

## *Thermococcus kodakarensis* Genetics: TK1827-Encoded $\beta$ -Glycosidase, New Positive-Selection Protocol, and Targeted and Repetitive Deletion Technology<sup>∇†</sup>

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**Inactivation of TK1761, the reporter gene established for *Thermococcus kodakarensis*, revealed the presence of a second  $\beta$ -glycosidase that we have identified as the product of TK1827. This enzyme (pTK1827) has been purified and shown to hydrolyze glucopyranoside but not mannopyranoside, have optimal activity at 95°C and from pH 8 to 9.5, and have a functional half-life of ~7 min at 100°C. To generate a strain with both TK1761 and TK1827 deleted, a new selection/counterselection protocol has been developed, and the levels of  $\beta$ -glycosidase activity in *T. kodakarensis* strains with TK1761 and/or TK1827 deleted and with these genes expressed from heterologous promoters are described. Genetic tools and strains have been developed that extend the use of this selection/counterselection procedure to delete any nonessential gene from the *T. kodakarensis* chromosome. Using this technology, TK0149 was deleted to obtain an agmatine auxotroph that grows on nutrient-rich medium only when agmatine is added. Transformants can therefore be selected rapidly, and replicating plasmids can be maintained in this strain growing in rich medium by complementation of the TK0149 deletion.**

Members of the *Thermococcales*, hyperthermophilic *Euryarchaea*, grow readily under laboratory conditions and are the focus of many basic and applied research projects (2, 8). Their investigation has, however, been limited by the lack of genetics, and it was a seminal advance therefore when *Thermococcus kodakarensis* (formerly *Thermococcus kodakaraensis* [1]) was shown to be naturally competent and to recombine added DNA into its genome (21, 23). *T. kodakarensis* is attractive as an experimental system as a fermentative heterotroph that grows rapidly on a variety of different substrates (1), optimally at 85°C, including substrates from which it generates substantial levels of hydrogen (11). The *T. kodakarensis* genome sequence and genome microarray assays are established (7, 12, 18) and, since the discovery of transformation (21), inactivation and manipulation of chromosomal genes has revealed novel biochemical pathways, facilitated *in vivo* evaluations of the archaeal gene expression machinery, and simplified enzyme purifications (3, 5, 6, 9, 10, 12, 15–20, 22–24, 28). For complementation assays and to facilitate heterologous gene expression in *T. kodakarensis*, shuttle plasmids have been constructed that replicate and confer selectable phenotypes in both *T. kodakarensis* and *Escherichia coli* (19), and TK1761 expression has been established as a reporter system that can be used to identify and quantify regulatory elements in *T. kodakarensis* (18, 20).

During the development of the TK1761 reporter system, a *T. kodakarensis* strain, designated TS416, was constructed with a nonsense mutation in TK1761 (18). This mutation had no discernible effects on growth, confirming that TK1761 was not

an essential gene, but lysates of *T. kodakarensis* TS416 retained a low level  $\beta$ -glycosidase activity. *T. kodakarensis* apparently, therefore, had a second  $\beta$ -glycosidase, but since this activity was very low and remained constant with changes in TK1761 expression, its presence did not compromise the use of TK1761 expression as a reporter system in the laboratory media used. The existence of a second  $\beta$ -glycosidase did, nevertheless, raise a potential concern for TK1761 assays in cells grown under different conditions in which expression of the gene encoding this second  $\beta$ -glycosidase might not be constant. To address this, we purified and characterized the second  $\beta$ -glycosidase and, to eliminate the concern for the reporter assay, we constructed a *T. kodakarensis* strain with both TK1761 and the gene (TK1827) that encodes the second  $\beta$ -glycosidase deleted.

To construct the double-deletion strain, we developed a new selection/counterselection protocol and have extended this into a procedure that can be used to delete any nonessential gene from the *T. kodakarensis* genome. A two-gene cassette has been constructed that can be integrated into the *T. kodakarensis* chromosome at any desired locus by homologous recombination of flanking genes. Expression of the cassette provides a positive selection for transformants and confers sensitivity to 6-methyl purine (6MP<sup>s</sup>). Mutants then isolated that are spontaneously resistant to 6-methyl purine (6MP<sup>r</sup>) have both the cassette and the adjacent target gene(s) precisely deleted.

Sato et al. (21, 23) established the *T. kodakarensis* transformation protocol by selecting transformants of tryptophan (*trpE*) and uracil (*pyrF*) auxotrophs that grew on minimal medium without tryptophan or uracil. Overexpression of the hydroxy-methylglutaryl-coenzyme A reductase encoded by PF1848, cloned from *Pyrococcus furiosus*, was later found to confer to resistance to simvastatin (15) and mevinoлин (19), allowing the selection of *T. kodakarensis* transformants on rich media that contain either antibiotic. Mutants spontaneously resistant to these antibiotics do, however, occur at experiment-

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TABLE 1. *T. kodakarensis* strains and plasmids used and/or constructed in this study

Strain or plasmid	Origin and/or genetic features <sup>a</sup>	Source or reference <sup>f</sup>
<i>T. kodakarensis</i> strains		
KW128	$\Delta pyrF$ ; $\Delta trpE::pyrF$	21
TS372	KW128 with $trpE$ -P <sub>hmtB</sub> -TK1761 insertion	18
TS416	TS372 with nonsense mutation at codon 3 of TK1761	18
TS502	KW128 with $trpE$ -P <sub>hmtB</sub> -(His) <sub>10</sub> TK1827 insertion	This study
TS503	KW128 with $trpE$ -P <sub>hmtB</sub> -TK1827 insertion with TAG at codon 5	This study
TS517	KW128 with $\Delta$ TK0664 <sup>b</sup>	This study
TS521	KW128 with $\Delta$ TK0664:: $trpE$	This study
TS538	TS517 with $\Delta$ TK1761-TK1762-TK1763	This study; Fig. S2
TS541	TS517 with $\Delta$ TK1827	This study; Fig. S2
TS1079	TS541 with $\Delta$ TK1761-TK1762-TK1763	This study
TS559	TS517 with $\Delta$ TK0149 <sup>c</sup>	This study
TS561	TS517 with $\Delta$ TK0148-TK0149	This study
PdaD	KU216 $\Delta pyrF$ with $\Delta$ TK0148- <i>pdaD</i>	6
Plasmids		
pTS502	pUC118::P <sub>hmtB</sub> -(His) <sub>10</sub> TK1827- $trpE$ -TK1828	This study
pTS503	pUC118::P <sub>hmtB</sub> -TK1827- $trpE$ -TK1828 with TAG at codon 5 of TK1827	This study
pTS517	pUC118::TK0662-TK0663-TK0664- $trpE$ -TK0662-TK0663-TK0665	This study
pTS521	pUC118::TK0662-TK0663- $trpE$ -TK0665	This study
pTS535	pUC118 with MCS1-( $trp$ -6MP <sup>s</sup> )-MCS2 <sup>d</sup>	This study; Fig. S1
pTS538	pTS535::TK1760-TK1764-( $trp$ -6MP <sup>s</sup> )-TK1761	This study; Fig. S2
pTS541	pTS535::TK1825-TK1826-TK1828-( $trp$ -6MP <sup>s</sup> )-TK1827-TK1828	This study; Fig. S2
pTS559	pTS535::TK0147-TK0148-TK0150-( $trp$ -6MP <sup>s</sup> )-TK0150-TK0151	This study
pTS561	pTS535::TK0147-TK0150-( $trp$ -6MP <sup>s</sup> )-TK0150-TK0151	This study
pLC70	$trpE$ ; P <sub>gdh</sub> -PF1848 <sup>e</sup>	19
pTS436	pLC70::P <sub>hmtB</sub> -TK1761	This study
pTS543	pLC70::P <sub>hmtB</sub> -TK0149	This study

