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Markerless Gene Deletion with Cytosine Deaminase in *Thermus thermophilus* Strain HB27

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We developed a counterselectable deletion system for *Thermus thermophilus* HB27 based on cytosine deaminase (encoded by *codA*) from *Thermaerobacter marianensis* DSM 12885 and the sensitivity of *T. thermophilus* HB27 to the antimetabolite 5-fluorocytosine (5-FC). The deletion vector comprises the pUC18 origin of replication, a thermostable kanamycin resistance marker functional in *T. thermophilus* HB27, and *codA* under the control of a constitutive putative trehalose promoter from *T. thermophilus* HB27. The functionality of the system was demonstrated by deletion of the *bglT* gene, encoding a β-glycosidase, and three carotenoid biosynthesis genes, *CYP175A1*, *crtY*, and *crtI*, from the genome of *T. thermophilus* HB27.

Thermus thermophilus HB27 is a Gram-negative, yellow-pigmented, aerobic bacterium growing at temperatures up to 85°C (1). It belongs to the phylum *Deinococcus-Thermus* and has a GC content of 69%. Due to its specific amenable characteristics, like high growth rates, cell yields, constitutive natural competence, and, not least, the availability of its genome sequence comprising the 1.90-Mb chromosome and a 0.23-Mb megaplasmid (2), *T. thermophilus* emerged as a laboratory model for studying the molecular basis of thermophilia (3, 4). The small genome of *T. thermophilus* contains few functional paralogues, and consequently, studying knockout mutants is one appropriate approach to elucidate specific gene functions in the organism (3).

A number of genetic tools have been developed for genetic manipulation of *T. thermophilus* HB27, including various *T. thermophilus-Escherichia coli* shuttle vectors and different deletion or integration systems (5–9). For plasmid maintenance or identification of desired mutants, only a few engineered thermostable antibiotic resistance markers, namely, kanamycin nucleotidyltransferase (10, 11), bleomycin binding protein (12), and hygromycin B phosphotransferase (13), are available. An alternative positive-selection strategy is complementation of gene defects of auxotrophic host strains by supplying gene functions, such as tryptophan synthetase (*trpB*) (14) or malate dehydrogenase (*mdh*) (15), in *trans*.

The limited number of selection markers and the demand for mutants whose construction does not irreversibly consume these rare markers pushed the development of alternative, counterselectable systems for markerless genome manipulation in *T. thermophilus*, including negative-selection systems based on *pyrE* (6, 16), the *rpsL1* allele (17), *bgl-lacZ* (18), or the *pheS* allele (5). These systems allow clearance of any positive-selection marker, thus enabling its reuse in the next step of sequential strain construction or maintenance of expression plasmids. The general principle of a counterselection strategy follows two steps: The first step includes the targeted chromosomal integration of a suicide plasmid carrying the desired allele to be exchanged, an antibiotic resistance marker, and a counterselectable marker by homologous recombination. Clones with integrated plasmids are identified by their antibiotic resistance. The second step employs the counterselectable trait, allowing excision of the plasmid, together with the selection markers, via homologous recombination, thereby leaving the allele to be exchanged in the chromosome.

A reliable counterselection strategy is inhibition of thymidylate synthetase by the uracil analog 5-fluorouracil (5-FU). The most commonly used marker of this kind is uracil phosphoribosyltransferase (*upp*) (UPRTase, Upp) (EC 2.4.2.9), which converts uracil to UMP (see Fig. S1 in the supplemental material). Transformation of the uracil analog 5-FU to 5-fluorouridine monophosphate (5-FUMP) by Upp and further conversion of 5-FUMP results in irreversible inhibition of thymidylate synthetase (19, 20). An alternative counterselectable marker is cytosine deaminase (*codA*) (EC 3.5.4.1), which has been applied for various bacteria (21–23). CodA (EC 3.5.4.1) catalyzes the deamination of cytosine and its analog, 5-fluorocytosine (5-FC), to uracil and 5-FU, respectively, which are subsequently converted to UMP and 5-FUMP by Upp (24). A deletion system developed for *T. thermophilus* employs *pyrE*, encoding orotic acid phosphoribosyltransferase (EC 2.4.2.10), as a negative-selection marker (6). In UMP *de novo* synthesis, PyrE catalyzes the synthesis of orotidine monophosphate (OMP) from orotic acid, which is then converted to UMP by PyrF. Conversion of 5-fluoroorotic acid (5-FO) finally also results in synthesis of 5-FUMP (see Fig. S1 in the supplemental material).

