and 5′-CTGCTGGGTGTTGTAGTCGGCA GTCGGGGCCATGT-3′, respectively). The probe within the *hmg* gene from *P. furiosus* (hmg_{Pf}) was amplified with the primers PfHMG-F and PfHMG-R (5′-AA AGCACATTGGCCACTACTCAATTGATCCAAACG-3′ and 5′-ACCCACTAA GTTCTTTAGGTAGTTTACTTCAGCGA-3′, respectively), and the probe corresponding to the promoter region of the glutamate dehydrogenase gene was amplified with the primers GDHp-F and GDHp-R (5′-ATATCCCACCTCCGAT TCCGTTGGTATTTAATCGG-3′ and 5′-TACCACCTCATTTCGGTAATCTGC GAGGTTAACTT-3′, respectively). Genomic DNA from the wild-type and gene disruption mutant strains was digested with PvuII.

Growth properties of *T. kodakaraensis* and mutant strains. *T. kodakaraensis* KOD1 and the mutant strains were grown in ASW-YT-S^0 medium at 85°C for 12 h and inoculated into 15 ml of ASW-YT-S^0 or ASW-YT medium supplemented with 0.5% (wt/vol) sodium pyruvate, a 0.5% (wt/vol) concentration of a specific maltooligosaccharide (3, 4, 5, 6, or 7 glucose units), 0.5% (wt/vol) amylose (polysaccharide consisting of glucose connected solely by α-1,4-glycosidic bonds), or 0.5% (wt/vol) pullulan (polysaccharide consisting of maltotriose units connected by α-1,6-glycosidic bonds). Cell densities (optical density at 660 nm) were measured at appropriate intervals with a UV spectrometer mini photo 518R (Taitec, Koshigaya, Japan). In order to estimate resistance toward simvastatin, cells were cultivated in 15 ml of ASW-YT-S^0 supplemented with 1, 5, 10, or 20 μM simvastatin.

Measurements of HMG-CoA reductase activity. Activity measurements were performed at 60°C in a final volume of 1 ml containing cell extracts, 200 μM NADPH, and 0.5 mM HMG-CoA (Sigma, St. Louis, MO) in 50 mM potassium phosphate buffer (pH 7.0). The consumption of NADPH was monitored at 340