<sup>a</sup>  $pyrF$  = TK2276 (21);  $trpE$  = P<sub>2279</sub>-TK0254 (18);  $pdaD$  = TK0149 (6).

<sup>b</sup> Loss of TK0664 results in resistance to 6-methyl purine (6MP<sup>s</sup>).

<sup>c</sup> Loss of TK0149, or loss of TK0148 plus TK0149, results in agmatine auxotrophy.

<sup>d</sup> The  $trp$ -6MP<sup>s</sup> cassette has P<sub>2279</sub> and P<sub>hmtB</sub> directing the divergent transcription of  $trpE$  (TK0254) and TK0664, respectively. TK0664 expression results in sensitivity to 6-methyl purine (6MP<sup>s</sup>). Flanking the cassette are multiple cloning sites 1 and 2 (MCS1 and MCS2) that have single cleavage sites for HindIII, SphI, BglII, BclI, ClaI, NheI, SpeI, NotI, SacII, ApaI, AccI, AscI, EcoRV, and BstEII and for EcoRI, KpnI, Acc65I, BamHI, XbaI, respectively (see Fig. S1 in the supplemental material).

<sup>e</sup> Expression of PF1848 confers resistance to simvastatin and mevinolin (15, 19).

<sup>f</sup> Figures S1 and S2 may be found in the supplemental material.

ally significant frequencies, and these are very expensive reagents for routine use and prohibitively expensive for incorporation into large preparative cultures. With this in mind, to develop an alternative selection that might be used in nutrient-rich media, we used the 6MP cassette system to delete TK0149. As predicted (6), the *T. kodakarensis*  $\Delta$ TK0149 strain generated was an agmatine auxotroph that only grows in nutrient-rich media when agmatine is added. When transformed with DNA expressing TK0149, transformants of this strain can be selected directly on standard nutrient-rich media, and complementation of the  $\Delta$ TK0149 mutation can be used to maintain the presence of an expression plasmid in *T. kodakarensis* cells grown in large-volume, rich-medium cultures for enzyme purification.

#### MATERIALS AND METHODS

**Reagents and enzymes.** Except where otherwise noted, all chemicals were purchased from Sigma Chemicals (St. Louis, MO), restriction enzymes from Invitrogen (Carlsbad, CA) and New England Biolabs (Waltham, MA), plasmid and PCR-product purification kits from Qiagen Inc (Valencia, CA) and Zymo Research (Orange, CA), and all chromatography columns from GE Healthcare (Piscataway, NJ).

**Strains and plasmids.** The origins and genetic features of the *T. kodakarensis* strains and plasmids used in the present study are listed in Table 1 (all of the *T. kodakarensis* strains and plasmids listed in Table 1 are available from T. J. Santangelo on request). The numerical and letter designations of the *T. kodakarensis* genes, and their genome annotations (7; <http://cmr.jcvi.org/>) are listed in

Table 2. Standard molecular biology procedures were used to PCR amplify DNA, construct plasmids, transform *E. coli* DH5 $\alpha$ , select  $\beta$ -lactamase-resistant transformants, and isolate plasmid preparations. DNA molecules PCR amplified from *T. kodakarensis* genomic DNA (the primer sequences are available from T. J. Santangelo) were cloned into pUC118 (27) or pLC70 (19). In all constructs,  $trpE$  (TK0254) was positioned for constitutive transcription from P<sub>TK2279</sub> (18–20). For TK1827 overexpression and for constitutive transcription of TK0664 in the  $trp$ -6MP<sup>s</sup> cassette, these genes were transcribed from P<sub>hmtB</sub> (18–20). In plasmid pTS535, the  $trp$ -6MP<sup>s</sup> cassette was positioned between two multiple cloning sites (MCS1 and MCS2; see Fig. S1 in the supplemental material). Plasmids pTS538, pTS541, pTS559, and pTS561 (Table 1) were constructed by ligating PCR-amplified fragments of the *T. kodakarensis* genome into MCS1 and MCS2 on both sides of the  $trp$ -6MP<sup>s</sup> cassette in pTS535 (see Fig. S2A and B in the supplemental material).

**Media and growth conditions.** *T. kodakarensis* cultures were grown anaerobically at 85°C in nutrient-rich ASW-YT-S° medium or minimal ASW-AA-S° medium that contained 2 g of sulfur/liter (1, 21, 23). Tryptophan (0.73 mM) and/or agmatine (6.7 mM) were added as required. Cells competent for DNA uptake were prepared as described previously (21). *T. kodakarensis*  $\Delta$ *trpE* transformants capable of growth without tryptophan were selected on ASW-AA plates, *T. kodakarensis*  $\Delta$ TK0149 transformants capable of growth without agmatine were selected on ASW-YT plates, and *T. kodakarensis* transformants resistant to mevinolin were selected on ASW-YT plates containing 15  $\mu$ M mevinolin (19). The media were solidified by incorporation of 1% Gelrite.