In *T. thermophilus* HB27, a *pyrE* gene (TT_C1380) and an *upp* gene (TT_C0946), but no *codA* gene or orthologs, have been identified. In contrast to *pyrE* and *upp*, application of heterologous *codA* as a counterselectable marker for *T. thermophilus* HB27 has the great advantage that the wild type can be used directly because prior construction of a *codA* deletion strain is not required. We constructed a new markerless deletion system for *T. thermophilus* HB27 using *codA* (Tmar_1477) from *Thermaerobacter marianensis* DSM 12885. *T. marianensis* DSM 12885 has a GC content of

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72.5%, an optimal growth temperature from 74°C to 76°C, and a pH optimum of 7.0 to 7.5 (25), properties that are comparable to the optimal growth parameters of *T. thermophilus* HB27. The *codA* deletion system was used to delete the *bglT* gene (TT_P0042), encoding a β-glycosidase, and three carotenoid biosynthesis genes, *CYP175A1*, *crtY*, and *crtI* (TT_P0059, TT_P0060, and TT_P0066), encoding a β-carotene hydroxylase of the P450 superfamily (26, 27), a lycopene β-cyclase, and a phytoene desaturase (2, 28) (see Fig. S2 in the supplemental material), on the megaplasmid pTT27 from the genome of *T. thermophilus* HB27.

MATERIALS AND METHODS

Plasmids, bacterial strains, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. *E. coli* DH5α was grown at 37°C with shaking in LB medium (10 g liter^{-1} tryptone, 5 g liter^{-1} yeast extract, 5 g liter^{-1} NaCl) supplemented with 50 μg ml^{-1} kanamycin (Km), as appropriate. *T. thermophilus* strains were cultured in *Thermus* broth (TB) medium (8 g liter^{-1} tryptone, 4 g liter^{-1} yeast extract, 3 g liter^{-1} NaCl, pH 7.5) (29) at 70°C with shaking. For selection, the medium was supplemented with 25 μg ml^{-1} kanamycin, as appropriate. *T. thermophilus* strains were also grown in minimal medium 162 (M162) (30) with a minor modification [100 mg liter^{-1} nitrilotriacetic acid, 0.4 mg liter^{-1} Bacto nutrient broth, 0.005 mM iron(III) citrate, 40 mg liter^{-1} $CaSO_4 \cdot 2H_2O$, 0.2 g liter^{-1} $MgCl_2 \cdot 6H_2O$, 0.1 g liter^{-1} $(NH_4)_2SO_4$, 15 mM Na_2HPO_4, 5 mM KH_2PO_4, pH 7.5], supplemented with 0.1 mg liter^{-1} biotin, 1 mg liter^{-1} thiamine, and 2.0 g liter^{-1} D-glucose. Negative selection was carried out on M162 agar plates containing 1.5% (wt/vol) agar with 15 μg ml^{-1} 5-FC (Sigma-Aldrich, Steinheim, Germany).

Transformation procedures. The electroporation method (31) was used to transform competent *E. coli* DH5α (32) with plasmids. The transformation method used for naturally competent *T. thermophilus* HB27 was principally derived from two methods described previously (4, 33, 34). The cells were first grown in TB medium at 70°C overnight. The culture was diluted 1:100 with fresh TB medium and incubated at 70°C until the optical density at 550 nm (OD_{550}) reached a value of 0.8. Next, 500 μl of cell culture was mixed with 50 to 100 ng of plasmid DNA and incubated further at 70°C. After 2 h of incubation, 150 μl of the cultures was plated on TB agar medium supplemented with 25 μg ml^{-1} kanamycin. The plates were incubated at 65°C overnight.

Plasmid construction. Standard protocols were used for recombinant DNA techniques (31). Plasmid DNA was prepared with the innuPrep Plasmid minikit (Analytik Jena, Jena, Germany). To isolate genomic DNA of *T. thermophilus* strains, 4×10^9 cells were harvested from an overnight culture by centrifugation (5 min; 4,500 × *g*) and resuspended in 180 μl lysis buffer (25 mM Tris-HCl, pH 8.0, 25 mM EDTA, 10% [wt/vol] sucrose, 0.5 mM glycine, 20 mg ml^{-1} lysozyme). Genomic DNA was extracted with the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) using the protocol "pretreatment for Gram-positive bacteria." Oligonucleotides (see Table S2 in the supplemental material) were synthesized by Eurofins MWG (Ebersberg, Germany). DNA sequencing was performed by GATC Biotech (Constance, Germany).