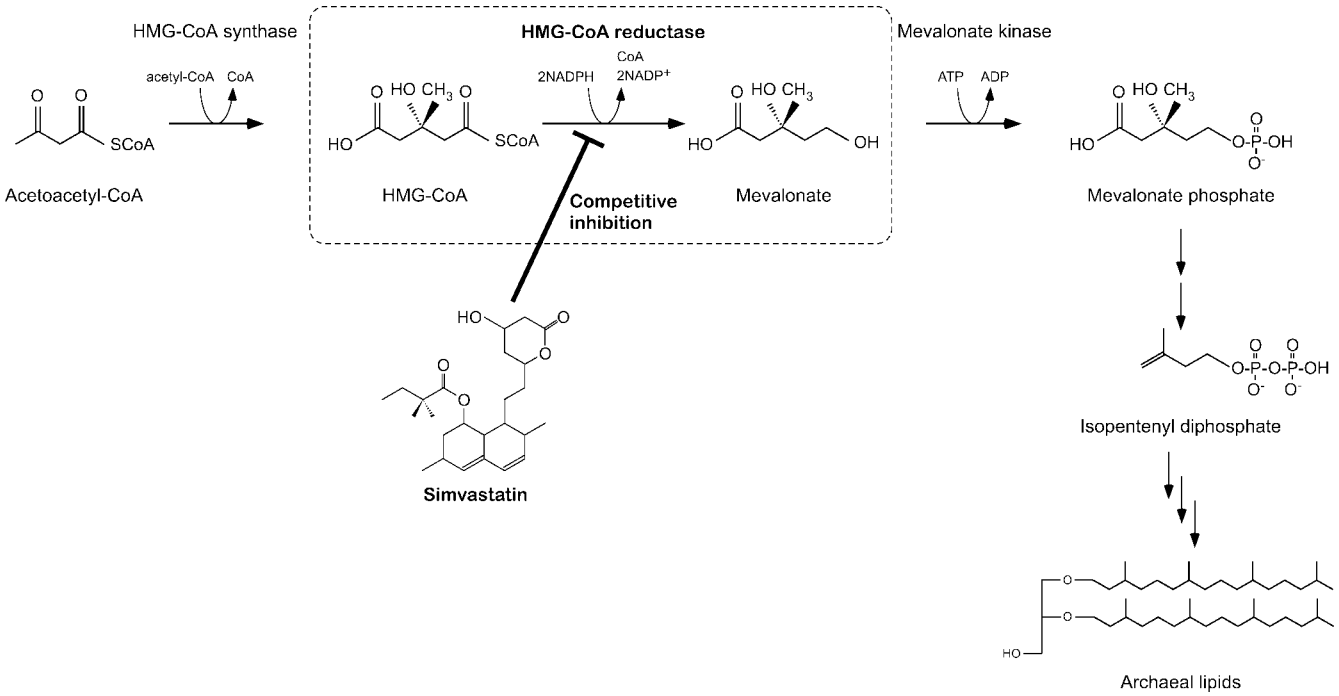

FIG. 1. The mevalonate pathway for isoprenoid lipid biosynthesis in *Archaea*. The reaction catalyzed by HMG-CoA reductase is boxed with dotted lines. It should be noted that the two reactions converting mevalonate phosphate to isopentenyl diphosphate are distinct from the reactions in the classical mevalonate pathway (26).

nm by a UV-visible light spectrophotometer (UV-1600PC; Shimadzu, Kyoto, Japan). Cell extracts were prepared as follows. *T. kodakaraensis* and the disruptants were cultivated in ASW-YT-S^0 medium at 85°C for approximately 8 h. Cells were collected and sonicated on ice, and the supernatant after centrifugation (20,000 × *g* for 30 min at 4°C) was used as the cell extract. Protein concentrations were determined with a protein assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard.

RESULTS

Effect of various concentrations of simvastatin on the growth of *T. kodakaraensis*. As isopentenyl diphosphate is the major precursor for archaeal lipid membranes, we supposed that inhibition of HMG-CoA reductase would have severe effects on the growth of *T. kodakaraensis* (Fig. 1). Our main concerns were whether the uptake and inhibitory effects of simvastatin were sufficient to allow use of the antibiotic at realistic concentrations and whether the compound was stable enough at temperatures of >80°C to inhibit growth for several days, which is necessary for the formation of colonies.

We examined the growth of *T. kodakaraensis* KOD1 in the presence of various concentrations of simvastatin in the nutrient-rich medium ASW-YT-S^0. Simvastatin was dissolved in ethanol, and the amount of ethanol added to the medium was constant at 0.1% (vol/vol). In ASW-YT-S^0 medium, *T. kodakaraensis* KOD1 cells reach the stationary phase within 24 h. No effect on growth was observed with the addition of ethanol alone. In the presence of 1 or 2 μM simvastatin, growth was observed only after 24 h, while 48 h was necessary for growth with 3 μM simvastatin. At concentrations of 4 or 5 μM simvastatin, growth was not observed for at least 5 days, indicating that these concentrations would be suitable for selecting transformants with resistance against simvastatin. We also confirmed that these concentrations were sufficient to prevent colony formation of *T. kodakaraensis* KOD1 on nutrient-rich plate medium.

A cassette for the overexpression of the HMG-CoA reductase gene. As simvastatin is a competitive inhibitor of HMG-CoA reductase, we expected that overexpression of its gene from *T. kodakaraensis* (hmg_{Tk}) would reduce the inhibitory effects of simvastatin on cell growth. Previous studies have indicated that the enzyme glutamate dehydrogenase is abundant in *T. kodakaraensis* cells grown in various media (49), suggesting that the gene (gdh_{Tk}) is under the control of a strong promoter. We therefore utilized a 551-bp intergenic region between the coding regions of gdh_{Tk} (TK1431) and the adjacent gene TK1432 and fused the region upstream of hmg_{Tk} (see Materials and Methods). This overexpression cassette (P_{gdh}-*hmg*) was used as the marker gene for construction of disruption plasmids (Fig. 2A).

Design and construction of the gene disruption plasmids. The genes disrupted in this study were a putative amylopullulanase gene (apu_{Tk}, or TK1774) and a gene cluster including apu_{Tk} and three additional genes encoding the components of a sugar transporter (TK1771 to TK1773) of *T. kodakaraensis* (Fig. 2B) (24). In the latter stages of this study, we discovered an error in the original genome sequence of TK1774 (an excess A at position 1,581,978 of the genome). The correct sequence leads to a protein with a change and elongation in sequence in the C-terminal region from residue Asn1070 (see Discussion). In this report we will refer to the corrected apu_{Tk} gene as TK1774* and, for simplicity, to the four-gene cluster (TK1771 to TK1774*) as mal_{Tk}.