**Isolation and identification of the  $\beta$ -glycosidase (pTK1827) encoded by TK1827.** *T. kodakarensis* KW128 cells (~320 g [wet weight]) were resuspended, under aerobic conditions, in ~1 liter of lysis buffer (25 mM Tris-HCl [pH 8], 100 mM NaCl, 10% [vol/vol] glycerol, 10 mM  $\beta$ -mercaptoethanol) and lysed by sonication. Particulates were removed by centrifugation (JA25-50 rotor; 25,000 rpm for 20 min at 4°C), and aliquots of the clarified lysate were loaded onto a 15-ml HiTrap heparin column equilibrated with lysis buffer. Proteins

TABLE 2. Correlation of numerical and symbol designations and genome annotations of the *T. kodakarensis* genes examined in this study

Gene		Genome annotation <sup>a</sup>
TK no.	Name	
0147	<i>speE</i>	Spermidine synthase
0148		Hypothetical protein
0149	<i>pdaD</i>	Arginine decarboxylase
0150	<i>gcvH</i>	Glycine cleavage system protein H
0151		Transcription regulator
0254	<i>trpE</i>	Anthranilate synthase
0661		ABC-type ion transport system
0662		Hypothetical protein
0663		Hypothetical protein
0664		Hypoxanthine/guanine phosphoribosyltransferase
0665	<i>acdA</i>	Acetyl-coenzyme A synthetase I ( $\alpha$ -subunit)
1760		ABC-type peptide transport system
1761		$\beta$ -Glycosidase
1762		Hypothetical protein
1763		Nucleic acid-binding protein
1764	<i>Tk-Dac</i>	<i>N</i> -Acetylchitobiose deacetylase
1825		Hypothetical protein
1826		Transcription regulator
1827	<i>bglA</i>	$\beta$ -Glycosidase
1828	<i>galT</i>	Galactose-1-phosphate uridylyltransferase
1829		Permease
2276	<i>pyrF</i>	Orotidine-5-phosphate decarboxylase
2279		Sugar nucleotidyltransferase

<sup>a</sup> As presented elsewhere (7; <http://cmr.jcvi.org/>).

were eluted by using a linear 0.1 to 1 M NaCl gradient in lysis buffer. An aliquot of each fraction was assayed for  $\beta$ -glycosidase activity by incubation with 2.8 mM *ortho*-nitrophenyl- $\beta$ -D-glucopyranoside (ONP-gluco) at 85°C in 50 mM sodium phosphate buffer (pH 6.5). Increases in the  $A_{405}$  revealed two peaks of  $\beta$ -glycosidase activity that were separated by the heparin chromatography. Comparison with the activity in a lysate of *T. kodakarensis* TS416, the strain with a nonsense mutation in TK1761 (Table 1) (18), identified the fractions that contained the  $\beta$ -glycosidase not encoded by TK1761. These were pooled, dialyzed into 25 mM Tris-HCl (pH 8.8)–50 mM NaCl–10% (vol/vol) glycerol, clarified by centrifugation (JA16-250 rotor; 16,000 rpm for 40 min at 4°C), and loaded onto a 5-ml HiTrapQ-FF column. Fractions were eluted by using a linear 0.1 to 1 M NaCl gradient. The fractions that contained  $\beta$ -glycosidase activity were pooled and diluted with 25 mM Tris-HCl (pH 8.8)–10% (vol/vol) glycerol to a conductivity below that of 100 mM NaCl. This solution was loaded onto a 1-ml MonoQ column and eluted with a linear 0.1 to 1 M NaCl gradient in 20 mM Tris-HCl (pH 8.8). The fractions containing  $\beta$ -glycosidase were pooled, and the polypeptides present were visualized by Coomassie blue staining, and identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) after separation by denaturing polyacrylamide gel electrophoresis (SDS-PAGE).

**Purification of recombinant His<sub>10</sub>-tagged pTK1827 from *T. kodakarensis* TS502.** The purification was carried out at room temperature, with equipment in a Coy anaerobic chamber in an atmosphere of 5% H<sub>2</sub> and 95% N<sub>2</sub>. *T. kodakarensis* TS502 cells (~6 g [wet weight]) were harvested near the end of exponential growth from cultures incubated at 85°C in ASW-YT-S<sup>o</sup> medium. The cells were resuspended (200 mg/ml) in 20 mM Tris-HCl (pH 8.8)–250 mM NaCl and frozen and thawed three times by immersion of the tube in liquid N<sub>2</sub>, and lysis was completed by repetitive passage through a small-gauge needle. Particulates were removed by centrifugation (JA25-50 rotor, 25,000 rpm for 20 min at 4°C), and the clarified lysate was loaded onto a 5-ml HiTrap chelating column charged with NiSO<sub>4</sub> and equilibrated with 250 mM NaCl dissolved in 20 mM Tris-HCl (pH 8.8). Proteins were eluted by using a linear 0 to 250 mM imidazole gradient in 20 mM Tris-HCl (pH 8.8)–250 mM NaCl. An aliquot of each fraction was assayed for  $\beta$ -glycosidase activity by measuring ONP-gluco hydrolysis. Fractions that contained this activity were pooled and diluted with 20 mM Tris-HCl (pH 8.8) until the conductivity was below that of 0.1 M NaCl. The solution was loaded on a 1-ml MonoQ column, and fractions were eluted by using a linear 0.1 to 1 M NaCl gradient in 20 mM Tris-HCl (pH 8.8). The fractions containing  $\beta$ -glycosidase activity were pooled and dialyzed against 1 M NaCl–20 mM Tris-HCl (pH

8.8), and this solution was loaded onto a 5-ml HiTrap chelating column charged with NiSO<sub>4</sub> and equilibrated with 1 M NaCl–20 mM Tris-HCl (pH 8.8). Fractions were eluted with a linear gradient from 0 to 250 mM imidazole in 250 mM NaCl–20 mM Tris-HCl (pH 8.8). The fractions that contained  $\beta$ -glycosidase activity were pooled, concentrated, and the buffer exchanged by using 30-kDa cutoff centrifugal concentrators (Millipore, Danvers, MA). The purified activity (~1-mg yield) was dialyzed into 25 mM sodium phosphate (pH 7), 100 mM NaCl, and 50% (vol/vol) glycerol. The polypeptides present in an aliquot were visualized by Coomassie blue staining after separation by SDS-PAGE.

**Characterization and optimization of the TK1827-encoded  $\beta$ -glycosidase.** Purified pTK1827 (1  $\mu$ g) was added to reaction mixtures (500  $\mu$ l) that contained 0 to 10 mM ONP-gluco, *ortho*-nitrophenyl- $\beta$ -D-mannopyranoside (ONP-manno), or *ortho*-nitrophenyl- $\beta$ -D-galactopyranoside (ONP-galacto) in 25 mM sodium phosphate (pH 6.5). After incubation at 85°C for 10 min, the increase in  $A_{405}$  was measured. A small change in  $A_{405}$  resulted from chemical hydrolysis of these substrates in the absence of pTK1827 that was subtracted from the experimental values.