The basic integrative vector pLEI269.1 (Fig. 1) is based on pUC18 (35) and the *Thermus* vector pMY1 (33, 36). It was constructed by ligation of several PCR fragments carrying a different restriction site on each end. The pUC18 origin of replication was amplified with primers s10346 and s9162b. The PCR fragment contains an NcoI site and a multiple cloning site (MCS) comprising restriction sites for PstI, PvuII, ScaI, SpeI, AgeI, and KpnI. The promoter region (P_{treha}) (see Table S3 in the supplemental material) of the putative trehalose operon (TT_C0611 to TT_C0615) (2) was amplified using primers s9549 (NcoI) and s9551 (BamHI) and genomic DNA of *T. thermophilus* HB27 as the template. The *codA* gene (Tmar_1477) of *T. marianensis* was synthesized by GenScript (Piscataway, NJ, USA). Eleven undesirable restriction sites within the gene were removed in consideration of codon usage (*codA*) (see Table S4 in the supplemental material), and BamHI and HindIII sites were added on both ends for cloning. In pLEI269.1, *codA* is constitutively expressed under the control of P_{treha}. In order to prevent transcriptional readthrough from P_{treha}, the transcriptional terminator *rrnB* of *E. coli* was amplified with primers s9088 and s9436 from pJOE5751.1 (37) as the template and positioned downstream of *codA* using HindIII and NheI restriction sites. For plasmid maintenance in both *E. coli* and *T. thermophilus* HB27, pLEI269.1 carries a thermostable kanamycin resistance marker (38) under the control of the S-layer protein A promoter (P_{slpA}) (39). P_{slpA}, together with the thermostable kanamycin resistance (Km^r) gene, was amplified with s9289 and s9290 (NheI and PstI) from pMY1. The NdeI restriction site between P_{slpA} and the Km^r gene was deleted by PCR using primers s9289 and s9400 (NheI and AseI).

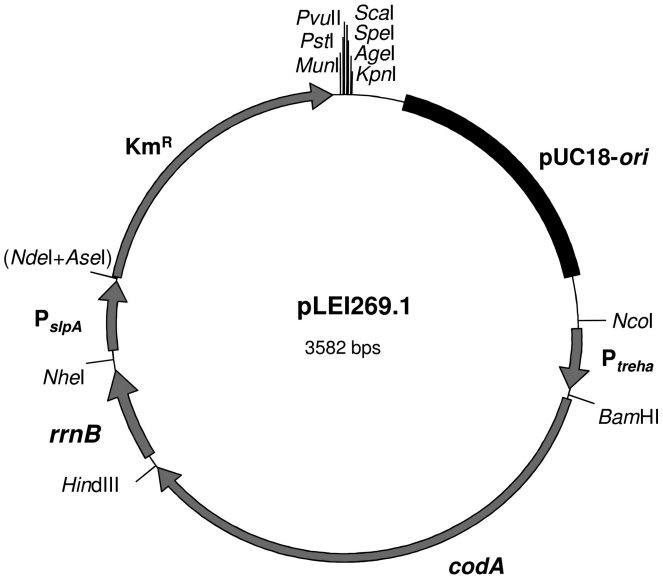

FIG 1 The basic integrative vector pLEI269.1 for targeted gene deletion in *T. thermophilus* HB27. The vector consists of the pUC18 origin of replication, the promoter region of a putative trehalose operon (P_{treha}) responsible for constitutive expression of *codA* (encoding cytosine deaminase from *T. marianensis*), a transcription terminator (*rrnB*), a Km^r cassette under the control of the S-layer protein A promoter (P_{slpA}), and a multiple cloning site for integration of flanking regions of the gene to be deleted. All the fragments were amplified by PCR and ligated with each other by their terminal restriction sites via several cloning steps (see Materials and Methods).