Amylopullulanases, or type II pullulanases, exhibit both

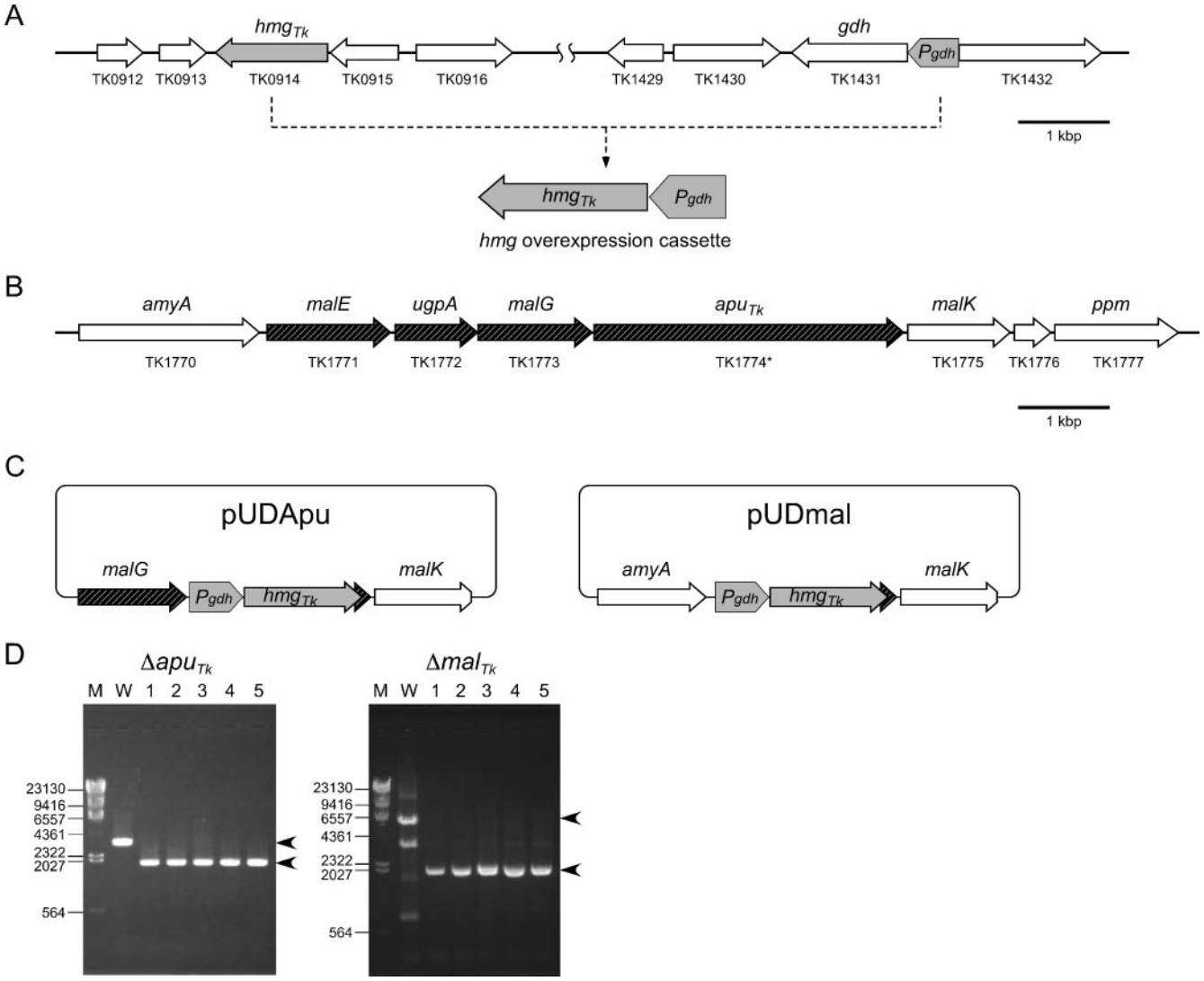

FIG. 2. Disruption of the apu_{Tk} and mal_{Tk} loci of *T. kodakaraensis*. (A) Design of the hmg_{Tk} overexpression cassette using the 5′ upstream flanking region of gdh_{Tk}. (B) Gene organization of the putative maltooligosaccharide transporter of *T. kodakaraensis*. TK1774* represents the correct sequence of the apu_{Tk} gene (see text). Black arrows indicate the gene(s) disrupted in this study. (C) The two plasmids constructed for the disruption of the apu_{Tk} and mal_{Tk} loci via double-crossover recombination. (D) PCR analyses of the apu_{Tk} and mal_{Tk} loci confirming gene disruption. Primers were designed in the 5′ and 3′ flanking regions of the gene(s) to be disrupted. DNA size markers were run in lane M, and their sizes (bp) are indicated to the left of the gels. The results of PCR with wild-type *T. kodakaraensis* KOD1 and five individual transformants are indicated in lane W and lanes 1 to 5, respectively. The arrowheads to the right of the gels indicate the amplified fragments expected before and after recombination. The decreases in lengths of the amplified fragments reflect the differences in length between apu_{Tk} (~3,500 bp) and P_{gdh}-*hmg* (~2,000 bp) and between mal_{Tk} (~7,000 bp) and P_{gdh}-*hmg*. Nonspecific amplifications of DNA fragments observed for the wild-type mal_{Tk} locus were due to the prolonged reaction time necessary to amplify the entire locus.