To establish the optimum temperature for catalysis, reaction mixtures containing 1  $\mu$ g of pTK1827 and 2.8 mM ONP-gluco were incubated for 10 min at different temperatures. The changes in  $A_{405}$  were measured, and the activity at each temperature was calculated as a percentage of the maximum activity observed. To establish the optimum pH for catalysis, reaction mixtures containing 1  $\mu$ g of pTK1827 and 2.8 mM ONP-gluco dissolved in 25 mM sodium citrate (pH 4 to 5.5), sodium phosphate (pH 6 to 7.5), sodium borate (pH 8 to 9), or glycine-NaOH (pH 9.5 to 10) were incubated for 10 min at 85°C. The changes in  $A_{405}$  were measured, and the activity at each pH was calculated as a percentage of the maximum activity observed. To determine the rate of inactivation of pTK1827 at 100°C, aliquots (250  $\mu$ l) that contained 1  $\mu$ g of pTK1827 in 25 mM sodium phosphate (pH 6.5) were incubated at 100°C. Over time, 250  $\mu$ l of 5.6 mM ONP-gluco in 25 mM sodium phosphate (pH 6.5) was added to each aliquot, and the reaction mixture was placed at 85°C for 10 min. Changes in  $A_{405}$  were measured, and the activity present was calculated as a percentage of the activity present in a reaction mixture that was not incubated at 100°C.

## RESULTS AND DISCUSSION

**Identification and properties of the  $\beta$ -glycosidase encoded by TK1827.** Purification by sequential column chromatography generated  $\beta$ -glycosidase-containing fractions that, based on Coomassie blue staining after SDS-PAGE, contained only two predominant polypeptides. Gel elution, trypsin digestion, and LC-MS/MS revealed that both polypeptides were the full-length product of TK1827. The migration of this hyperthermophilic protein as two bands during SDS-PAGE likely resulted from incomplete denaturation, and the same phenomenon, resolution by SDS-PAGE into two distinct bands, was also reported for purified recombinant pTK1761 (4). The identification of the purified protein as pTK1827 was consistent with the annotated function of the TK1827 gene product (7) (Table 2). The pTK1827 sequence is ~79% identical to that of a  $\beta$ -glycosidase, designated BglA, encoded by PF0442 in *P. furiosus*, a *Thermococcales* relative of *T. kodakarensis* (13, 14, 26).

The very low abundance of pTK1827 in *T. kodakarensis* KW128 made its purification from this strain, in quantities sufficient for detailed characterization, impractical. To avoid potential concerns for misfolding or lack of posttranslation modifications if pTK1827 were generated in a mesophile or in a nonarchaeal expression system, we chose to overexpress TK1827 in *T. kodakarensis*. To do so, we constructed *T. kodakarensis* TS502 in which the native TK1827 promoter was replaced by the strong constitutive promoter P<sub>hmtB</sub> (19, 20) and, to simplify the purification, 10 histidine codons were added to the 5' terminus (N-terminal His<sub>10</sub>-tag) of TK1827 (Fig. 1A). As a control, *T. kodakarensis* TS503 was constructed with TK1827 identically transcribed from P<sub>hmtB</sub> but with a

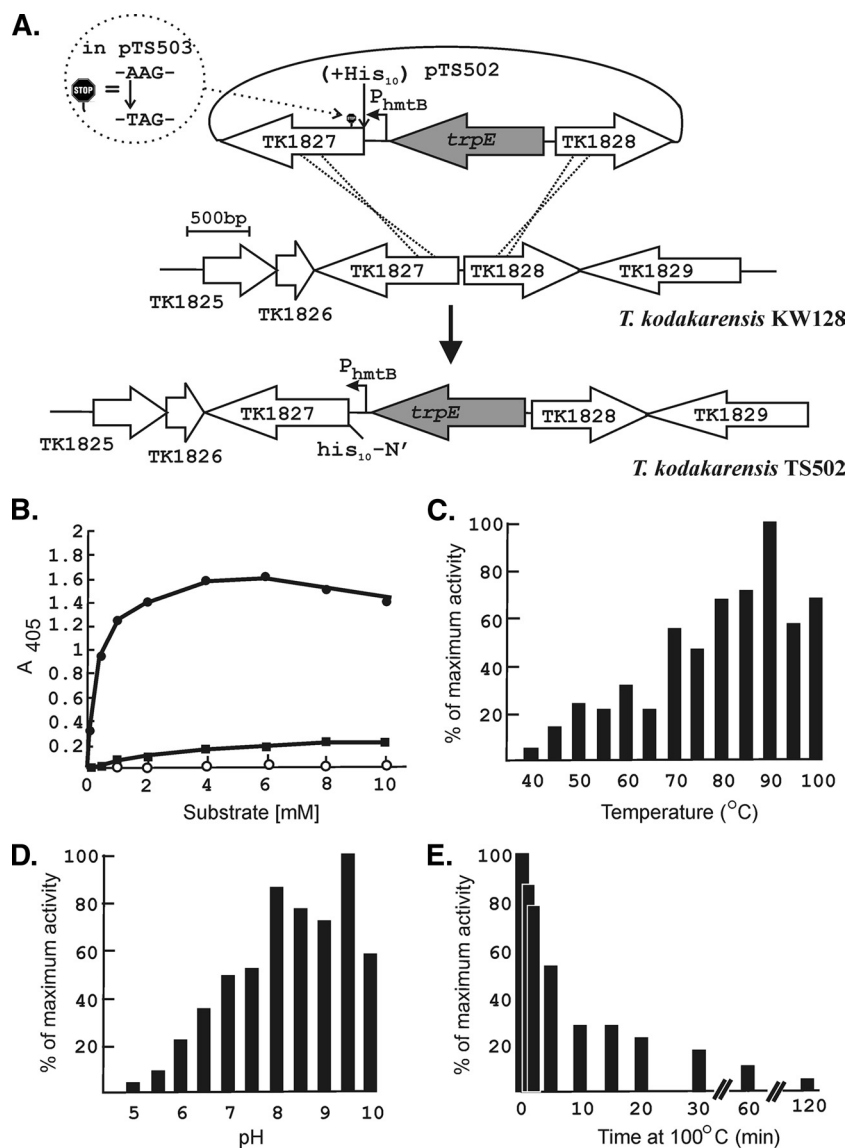


FIG. 1. Construction of *T. kodakarensis* TS502 and TS503 and properties of pTK1827. (A) Plasmids pTS502 and pTS503 were constructed by insertion of TK1827-*trpE*-TK1828 into pUC118 (27). As illustrated, in pTS502, 10 histidine codons were inserted in-frame at the 5' terminus of TK1827 and, in pTS503, the TK1827 reading frame was terminated at codon 5 by a nonsense mutation. Aliquots of pTS502 and pTS503 DNA were used to transform *T. kodakarensis* KW128 and transformants, designated *T. kodakarensis* TS502 and TS503, were selected by growth on plates lacking tryptophan. PCR amplification and sequencing confirmed that *T. kodakarensis* TS502 had the genome structure shown, and that *T. kodakarensis* TS503 similarly had the predicted genome structure and retained the nonsense mutation in TK1827. (B) Hydrolysis of ONP-gluco (●), ONP-galacto (■), and ONP-manno (○) during incubation with pTK1827 for 10 min at 85°C. (C) Effect of temperature on ONP-gluco hydrolysis expressed as a percentage of the maximum hydrolysis observed at 90°C. (D) Effect of pH on ONP-gluco hydrolysis. The results shown are average values of the percentages of the maximum hydrolysis observed at pH 9.5 measured in six replicates of this experiment. (E) Inactivation of pTK1827 by incubation at 100°C. The pTK1827 activity present after incubation at 100°C is shown as a percentage of the activity present before incubation at 100°C. The results shown are average values from two replicates of the experiment.