For the construction of the different plasmids carrying deletion cassettes, flanking sequences (each about 800 bp) of the genes to be deleted were amplified by PCR and integrated into pLEI269.1 using its MCS. The up- and downstream regions of the carotenoid biosynthesis genes *CYP175A1*, *crtY*, and *crtI* were amplified with the primers listed in Table S2 in the supplemental material and genomic DNA of *T. thermophilus* HB27 as the template. The PCR fragments were fused by their PstI (*CYP175A1* and *crtY*) or SalI (*crtI*) restriction sites and inserted into pLEI269.1 via MunI and SpeI restriction sites, thereby creating pLEI270.5, pLEI271.7 (Fig. 2), and pLEI273.2, respectively. The flanking regions of *bglT* were amplified with primers s9978, s10236, s9980, and s9981, fused by their PstI sites, and integrated into the basic vector by MunI and KpnI restriction sites to create pLEI257.1. All the plasmids were verified by DNA sequencing.

Deletion strain construction. *T. thermophilus* HB27 was transformed with the plasmids carrying the constructed deletion cassettes as described above. Due to their inability to autonomously replicate in *T. thermophilus* HB27, the plasmids can be maintained only by integration into the megaplasmid pTT27 via homologous recombination (Fig. 2). Cells with integrated plasmids were selected on TB agar with kanamycin at 65°C over-

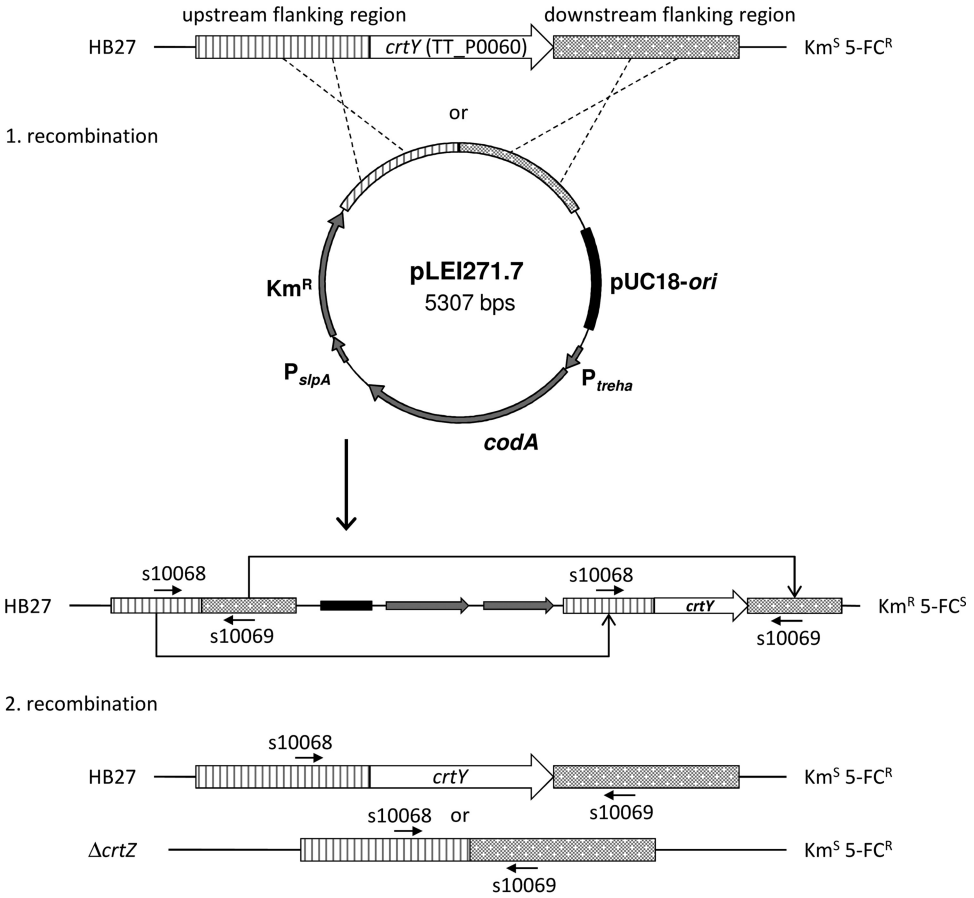

FIG 2 Principle of the counterselection system for targeted gene deletion in *T. thermophilus* HB27 demonstrated with pLEI271.7. The binding sites of the primers s10068 and s10069 used for analytical PCR of the mutant strains are indicated. The integrative vector pLEI271.7 contains the flanking regions of *crtY* (encoding lycopene β-cyclase), a kanamycin resistance determinant, and *codA* (encoding cytosine deaminase) as a counterselectable trait. The plasmid integrates into the genome by homologous recombination with the upstream or downstream flanking region of *crtY*. The transformants are resistant to Km and sensitive to 5-FC. The cell loses the integrated plasmid by a second homologous recombination. Depending on where the recombination occurs, *crtY* is cut out, together with the plasmid, or the wild-type situation is restored. The Δ*crtY* strain, as well as the reconstituted wild type, is resistant to 5-FC and sensitive to Km.