α-amylase and pullulanase activities and can therefore cleave both α-1,4- and α-1,6-glucosidic bonds (20). There are a number of other homologs on the genome (24), some with putative signal sequences for secretion, expected to harbor the ability to degrade α-linked polysaccharides. In particular, the TK1884 protein has been experimentally confirmed to exhibit α-amylase activity (59). On the other hand, in contrast to the two sugar transporters present in *Pyrococcus furiosus* (Mal-I, PF1739 to PF1744, and Mal-II, PF1933 to PF1938) (33), only one putative gene cluster is found on the *T. kodakaraensis* genome (TK1771 to TK1775) (24). Based on primary structure similarity, the transporter from *T. kodakaraensis* corresponds to Mal-II, suggesting that it is specific to maltooligosaccharides with three or more glucose units. By disrupting apu_{Tk}, we expected to gain insight into the actual degree of influence apu_{Tk} has, among the multiple amylase homologs of *T. kodakaraensis*, on the degradation of various extracellular polysaccharides. Growth characteristics of the mal_{Tk} disruptant were expected to clarify the presence or absence of other sugar transporters as well as to provide information on the substrate specificity of the Mal_{Tk} transporter in vivo.

Similar to the design of gene disruption plasmids in a previously described system using *pyrF* or *trpE* as selectable markers (52), P_{gdh}-*hmg* was inserted between the 5′ and 3′ flanking

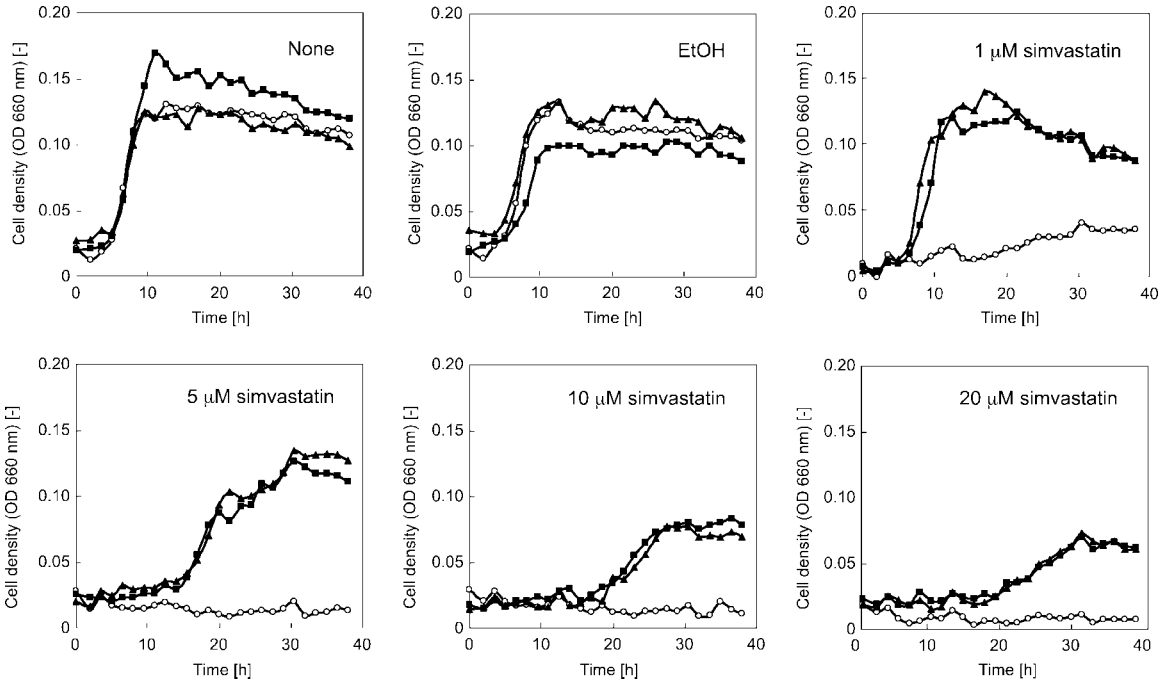

FIG. 3. Growth of wild-type *T. kodakaraensis* KOD1 and Δapu_{Tk} and Δmal_{Tk} mutant strains in the presence of various concentrations of simvastatin. Open circle, wild-type strain; filled square, Δapu_{Tk} strain; filled triangle, Δmal_{Tk} strain; OD, optical density.

regions (~1,000 bp) of the target gene(s) (Fig. 2C). The plasmids pUDapu and pUDmal were used to transform wild-type *T. kodakaraensis* KOD1, and transformants were selected based on their resistance toward simvastatin.