nonsense mutation introduced at codon 5 of the TK1827 reading frame (Fig. 1A).  $P_{hmtB}$ -directed expression of TK1827 resulted in a higher level of  $\beta$ -glycosidase activity in *T. kodakarensis* TS502 than in *T. kodakarensis* KW128, and this increase did not occur in *T. kodakarensis* TS503 (see below and Fig. 3). As observed for native pTK1827, aliquots of the His<sub>10</sub>-tagged pTK1827 purified from *T. kodakarensis* TS502 by Ni<sup>2+</sup>-affinity chromatography migrated to form two bands during SDS-PAGE.

Purified His<sub>10</sub>-tagged pTK1827 hydrolyzed ONP-gluco and had lower but detectable activity with ONP-galacto but did not hydrolyze ONP-manno (Fig. 1B). In contrast, pTK1761 was shown to have high activity with both ONP-gluco and ONP-manno and much lower activity with ONP-galacto (4). In common with pTK1761, pTK1827 was active *in vitro* at temperatures ranging from 45 to 100°C, optimally at ~90°C (Fig. 1C), and hydrolyzed ONP-gluco from pH 5 to 10 and optimally at pH 8 and 9.5 (Fig. 1D). By extrapolation from *Pyrococcus*



*woesii* (25), a related *Thermococcales*, the cytoplasm of *T. kodakarensis* is predicted to contain  $\sim 800$  mM  $K^+$  and the activity of pTK1827 was stimulated by 400 to 650 mM KCl addition, but no specific salt or salt concentration was required for activity. Under optimized conditions, the  $K_m$  of pTK1827 for ONP-gluco was  $\sim 0.5$  mM, similar to the  $K_m$  of 1.79 mM reported for this substrate for pTK1761 (4). Although pTK1827 was very active at 100°C (Fig. 1C), the activity was lost, with a half-life of  $\sim 7$  min during incubation at 100°C (Fig. 1E). Recombinant pTK1761 was similarly very active but also rapidly inactivated by incubation at 100°C (4).

**Inactivation of TK0664 confers resistance to 6-methyl purine (6MP<sup>r</sup>).** The nonsense mutations in TK1761 and TK1827, in strains *T. kodakarensis* TS416 (18) and *T. kodakarensis* TS503 (Fig. 1A), respectively, had no discernible effects on viability or growth. Construction of a *T. kodakarensis* strain with both genes deleted therefore seemed an attractive possibility to optimize the TK1761 reporter system. We tried to delete these genes by complementation of the uracil auxotrophy in *T. kodakarensis* KU216 to obtain targeted integration (pop-in), followed by selection of 5-fluoroorotic acid resistant mutants (pop-out/counterselection), as described by Sato et al. (23), but were unsuccessful. We therefore chose to use *trpE* complementation to direct integration in *T. kodakarensis* KW128 (Table 1) and screened additional nucleotide analogues to develop a different counterselection. The growth of *T. kodakarensis* KW128 was inhibited on plates containing 10  $\mu$ M 6MP, 1 mM 6-thioguanine, or 1 mM 8-azaguanine and, based on colony formation, spontaneous resistance to these inhibitors occurred at a frequency of  $\sim 1$  per  $10^8$  cells. This frequency was consistent with mutations that resulted in the loss of a single enzyme involved in purine scavenging that conferred resistance by preventing the lethal incorporation of these toxic purine analogues. Based on the *T. kodakarensis* genome annotation (7), genes predicted to encode such enzymes were PCR amplified and sequenced from many analogue-resistant mutants. Resistance to 6MP correlated consistently with mutations in TK0664, and different missense and nonsense mutations, single- and multiple-base-pair insertions and deletions were identified in the TK0664 sequences amplified from different 6MP<sup>r</sup> mutants. To confirm that the loss of TK0664, annotated as encoding a hypoxanthine-guanine phosphoribosyl-transferase (7) (Table 2), resulted in 6MP<sup>r</sup>, TK0664 was replaced in the *T. kodakarensis* KW128 genome by *trpE* (Fig. 2A). The resulting strain, *T. kodakarensis* TS521, grew normally without added tryptophan and in the presence of up to  $\sim 5$  mM 6MP.

**Use of 6MP<sup>r</sup> as a counterselection to isolate mutants with chromosomal deletions.** To determine whether 6MP<sup>r</sup> could be used to select *T. kodakarensis* mutants with targeted deletions and to obtain a strain with TK0664 precisely deleted and not replaced by *trpE*, plasmid pTS517 was constructed (Fig. 2B) and used to transform *T. kodakarensis* KW128. Transformants were selected by growth on minimal medium plates lacking tryptophan. PCR amplification and sequencing of genomic DNA confirmed that *trpE* (TK0254) was integrated into the genomes of several transformants adjacent to TK0664 and flanked by directly repeated copies of TK0662 and TK0663, as illustrated in Fig. 2B. Dilutions of a representative transformant, designated *T. kodakarensis* TS517i (strains intermediate

in each construction are denoted by an “i” in the designation), were spread on plates that contained 6MP and tryptophan. Colonies grew at a frequency of  $\sim 1$  per  $10^3$  plated cells, and all of the spontaneous 6MP<sup>r</sup> mutants screened were also tryptophan auxotrophs. PCR amplification and sequencing of genomic DNA from several of these mutants confirmed that the *trpE*-TK0664 sequence was deleted, that only one copy of TK0662 and TK0663 remained, and that the intergenic sequence between TK0663 and TK0665 was identical to that in pTS517 (Fig. 2B). One isolate was retained for future studies and designated *T. kodakarensis* TS517 (Table 1).