night. Several clones were isolated and streaked out three times on TB agar plates supplemented with kanamycin. From the last plate, kanamycin-resistant clones were checked for 5-FC sensitivity on M162 agar plates at 65°C for 72 h. The presence of *codA* causes sensitivity to 5-FC. Next, a kanamycin-resistant and 5-FC-sensitive clone was incubated in TB medium without antibiotics at 70°C overnight. The overnight culture was diluted 1:10^4 with TB medium containing 15 μg ml^{-1} 5-FC and incubated further at 70°C for 6 h. Meanwhile, the integrated plasmid should be excised via a second homologous recombination causing either deletion of the desired marker or reconstitution of the wild type (Fig. 2). In both cases, loss of the plasmid causes resistance to 5-FC and sensitivity to kanamycin. The culture was diluted 1:10 or 1:10^2 with H_2O, and 100 μl was plated on M162 agar with 5-FC and incubated at 65°C for 72 h. M162 media were necessary, as TB media obviously contain pyrimidines competing with 5-FC uptake and use and allowing *Thermus*, despite containing the integrative deletion vector, to grow on TB plates supplemented with 5-FC. Several 5-FC-resistant clones were isolated, streaked out three times on M162 plates with 5-FC, and finally transferred onto TB plates to obtain quick growth at 65°C. Sensitivity to kanamycin and resistance to 5-FC of the clones were tested on TB agar with kanamycin and M162 agar with 5-FC. Clones carrying the desired deletions were identified by PCR (see below). The numbers of colonies used to delete the four genes and the numbers of mutants obtained are summarized in Table S5 in the supplemental material.

Identification of deletion mutants by PCR. The deletion mutants grown on TB plates were scraped off and treated with the DNeasy blood and tissue kit as described above. The prepared genomic DNAs were applied as templates for PCRs with primers s10068/s10069, s10363/s10364, s10365/s10366, and s10369/s10370 (see Table S2 in the supplemental material) for verification of *bglT*, *CYP175A1*, *crtY*, and *crtI* deletion, respectively. The primers were designed with AT-rich sequences of the flanking regions in order to minimize nonspecific binding with the GC-rich genomic DNA of *T. thermophilus* HB27. Genomic DNA of the *T. thermophilus* HB27 wild type and the plasmid DNAs used for deletion strain construction served as controls.

β-Glycosidase assay. The *bglT* gene from *T. thermophilus* HB27 encodes a β-glycosidase with a broad substrate specificity for the β-anomeric linkage and catalyzes the hydrolysis of β-galacto-, β-gluco-, and β-fucopyranosides (40). We used *p*-nitrophenyl β-galactopyranoside (pNPGal) as the substrate to measure its enzyme activity.

Cell cultures grown in TB medium at 70°C for 6 h were used for the assay. The cells were harvested by centrifugation (5 min; 4,500 × *g*), washed with 0.1 M potassium phosphate buffer (pH 6.5), and then resuspended in the same buffer. An ultrasonic homogenizer (Sonopuls HD2070; Bandelin, Berlin, Germany) was applied for cell disruption. The supernatant containing soluble proteins was assayed after centrifugation (15 min; 16,100 × *g*). The reaction mixture, containing 25 μl of the supernatant and 450 μl of 0.1 M potassium phosphate buffer (pH 6.5), was

preincubated at 70°C for 5 min. Then, 25 μl pNPGal (4 mg ml^{-1}; Sigma-Aldrich, Steinheim, Germany) was added to the mixture. After 2 to 5 min incubation at 70°C, the reaction was stopped by adding 1 ml 400 mM sodium borate buffer (pH 9.4). The release of *p*-nitrophenol (pNP) was measured at 405 nm. One unit of enzyme activity was defined as the amount of enzyme catalyzing the liberation of 1 μmol of pNP per minute, using an extinction coefficient (ε_{405}; pH 10) of 18.5×10^3 M^{-1} cm^{-1} for pNP. The amount of total soluble protein was determined by the Bradford method (41). Specific activity was expressed as units per milligram of protein.