Isolation of the gene disruption strains Δapu_{Tk} and Δmal_{Tk}. After transformation, cells were grown in ASW-YT-S^0 liquid medium in the presence of 4 μM simvastatin. Growth was observed with cells transformed with pUDapu and pUDmal but not for cells treated without plasmid. Cells were further inoculated in the same liquid medium with 8 μM simvastatin and then spread on plate medium with 4 μM simvastatin. Five colonies were selected for each gene disruption and grown in ASW-YT-S^0 medium. We examined the apu_{Tk} and mal_{Tk} loci by PCR (Fig. 2D). As expected, we observed shorter amplified fragments from the transformants than from the wild-type strain, corresponding to the decrease in length brought about by the replacement of apu_{Tk} and mal_{Tk} by P_{gdh}-*hmg*.

Extent of simvastatin resistance of the transformants. As the transformants harbored P_{gdh}-*hmg* on their genomes, we examined their resistance against various concentrations of simvastatin (Fig. 3). One Δapu_{Tk} and one Δmal_{Tk} transformant were grown in the presence of 1, 5, 10, and 20 μM simvastatin, and their growth characteristics were compared with those observed in medium without simvastatin. Although the wild-type strain could not grow at all with 5 μM simvastatin, specific growth rates and cell yields of the transformants were still comparable to those in the absence of simvastatin. The degree of inhibition became prominent at higher concentrations, but we found that concentrations over 20 μM were necessary to completely inhibit growth of the transformants. We further examined the levels of HMG-CoA reductase activity in the wild-type and transformant cells. Specific activity in the cell extracts of wild-type cells was approximately 25 nmol min^{-1} mg^{-1}. In contrast, the level observed in the extracts of the Δapu_{Tk} strain was 760 nmol min^{-1} mg^{-1}, indicating an increase in activity of over 30-fold. The resistance against simvastatin and the increase in HMG-CoA reductase activity in the transformants are consistent with the presumption that simvastatin inhibits growth of *T. kodakaraensis* KOD1 by specifically inhibiting the activity of HMG-CoA reductase.

Phenotype analyses of the gene disruption strains. We examined the growth characteristics of the Δapu_{Tk} and Δmal_{Tk} strains in various media and compared them with those of the wild-type strain (Fig. 4). No change in phenotype was observed when the three strains were grown on amino acids (ASW-YT-S^0) or amino acids and pyruvate (ASW-YT-pyruvate) as carbon sources. However, disruption of apu_{Tk} and mal_{Tk} brought about dramatic changes in phenotype when the strains were grown on various sugars. Disruption of the Mal_{Tk} transporter abolished growth on all sugars examined. Although the Δapu_{Tk} strain displayed growth on a number of maltooligosaccharides, the strain could not grow on pullulan (Fig. 4I). Interestingly, while several α-amylase homologs (including TK1884) are present on the genome, we found that disruption of apu_{Tk} led to a significant decrease in growth rates when strains were grown on amylose, a maltopolysaccharide consisting of only α-1,4-linkages (Fig. 4H). Another intriguing finding was that the disruption of apu_{Tk} had a greater detrimental effect on growth with shorter maltooligosaccharides, which was rather surprising as amylopullulanases are presumed to function in the breakdown of poly- or oligosaccharides. In contrast to the wild-type strain, no growth was observed for Δapu_{Tk} in the medium supplemented with maltotriose (Fig. 4C).