**Construction of the trp-6MP<sup>s</sup> cassette and 6MP<sup>r</sup> selection of TK1761 and TK1827 deletions.** Plasmid pTS535 was constructed with *trpE* (TK0254) and TK0664 adjacent but transcribed divergently from two strong constitutive promoters, P<sub>TK2279</sub> and P<sub>hmtB</sub> (18–20), respectively, and flanked by multiple cloning sites designated MCS1 and MCS2 (see Fig. S1 in the supplemental material). When expressed in *T. kodakarensis* TS517 ( $\Delta trpE$ ;  $\Delta TK0664$ ), these genes, designated the trp-6MP<sup>s</sup> cassette, conferred tryptophan-independent growth and sensitivity to 6MP. Plasmids pTS538 and pTS541 were generated from pTS535 by cloning PCR-amplified fragments of the *T. kodakarensis* genome into MCS1 and MCS2 and so flanking the trp-6MP<sup>s</sup> cassette (see Fig. S2A and B in the supplemental material; Table 1). The flanking sequences facilitated homologous recombination and directed the integration of the trp-6MP<sup>s</sup> cassette adjacent to TK1761 (see Fig. S2A) and TK1827 (see Fig. S2B) in pTS538 and pTS541 transformants of *T. kodakarensis* TS517, respectively. PCR amplification and sequencing of genomic DNA from several transformants confirmed the presence of the trp-6MP<sup>s</sup> cassette positioned between the designed duplications of chromosomal genes (see Fig. S2A and B). Dilutions of two representative transformants, designated *T. kodakarensis* TS538i and TS541i, were spread on plates containing 6MP and tryptophan, and sequencing of genomic DNA, PCR amplified from spontaneous 6MP<sup>r</sup> mutants, confirmed the absence of the trp-6MP<sup>s</sup> cassette. In the 6MP<sup>r</sup> mutants obtained from *T. kodakarensis* TS538i, the TK1761-TK1762-TK1763 operon was also deleted, and a representative was designated *T. kodakarensis* TS538 (see Fig. S2A in the supplemental material). In the 6MP<sup>r</sup> mutants obtained from *T. kodakarensis* TS541i, in addition to the loss of the trp-6MP<sup>s</sup> cassette, TK1827 was deleted, and a representative was designated *T. kodakarensis* TS541 (see Fig. S2B in the supplemental material). Consistent with deletion of the trp-6MP<sup>s</sup> cassette, all of the spontaneous 6MP<sup>r</sup> mutants isolated and screened from *T. kodakarensis* TS538i and TS541i were also tryptophan auxotrophs.

To construct a strain with both TK1761 and TK1827 deleted, *T. kodakarensis* TS541 (Fig. S2B; Table 1) was transformed with pTS538 DNA (Fig. S2A). Dilutions of a transformant selected by growth on plates lacking tryptophan were spread on plates that contained 6MP and tryptophan. PCR amplification and sequencing of genomic DNA from several spontaneous 6MP<sup>r</sup> mutants confirmed that both the trp-6MP<sup>s</sup> cassette and the TK1761-TK1762-TK1763 operon had been deleted, as illustrated for the construction of *T. kodakarensis* TS538 in Fig. S2A in the supplemental material. Screening confirmed that  $\sim 80\%$  of these 6MP<sup>r</sup> mutants were also tryptophan auxotrophs. PCR amplification and sequencing recon-

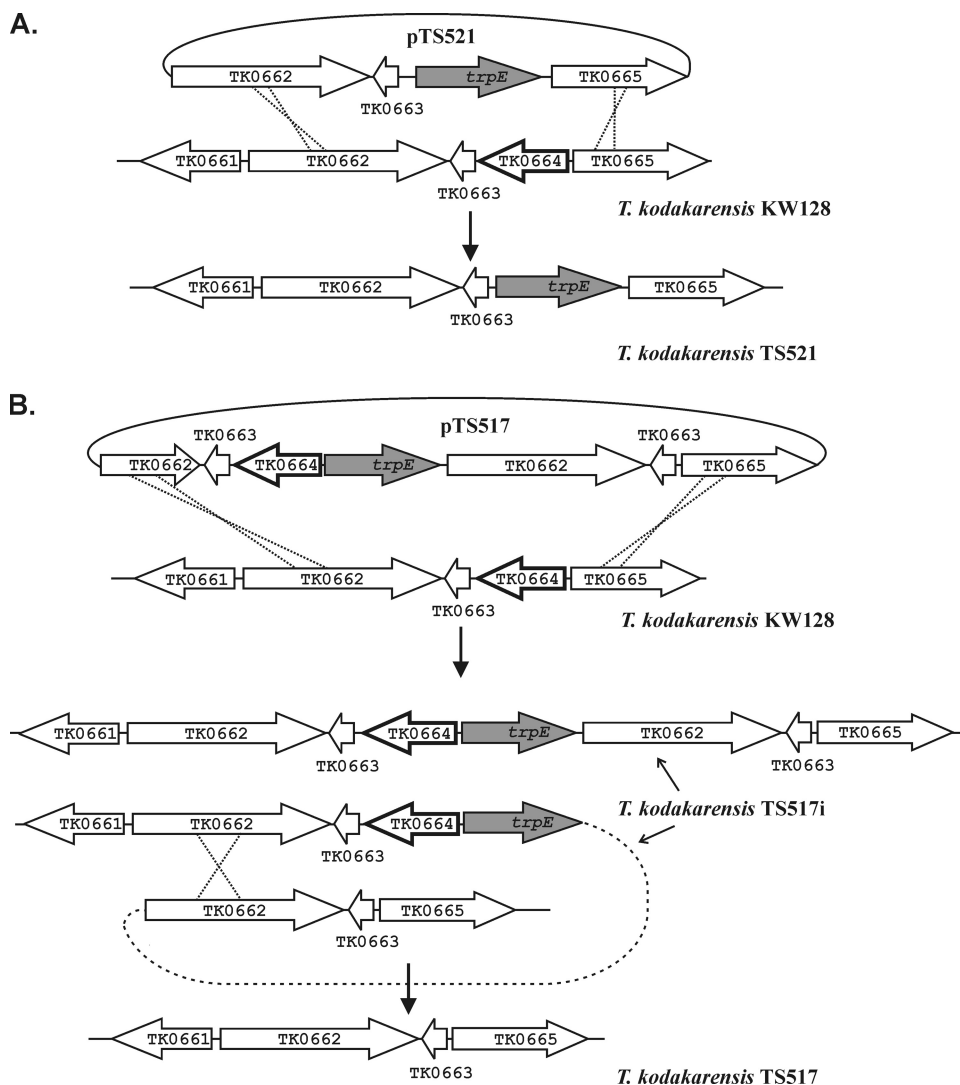


FIG. 2. Construction of *T. kodakarensis* TS521 and TS517. In this figure, as well as in Fig. S2 in the supplemental material and Fig. 4, the gene(s) targeted for deletion are identified by a bold outline. (A) Plasmid pTS521 was constructed by cloning the genes, as shown, into pUC118 and used to transform *T. kodakarensis* KW128. A representative transformant, designated *T. kodakarensis* TS521, selected by growth on plates lacking tryptophan, had the genome structure shown. (B) Plasmid pTS517 was constructed by cloning the genes, as shown, into pUC118 and used to transform *T. kodakarensis* KW128. A representative transformant, designated *T. kodakarensis* TS517i, was selected by growth on plates lacking tryptophan and had the genome structure shown. Dilutions of a culture of *T. kodakarensis* TS517i were spread on plates containing 6MP, and a 6MP<sup>r</sup> tryptophan auxotroph, designated *T. kodakarensis* TS517, had the genome structure shown.

firmed that TK1827 was also deleted in one such isolate that was retained for future studies and designated *T. kodakarensis* TS1079 (Table 1).