TLC of carotenoid extracts from *T. thermophilus* strains. *T. thermophilus* HB27 wild type and mutants were cultivated overnight in TB medium at 70°C. The next day, 2.6×10^{10} cells were harvested by centrifugation (5 min; 4,500 × *g*), washed with 1 ml H_2O, and stored at −20°C until use. For carotenoid extraction, the cells were resuspended in 1 ml potassium phosphate buffer (0.1 M; pH 6.5) with 10 mg ml^{-1} lysozyme and incubated at 37°C for 1 h. The suspensions were centrifuged (5 min; 16,100 × *g*), and the cells were washed with 1 ml H_2O and extracted with 600 μl acetone. The acetone extracts were mixed with 200 μl H_2O and 200 μl hexane for reextraction. The samples were mixed and centrifuged (5 min; 16,100 × *g*), and the upper hexane phase was isolated. Reextraction with 200-μl portions of hexane was repeated until the hexane phase remained colorless. The hexane extracts were pooled and dried in a vacuum centrifuge. The extracts were dissolved in 20 μl hexane and applied for thin-layer chromatography (TLC) (silicagel 60 F_{254}; Merck, Darmstadt, Germany) with petroleum-diethyl ether-acetone (40:10:10) as the mobile phase. β-Carotene (Sigma-Aldrich, Steinheim, Germany) and lycopene (extracted from tomato puree) were used as authentic standards.

RESULTS

The integrative deletion vector. The basis of the presented *codA* counterselection system for *T. thermophilus* HB27 is an integrative plasmid derived from pUC18 and the *Thermus* vector pMY1 (33). Only a few constitutive and even fewer regulated promoters are available for gene expression in *T. thermophilus* HB27. We have observed that the promoter region of the putative trehalose operon (P_{treha}) shows a constitutive promoter activity together with *bglT* as a reporter gene in *T. thermophilus* HB27 (data not shown). Thus, P_{treha} was applied for *codA* expression on the integrative deletion vector. Expression of *codA* from P_{treha} in *T. thermophilus* HB27 was verified on M162 agar supplemented with 5-FC. The presence of the genomically integrated vector caused sensitivity to 5-FC when a concentration of 15 μg ml^{-1} was applied.

Deletion of selected genes from the *T. thermophilus* HB27 genome. In order to demonstrate the functionality of the new *codA* counterselection system, we chose *bglT* as the first target for deletion. BglT is a widely used reporter protein for studying promoter activities in *T. thermophilus* HB27 (16, 42). The additional copy of *bglT* on the megaplasmid pTT27 of *T. thermophilus* HB27 interferes with these reporter assays. Therefore, a *bglT*-deficient strain is a desirable host for promoter studies using *bglT* as a reporter gene. We deleted *bglT* with pLEI257.1 carrying the homologous up- and downstream regions of *bglT* following the procedure described in Materials and Methods. In order to control the substeps of the deletion procedure, analytical PCRs were performed using primers s10068/s10069 and genomic DNA of *T. thermophilus* HB27 (wild type), *T. thermophilus* LW14 (with pLEI257.1 integrated into the genome after the first recombination), *T. thermophilus* LW19 (Δ*bglT*), or pLEI257.1 (deletion plasmid) as the template (Fig. 3). The primers used bind within the up- and downstream regions of *bglT*. The wild type should show a

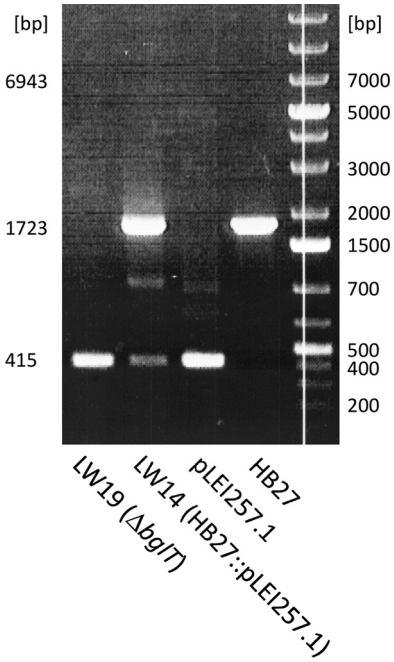

FIG 3 PCR analysis with primers s10068/s10069 and genomic DNA of *T. thermophilus* HB27 (wild type), *T. thermophilus* LW14 (with pLEI257.1 integrated into the genome), *T. thermophilus* LW19 (Δ*bglT*), or pLEI257.1. The fragments were separated by 0.7% agarose gel electrophoresis and stained with ethidium bromide. The 415-bp, 1,723-bp, and 6,943-bp markers on the left correspond to the expected fragment sizes.