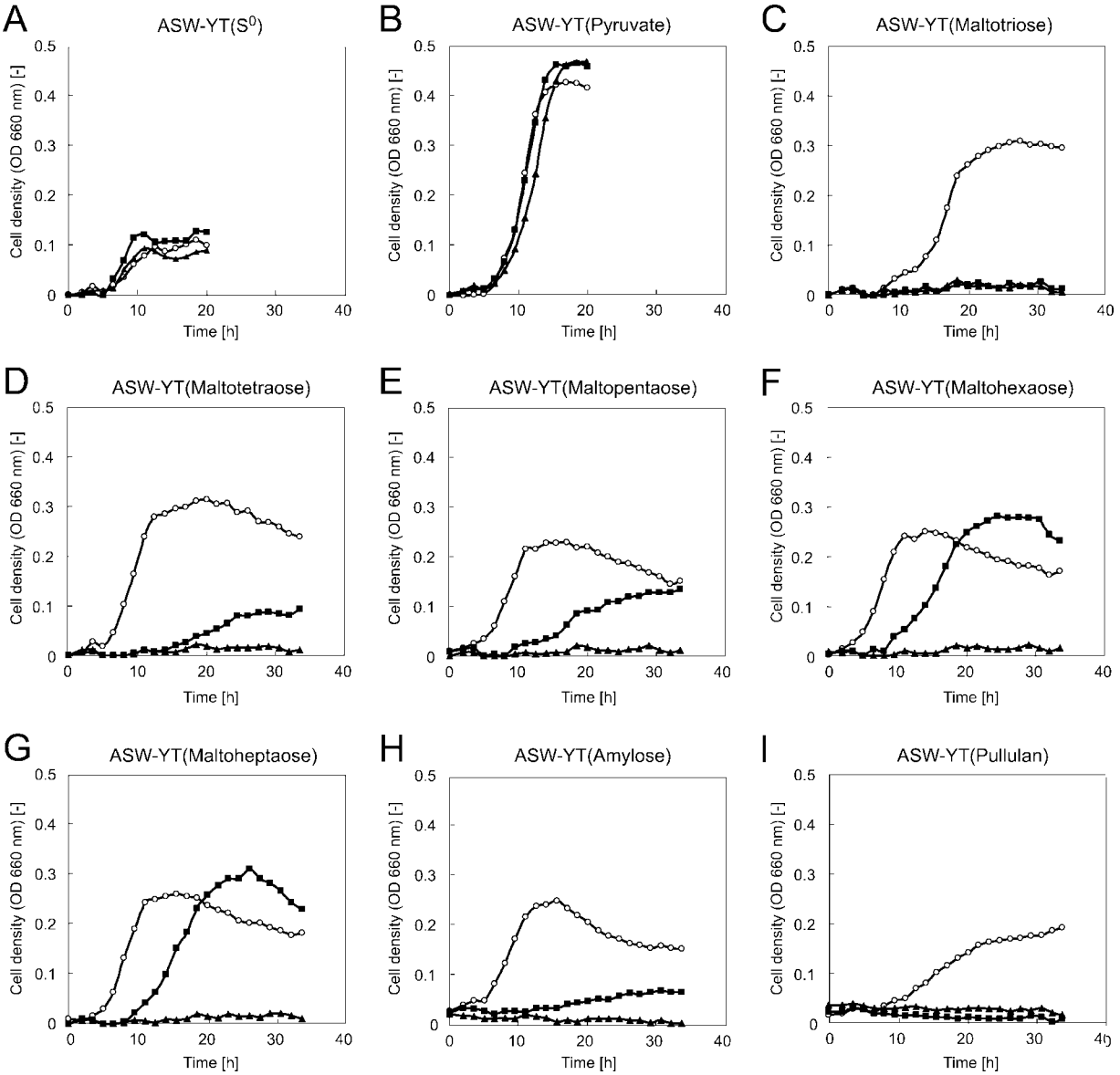

FIG. 4. Growth of wild-type *T. kodakaraensis* KOD1 and Δapu_{Tk} and Δmal_{Tk} mutant strains on various carbon sources. The carbon sources examined are indicated above each panel. Glucose and maltose were not examined, as the wild-type strain cannot utilize these sugars. Open circle, wild-type strain; filled square, Δapu_{Tk} strain; filled triangle, Δmal_{Tk} strain; OD, optical density.

Recombination at the hmg_{Tk} locus. As the plasmid used in this study harbors the endogenous hmg_{Tk} and P_{gdh}, there will always be a possibility of these regions recombining with the corresponding native loci present on the genome. We have actually applied this property in developing a single-crossover insertion/pop-out recombination system using the *pyrF* gene as a selective marker (52). We therefore examined whether recombination events had occurred at the hmg_{Tk} and/or P_{gdh} glutamate dehydrogenase locus. We observed that in some strains the native hmg_{Tk} locus had been disturbed (data not shown). As the locus of the target gene is stable in a disrupted form via double-crossover recombination, the recombination at the hmg_{Tk} locus does not directly pose a problem. Nevertheless, we examined possibilities to prevent or decrease the frequencies of unintended recombination. Linearizing the plasmids prior to introducing them into the cells would prevent single-crossover recombination. Utilizing an *hmg* gene from a heterologous host would also decrease the possibilities of single-crossover recombination as well as further recombination due to the presence of two identical regions on the genome. We found that both methods could be used for gene disruption. In examining the latter possibility, we used the *hmg* gene from the closely related *P. furiosus* and constructed plasmids to disrupt the apu_{Tk} gene. In five randomly chosen transformants with resistance against simvastatin, we clearly observed that double-crossover recombination had occurred at the apu_{Tk} locus (Fig. 5A and B) along with the appearance of a single copy of the hmg_{Pf} gene (Fig. 5C) and an additional copy of the *gdh* promoter (Fig. 5E), whereas neither the native hmg_{Tk} locus nor the *gdh* promoter regions were disturbed (Fig. 5D