#### **$\beta$ -Glycosidase activity in *T. kodakarensis* deletion mutants.**

Lysates of cells harvested at an optical density at 600 nm (OD<sub>600</sub>) of ~0.6 from three independent exponentially growing cultures of *T. kodakarensis* KW128, TS372, TS416, TS502, TS503, TS538, TS541, and TS1079 (Table 1) were assayed for  $\beta$ -glycosidase activity. Expression of TK1761 from P<sub>hmtB</sub> in *T. kodakarensis* TS372 resulted in a three- to fivefold increase in ONP-gluco and ONP-manno hydrolyzing activity relative to the activity in *T. kodakarensis* KW128 (Fig. 3). This increase did not occur in *T. kodakarensis* TS416 that differs from *T. kodakarensis* TS372 only by the presence of a nonsense mutation in TK1761 (18). Transcription of TK1827 from P<sub>hmtB</sub> in *T.*

*kodakarensis* TS502 (Fig. 1A) also resulted in an increase in ONP-gluco but not in ONP-manno hydrolyzing activity, and this increase similarly did not occur in *T. kodakarensis* TS503 that has a nonsense mutation in TK1827. Surprisingly, however, in contrast to these increases in activity that resulted from increased expression of TK1761 and TK1827, deletion of these genes did not significantly reduce the levels of ONP-gluco or ONP-manno hydrolyzing activity in *T. kodakarensis* TS538, TS541, and TS1079 below the levels in *T. kodakarensis* KW128, TS416, and TS503 (Fig. 3). Purification of pTK1827 provided no evidence for a third  $\beta$ -glycosidase, and it seemed possible therefore that this residual substrate hydrolysis was not enzyme catalyzed. To pursue this, knowing that both pTK1761 (4) and pTK1827 (Fig. 1E) were inactivated by incubation at 100°C, aliquots of *T. kodakarensis* KW128, TS372, and TS1079 lysates

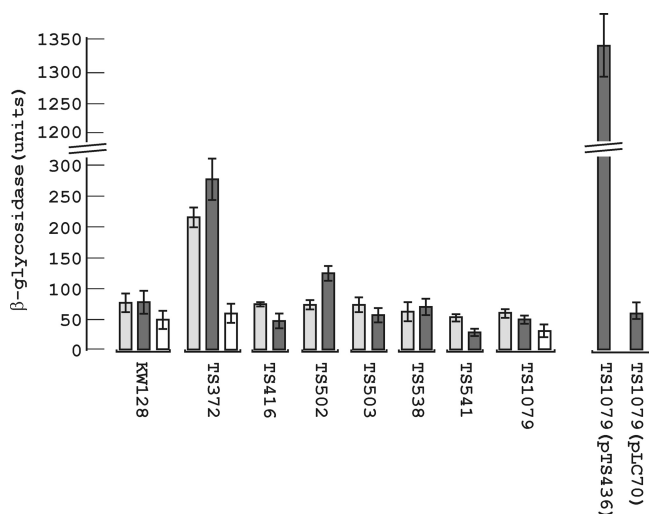


FIG. 3. Assays of  $\beta$ -glycosidase activity in *T. kodakarensis* cell lysates. Cells were harvested from cultures of the different *T. kodakarensis* strains (see Table 1) grown in ASW-YT-S<sup>o</sup> medium to an OD<sub>600</sub> of ~0.6. The cells were lysed, and aliquots of the cleared lysates were incubated with ONP-manno (light gray bars) or ONP-gluco (dark gray bars) for 10 min at 85°C. The release of ONP was measured by the increase in A<sub>405</sub>. The results shown above each strain designation are the averages (with error bars) of triplicate assays of lysates from three separate cultures of that strain. The open bars show the results of assays with aliquots of *T. kodakarensis* KW128, TS372, and TS1079 lysates that were autoclaved for 20 min at 121°C before incubation with ONP-gluco.

were autoclaved for 20 min at 121°C and then assayed for ONP-gluco hydrolyzing activity. Consistent with inactivation of pTK1761, autoclaving substantially reduced the  $\beta$ -glycosidase activity present in *T. kodakarensis* TS372 lysates but did not totally eliminate substrate hydrolysis and only marginally reduced the activity present in *T. kodakarensis* KW128 and TS1079 lysates (Fig. 3). Although this residual hydrolysis could still be catalyzed by one or more unidentified, very-heat-resistant  $\beta$ -glycosidases, it seems most likely that these colorimetric substrates undergo slow chemical hydrolysis when incubated at 85°C with components in a *T. kodakarensis* cell lysate. In either case, this very low level of background activity can be readily determined and subtracted, if necessary (see below), from experimental results.

To investigate the range of the TK1761 reporter signal, *T. kodakarensis* TS1079 was transformed with expression plasmids that replicate in the *T. kodakarensis* cytoplasm at a copy number of ~3 per chromosome (19). A DNA molecule with TK1761 transcribed from P<sub>hmtB</sub> was PCR amplified from pTS372 (18) (Table 1) and cloned into pLC70 (19), generating pTS436 (Table 1). The ONP-gluco hydrolyzing activity in *T. kodakarensis* TS1079(pTS436) was ~30-fold higher than in the control *T. kodakarensis* TS1079(pLC70) and ~5-fold higher than in *T. kodakarensis* TS372 (18) that has a single copy of the P<sub>hmtB</sub>-TK1761 construct present in its genome (Fig. 3). In experiments with such large positive signals, the low background of substrate hydrolysis (see above) would not pose a serious experimental concern.