PCR fragment (1,723 bp) that contains *bglT* between these flanking sequences. The PCR fragment of the *bglT* deletion strain should have a size reduced by 1,308 bp, based on the size of *bglT*. The strain with the integrated plasmid should show both PCR fragments and an additional fragment corresponding to the whole plasmid (Fig. 2).

As expected, the PCR with *T. thermophilus* HB27 wild-type DNA resulted in a fragment of 1,723 bp, and the PCR with pLEI257.1 yielded a fragment of 415 bp (Fig. 3). The PCR with *T. thermophilus* LW14 DNA showed both fragments. The large fragment (6,943 bp) corresponding to the whole integrated plasmid could not be detected under the PCR conditions used. PCR with DNA of the deletion strain *T. thermophilus* LW19 exhibited only the small fragment of 415 bp, suggesting the deficiency of *bglT*. The β-glycosidase assay with crude extracts of *T. thermophilus* HB27 and LW19 with pNPGal as the substrate revealed reduced β-glycosidase activity of the Δ*bglT* strain (3 mU mg^{-1} of total soluble proteins) compared to the wild type (8 mU mg^{-1}). This result is in agreement with the data published previously (42) and further substantiates the loss of *bglT*.

T. thermophilus HB27 produces carotenoids (43), especially thermozeaxanthin (44, 45). The carotenoid biosynthesis genes are located on the megaplasmid pTT27. Deletion of genes of the carotenoid-biosynthetic pathway is a very colorful way to demonstrate the applicability of the *codA* counterselection system, because removal of a gene involved in the multistep process of carotenoid biosynthesis results in change of color of the mutant strain.

Similar to the *bglT* deletion, the three genes *CYP175A1*, *crtY*, and *crtI* participating in the carotenoid synthesis pathway were

deleted with the three plasmids pLEI270.5, pLEI271.7, and pLEI273.2 carrying the appropriate deletion cassettes. The Δ*CYP175A1*, Δ*crtY*, and Δ*crtI* strains are referred to as *T. thermophilus* LW35, LW37, and LW40, respectively. For carotenoid analyses, the strains were incubated in TB medium at 70°C for 24 h. Cells were collected by centrifugation and suspended in potassium phosphate buffer. All of the cell suspensions had the same cell concentration (see Fig. S3 in the supplemental material). *T. thermophilus* HB27 showed the typical yellow color, whereas *T. thermophilus* LW35 (Δ*CYP175A1*) lacking β-carotene hydroxylase presented an orange color due to the enrichment of β-carotene in the cell membrane. The deletion of *crtY*, coding for lycopene β-cyclase, led to an accumulation of red lycopene (*T. thermophilus* LW37), which could be observed as a light-rose color of the cell suspension. The phytoene desaturase CrtI converts colorless phytoene to lycopene. Therefore, the cells of *T. thermophilus* LW40 (Δ*crtI*) appeared white. The development of cell colors caused by the gene deletions corresponds to those of the accumulated intermediates and is summarized in Fig. S2 in the supplemental material. The carotenoids of *T. thermophilus* HB27, LW35, and LW37 were extracted as described in Materials and Methods and analyzed by thin-layer chromatography, together with authentic standards of β-carotene and lycopene (see Fig. S4 in the supplemental material). *T. thermophilus* LW37 (Δ*crtY*) accumulated lycopene, as expected. β-Carotene and smaller amounts of lycopene could be detected in the extract of *T. thermophilus* LW35 (Δ*CYP175A1*). The obtained R_f values of the samples were 0 (*T. thermophilus* HB27), 0.97 (β-carotene, authentic standard), 0.97 (*T. thermophilus* LW35 Δ*CYP175A1*), 0.91 (lycopene, authentic standard), and 0.91 (*T. thermophilus* LW37 Δ*crtY*).