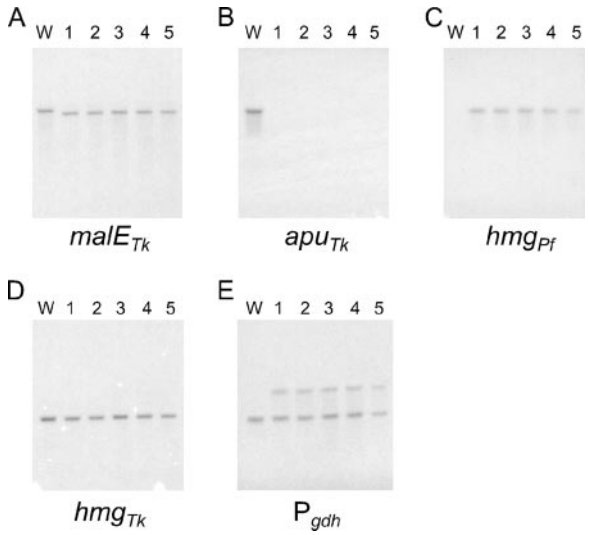

FIG. 5. Southern blot analyses on Δapu_{Tk} strains obtained with the hmg_{Pf} gene as a selectable marker. Genomic DNA from five selected Δapu_{Tk} strains and from wild-type *T. kodakaraensis* KOD1 (W) was subjected to Southern blot analyses using probes within the regions indicated below each membrane.

and E). We further confirmed that the strains obtained using hmg_{Pf} as a marker gene (i) were able to grow in the presence of 20 μM simvastatin, (ii) exhibited significant HMG-CoA reductase activities in the cell extracts (720 nmol min^{-1} mg^{-1}), and (iii) displayed the same growth characteristics toward various carbon sources as those shown in Fig. 4 (data not shown).

DISCUSSION

In this study, we have developed in *T. kodakaraensis* a gene disruption system based on resistance against antibiotics using simvastatin and an overexpression cassette of hmg_{Tk}. The system has many advantages for initiating gene disruption studies in hyperthermophilic archaea. First, one does not need to construct a host strain with a particular defect or auxotrophy toward an amino acid. There is also no need for selection to be carried out in minimal medium. Positive selection of mutant strains is possible in nutrient-rich medium. If the genome sequence is available, the possibilities to disrupt genes with this system can be examined immediately. It should be noted that we have not examined the stability of simvastatin under acidic conditions, which should be examined prior to application of the method on strains such as *Sulfolobus*. When the genome sequence is not available, it may be possible to use the genes of a closely related strain whose genome has been sequenced. As demonstrated in this study, the heterologous hmg_{Pf} gene was applicable for gene disruption in *T. kodakaraensis*. In order to avoid initial single-crossover recombination and possible recombination events afterwards due to the presence of two identical regions on the same chromosome, a heterologous marker may be more advantageous than an endogenous marker. We hope this methodology will promote gene disruption studies in a broader range of hyperthermophilic archaea.

The phenotypes of the Δapu_{Tk} and Δmal_{Tk} strains in various media not only provide valuable information on the physiological roles of the disrupted genes themselves but also allow us to estimate the contribution of other genes on the genome. The growth characteristics of the Δmal_{Tk} strain indicated that it is the only transporter in *T. kodakaraensis* involved in the uptake of the poly- and oligosaccharides examined in this study. As the Δapu_{Tk} strain could not grow at all on pullulan, there is a possibility that Apu_{Tk} is the only relevant enzyme responsible for the initial hydrolysis of pullulan, meaning that TK0977, annotated as a type II pullulan hydrolase, is not directly involved in the degradation of extracellular pullulan. Another possibility is that Apu_{Tk} and the TK0977 product display activity specified toward pullulan substrates of different lengths and are unable to complement each other. A third possibility would be that the lack of growth is due to the fact that the Δapu_{Tk} strain is defective in the uptake of maltotriose (Fig. 4C), a major product of pullulan hydrolysis. From the results shown in Fig. 4H, we also found that Apu_{Tk} plays a much greater role than expected in the cleavage of α-1,4-glycosidic linkages, suggesting that the experimentally verified α-amylase TK1884 protein (59) may not be the major amylose-degrading enzyme in *T. kodakaraensis*. This agrees well with the results of a transcriptome analysis of *P. furiosus* grown on starch, which revealed that PF1935*, the homolog of TK1774*, is the protein most up-regulated in the presence of starch (39).