**Deletion of TK0149, agmatine auxotrophy, and selection of transformants on rich media.** The observation that disruption

of TK0149 (designated *pdaD*; Table 2) resulted in agmatine auxotrophy (6) suggested a positive selection that might be imposed on cells growing in nutrient rich media. We confirmed that the *T. kodakarensis pdaD* mutant grew in ASW-YT · S<sup>o</sup> medium only with agmatine added, but PCR amplification and sequencing revealed that both TK0149 and an adjacent 3'-region of TK0148 were deleted in this strain (6). To determine whether TK0149 expression was sufficient to complement this mutation, TK0149 was cloned downstream from P<sub>hmtB</sub> in pLC70, resulting in plasmid pTS543 (Table 1). Transformation of *T. kodakarensis pdaD* with pTS543 resulted in transformants that grew and formed colonies within 24 h on plates that contained ASW-YT medium without added agmatine. The deletion into TK0148 did not therefore play a critical role in conferring agmatine auxotrophy, but to confirm that deletion of TK0149 was sufficient and to obtain defined strains for further use, we constructed mutants with precise deletions of TK0149 alone and of TK0148 plus TK0149. *T. kodakarensis* TS517 was transformed with pTS559 (Fig. 4) or pTS561 (Table 1), and transformants were selected by growth on minimal medium plates without tryptophan but containing agmatine. PCR amplification and sequencing of genomic DNA from several transformants confirmed the integration of the trp-6MP<sup>s</sup> cassette, deletion of TK0149 (pTS559 transformants; Fig. 4) or of TK0148 and TK0149 (pTS561 transformants), and duplication of TK0150. Transformation with pTS559 or pTS561 resulted directly in the deletion of TK0149 or the deletion of TK0148 plus TK0149, respectively, with integration (pop-in) of the trp-6MP<sup>s</sup> cassette, rather than at the subsequent counterselection (pop-out) step as in the *T. kodakarensis* TS538 and TS541 constructions (see Fig. S2A and B in the supplemental material). Representative transformants were designated *T. kodakarensis* TS559i (Fig. 4) and TS561i (Table 1), and dilutions of these strains were spread on plates that contained 6MP, tryptophan, and agmatine. Spontaneous 6MP<sup>r</sup> mutants were screened for growth with or without tryptophan and with or without agmatine. Genomic DNA was PCR amplified from several 6MP<sup>r</sup>, tryptophan- and agmatine-requiring mutants, and sequencing confirmed the absence of the trp-6MP<sup>s</sup> cassette and the presence of only one copy of TK0150 directly adjacent to TK0148 in mutants from *T. kodakarensis* TS559i (Fig. 4) and directly adjacent to TK0147 in mutants from *T. kodakarensis* TS561i. A representative from each construction was confirmed to require agmatine addition for growth in rich media and designated *T. kodakarensis* TS559 (Fig. 4) and *T. kodakarensis* TS561 (Table 1), respectively. Transformation of these strains with plasmid pTS543 resulted in transformants that grew rapidly on plates containing ASW-YT-S<sup>o</sup> medium without agmatine. Expression of TK0149 from pTS543 also ensured the maintenance of this plasmid in the cytoplasm of transformants grown in nutrient rich media lacking agmatine.

**Conclusions.** *T. kodakarensis* provides an attractive experimental system for investigations of hyperthermophily, archaeal physiology, and molecular biology (2, 8). The results reported here increase and accelerate the technologies available to genetically manipulate and exploit *T. kodakarensis* for such research and for production-scale applications. We have identified, characterized, and deleted the  $\beta$ -glycosidase encoded by TK1827 that might otherwise have compromised the use of the TK1761 reporter system in *T. kodakarensis* under some growth

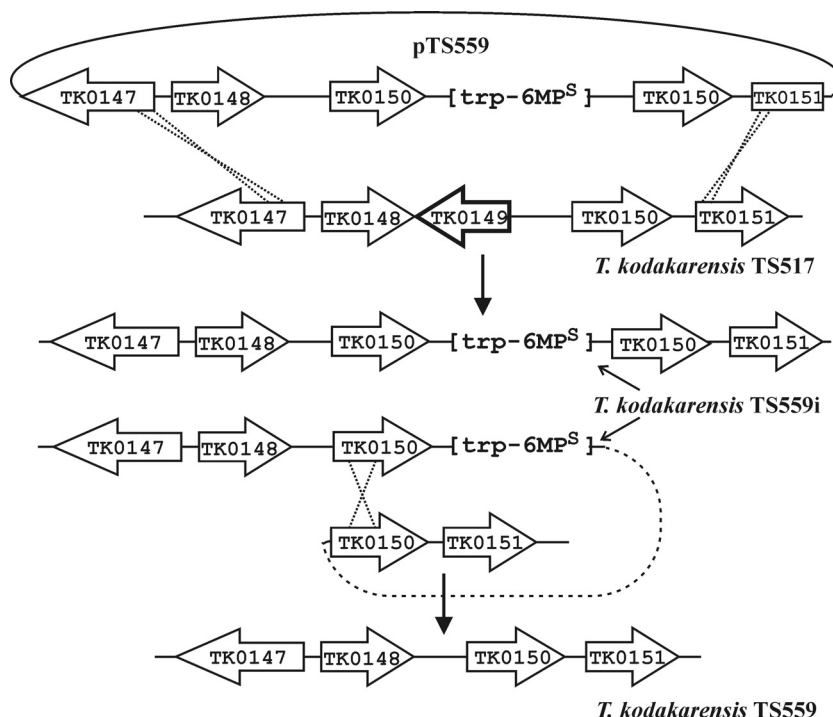


FIG. 4. Construction of *T. kodakarensis* TS559. Plasmid pTS559 was constructed by cloning the genes, as shown, into pTS535 (see Fig. S1 in the supplemental material) and was used to transform *T. kodakarensis* TS517 (Table 1). A transformant, designated *T. kodakarensis* TS559i, selected by growth on plates lacking tryptophan but containing agmatine, had the genome structure shown. Dilutions of a culture of *T. kodakarensis* TS559i were spread on plates containing 6MP, tryptophan, and agmatine, and a 6MP<sup>+</sup>, tryptophan, and agmatine auxotroph, designated *T. kodakarensis* TS559, had the genome shown. The same protocol was used to generate *T. kodakarensis* TS561 except that plasmid pTS561, which differed from pTS559 by the absence of TK0148 (Table 1), was used in the first step to transform *T. kodakarensis* TS517 to produce the intermediate strain, *T. kodakarensis* TS561i.

conditions and established that the TK1761 reporter  $\beta$ -glycosidase can accumulate to very different levels without effecting growth. Two new positive selections (6MP<sup>+</sup>, agmatine complementation) have been added for transformants, and a simple procedure has been established, based on TK0664 expression (6MP<sup>s</sup>), that makes it possible to delete multiple genes sequentially from the *T. kodakarensis* chromosome. By routine cloning into plasmid pTS535 (see Fig. S1 in the supplemental material) and transformation of *T. kodakarensis* TS517 (Fig. 2B and Table 1), the 6MP<sup>s</sup>-based selection can be used to delete any nonessential *T. kodakarensis* gene. Deletion of TK0149 results in agmatine auxotrophy (6) and complementation of a  $\Delta$ TK0149 mutation selects transformants on nutrient rich media, allowing much faster strain construction. Complementation of agmatine auxotrophy can also be used to maintain plasmids and so replace the need for antibiotics in large-volume preparative cultures.

#### ACKNOWLEDGMENTS

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