DISCUSSION

Commonly used reporter genes of expression vectors for *T. thermophilus* encode thermostable α-galactosidase and β-galactosidase (42, 46, 47), β-glycosidase (*bglT*) (16), or phytoene synthase (*crtB*) (9, 48). All the named reporter genes originate from *T. thermophilus* HB27 or a closely related species whose native enzyme activities interfere with reporter assays. Hence, mutant strains deficient in these interfering enzymes would facilitate promoter studies employing glucosidases or galactosidases as reporter enzymes. In addition, clarifying specific gene functions of *T. thermophilus* is another issue of great interest at this time, as it is an indispensable part of uncovering the molecular mechanisms underlying thermophilia.

Deletion systems allowing markerless genome manipulation provide powerful tools for genetic manipulation, enabling the construction of mutant strains for biotechnology or the study of specific gene functions with knockout mutants. Compared to other reported deletion methods for *T. thermophilus*, the *codA*-based deletion system described here has some advantages. (i) As a markerless and negative deletion system, the antibiotic resistance gene used for selection is excised from the genome. This allows the generation of multiple gene deletions/mutations in the parental strain to produce a final strain unmarked by an antibiotic resistance gene. (ii) The previously reported deletion systems based on *pyrE* (6), *bgl* (18), or *upp* need a *pyrE*-, *bgl*-, or *upp*-deficient host strain. In contrast, no *codA* ortholog exists in *T. thermophilus* HB27, and the wild type can be used directly as a parental strain in the reported deletion system. An additional disadvantage of the *pyrE* deletion system is the uracil auxotrophic feature of the *pyrE*-deficient host strain. (iii) Furthermore, the *codA* sequence used in the integrative plasmid should not cause unexpected homologous recombination with the genome sequence. However, *pheS* (5), *rpsL1* (17), or *bgl-lacZ* (18), as well as the two promoters P_{slpA} and P_{treha} that are also part of the integrative vector, originate from *T. thermophlilus* and could potentially recombine with the native genomic genes, leading to false-positive clones after the first recombination step. Using *pheS* as a counterselectable marker, a spontaneous large-scale deletion, including the carotenoid synthesis genes and the β-glycosidase gene in the megaplasmid, was observed, apparently mediated by insertion sequence (IS) elements (5). However, unexpected recombination or large-scale deletions have not been observed so far with the *codA* deletion system.

The Δ*bglT* strain *T. thermophilus* LW19 shows considerable β-galactosidase activity despite the lack of *bglT*. This is in agreement with the Δ*bglT* strain of *T. thermophilus* HB27 (*T. thermophiles* PPKU) constructed by Park and Kilbane (42), with a measured reduction of 50% of the β-galactosidase level compared to the wild type. Furthermore, inactivation of the identical *bglT* gene in the *T. thermophilus* strain TH125 led to 55% reduction of the pNP–β-galactoside-hydrolyzing activity (49). This background activity was probably due to the existence of two putative β-galactosidase genes (TT_P0220 and TT_P0222) (2). Despite the reduced β-galactosidase activity of *T. thermophilus* LW19, the deletion strain should allow the use of the β-galactosidase gene as a reporter gene. The purified BglT protein exhibits the highest catalytic efficiency for the substrate β-NP-glucopyranoside (40). Additionally, we used this substrate to measure the BglT activity and observed loss of activity in the deletion strain *T. thermophilus* LW19, but still with some background level, which is in agreement with the data published by Ohta et al. (16).

Until now, the genes *crtY* (TT_P0060) and *crtI* (TT_P0066) of *T. thermophilus* have been identified exclusively by genome sequence analysis. TLC analysis of carotenoid extracts revealed that lycopene is the main carotenoid produced by the Δ*crtY* strain. This result supports the sequence annotation of TT_P0060 as *crtY* (encoding lycopene β-cyclase). The colorless Δ*crtI* strain strongly suggests an interruption of the carotenoid synthesis pathway at a step before lycopene synthesis. In accordance with the sequence analysis, it is likely caused by a deficiency of phytoene desaturase. In this respect, *crtI* codes for phytoene desaturase of *T. thermophilus* HB27. The accumulation of β-carotene in the *T. thermophilus* Δ*CYP175A1* (TT_P0059) strain reflects the results obtained with enzyme assays with the *Thermus* β-carotene hydroxylase described previously (26). Interestingly, the carotenoid extract of the Δ*CYP175A1* strain revealed considerable amounts of lycopene, in addition to β-carotene, in TLC (see Fig. S4 in the supplemental material). This is most likely the result of an incomplete conversion of lycopene by CrtY.

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