An intriguing change in phenotype was observed in the Δapu_{Tk} strain grown on maltooligosaccharides. As the amylopullulanase was presumed to function in the degradation of poly- or oligosaccharides, we expected that gene disruption would have little effect on growth with maltooligosaccharides. Even if phenotypic changes were to be observed, the effects

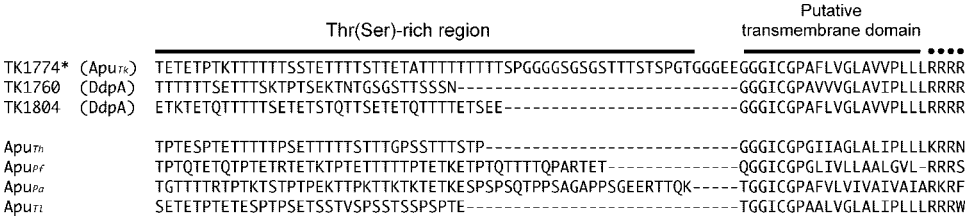

FIG. 6. Carboxy-terminal regions of various amylopullulanase proteins from the *Thermococcales*, along with the corresponding regions of periplasmic components of two putative ABC-type dipeptide transport systems of *T. kodakaraensis*. All of these proteins harbor a threonine (or serine)-rich region, followed by a putative transmembrane domain and a stretch of basic residues (indicated by circles) at the extreme C terminus. The subscripts of the amylopullulanase proteins identify the source organism as follows (accession number): Apu_{Tl}, *Thermococcus litoralis* (BAC10983); Apu_{Th}, *Thermococcus hydrothermalis* (AAD28552); Apu_{Pf}, *P. furiosus* (ABA33719); Apu_{Pa}, *P. abyssi* (CAB49104). Accession numbers for TK1760 and TK1804 are BAD85949 and BAD85993, respectively.

brought about by disrupting apu_{Tk} were expected to be greater with longer substrates. However, the results obtained with Δapu_{Tk} were just the opposite. This may be the result of polar effects brought about by insertion of the hmg_{Tk} overexpression cassette. With our disruption strategy, the downstream genes, in particular, $malK_{Tk}$ (TK1775), would be under the control of the *gdh* promoter and would thereby disturb the stoichiometric expression of the transporter subunits. Another possibility is that the up-regulation of transporter expression was disturbed by the absence of apu_{Tk}. Further, Apu_{Tk} itself may also be a component of the sugar transporter complex, resulting in a decrease in stability or efficiency of the complex when Apu_{Tk} is absent. This is consistent with the fact that the apu_{Tk} gene is clustered within the subunit genes of the transporter itself. The possibility that amylopullulanase resides on the cell surface has been proposed in closely related hyperthermophilic archaea (1, 23, 32). The enzyme from *Thermococcus hydrothermalis* has been reported to harbor a large C-terminal extension with three domains in addition to the central catalytic domain (23). One of the domains consists of two sequence repeats, each containing motifs with similarity to the S-layer homology signature (42), which is considered responsible for the anchoring of proteins to the cell surface in several bacteria (40, 42, 46, 51). The second region is extremely rich in threonine residues and is followed by the third, putative transmembrane domain. This architecture resembles the C-terminal regions of S-layer proteins of the haloarchaea, in which the Thr-rich regions are targets for O-linked glycosylation and the transmembrane domain serves as either a transmembrane anchor or hydrophobic cell wall anchor (38, 58). Moreover, as pointed out by Albers et al. (1), the two regions are also found in a number of proteins of the *Thermococcales* annotated as periplasmic components of ABC-type dipeptide transport systems, further supporting the involvement of these domains in cell surface attachment (Fig. 6). As amylopullulanases from *P. furiosus*, *P. abyssi*, and *T. kodakaraensis* also harbor these domains, it can be presumed that the amylopullulanases from the *Thermococcales* are attached to the cell surface. Further biochemical examination will be necessary to clarify whether these amylopullulanases have any additional function besides their roles in poly- and oligosaccharide hydrolysis.

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