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Polysaccharide-Degrading Thermophiles Generated by Heterologous Gene Expression in *Geobacillus kaustophilus* HTA426

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Thermophiles have important advantages over mesophiles as host organisms for high-temperature bioprocesses, functional production of thermostable enzymes, and efficient expression of enzymatic activities *in vivo*. To capitalize on these advantages of thermophiles, we describe here a new inducible gene expression system in the thermophile *Geobacillus kaustophilus* HTA426. Six promoter regions in the HTA426 genome were identified and analyzed for expression profiles using β-galactosidase reporter assay. This analysis identified a promoter region upstream of a putative amylose-metabolizing gene cluster that directed high-level expression of the reporter gene. The expression was >280-fold that without a promoter and was further enhanced 12-fold by maltose addition. In association with a multicopy plasmid, this promoter region was used to express heterologous genes. Several genes, including a gene whose product was insoluble when expressed in *Escherichia coli*, were successfully expressed as soluble proteins, with yields of 0.16 to 59 mg/liter, and conferred new functions to *G. kaustophilus* strains. Remarkably, cellulase and α-amylase genes conferred the ability to degrade cellulose paper and insoluble starch at high temperatures, respectively, generating thermophiles with the potential to degrade plant biomass. Our results demonstrate that this novel expression system expands the potential applications of *G. kaustophilus*.

Thermophiles enable microbial bioprocesses performed at high temperatures (high-temperature microbial processes [HTMPs]). Because HTMPs have many advantages compared to bioprocesses involving mesophiles, which require moderate temperatures, HTMPs have attracted attention over recent years. Two major advantages of HTMPs are that high temperature prevents the growth of human, animal, or plant pathogens and the thermophiles themselves are nonpathogenic. Pathogenic organisms, including all viruses, are killed or at least prevented from proliferating above 65°C (1), which is especially important in processes using sewage, municipal or agricultural waste, and materials from animal farms. A high temperature also facilitates easy removal of volatile products (e.g., ethanol and butanol) and maintenance of anaerobic conditions due to lower oxygen solubility. Because of these advantages, there are many practical examples of HTMPs, such as acetoin and 2,3-butanediol production by *Geobacillus* sp. strain XT15 (2), lactic acid production by *Bacillus coagulans* (3), hydrocarbon production by *Thermobrachium celere* (4), and ethanol production using *Geobacillus thermoglucosidasius* (5), *Thermoanaerobacterium saccharolyticum* (6), or *Thermoanaerobacter mathranii* (7, 8).

The genus *Geobacillus* comprises aerobic or facultative anaerobic, Gram-positive, thermophilic bacilli that have been reclassified from the genus *Bacillus* (9). Members of this genus are present in a wide range of environments and have many remarkable properties useful for HTMPs, such as ethanol tolerance (10), arsenate resistance (11), the ability to accumulate toxic metal ions (12), the ability to degrade either hydrocarbons (13), long-chain alkanes (14–16), herbicides (17), or polyvinyl alcohol (18), and the ability to utilize cellulose (19) or hemicelluloses (20). *G. kaustophilus* strain HTA426, isolated from deep-sea sediments of the Mariana Trench (21, 22), has also high potentials for various applications. This strain grows aerobically and at high cell density between 42 and 74°C (optimally at 60°C) as rapidly as either *Escherichia coli* or *Bacillus subtilis*. In addition, *G. kaustophilus* HTA426 can secrete proteins (23), utilize various carbon sources (24), and grow in media containing >3% NaCl (21, 22, 24). Whole-genome sequencing (25) has revealed that the HTA426 genome has medium GC content (52%) and 3,653 genes. The functions of many genes in strain HTA426 can be predicted on the basis of abundant knowledge of *Bacillus*-related species, such as *B. subtilis*.

With the goals of analyzing the properties of this genus and using it in practical applications, we established a plasmid transformation and counterselection system for the HTA426 strain (23, 24). Here we report the efficient and inducible expression of heterologous genes in HTA426 using the system that we have used to generate recombinant *G. kaustophilus* strains capable of digesting insoluble polysaccharides at high temperatures.

MATERIALS AND METHODS

Bacterial strains, media, plasmids, primers, and thermophile genes. *G. kaustophilus* strains are summarized in Table 1. See Table S1 in the supplemental material for primers used. Table 2 lists the thermophile genes used. *E. coli* JM109 (TaKaRa Bio) and pCR4Blunt-TOPO (Invitrogen) were used for DNA manipulation. *E. coli* BL21-CodonPlus(DE3)-RIL (Agilent Technology) and pET-16b (Novagen) were used for gene overexpression in *E. coli*. *E. coli* BR408 was used for conjugative DNA transfer

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TABLE 1 *Geobacillus kaustophilus* strains used in this study

Strain	Relevant description	Source or reference(s)
HTA426	Wild type	21, 22, 25
MK72	Δ*pyrF* Δ*pyrR*	23
MK93	Strain MK72 derivative constructed using pGAM48-*bgaB*, *GK0707*::P_{gk704}-*bgaB*	This study
MK95	Strain MK72 derivative, *GK0707*::P_{sigA}-*bgaB*	23
MK96	Strain MK72 derivative, *GK0707*::*bgaB*	23
MK111	Strain MK72 derivative constructed using pGAM50-*bgaB*, *GK0707*::P_{gk1894}-*bgaB*	This study
MK112	Strain MK72 derivative constructed using pGAM51-*bgaB*, *GK0707*::P_{gk1899}-*bgaB*	This study
MK115	Strain MK72 derivative constructed using pGAM53-*bgaB*, *GK0707*::P_{gk2150}-*bgaB*	This study
MK116	Strain MK72 derivative constructed using pGAM49-*bgaB*, *GK0707*::P_{gk1859}-*bgaB*	This study
MK118	Strain MK72 derivative constructed using pGAM52-*bgaB*, *GK0707*::P_{gk1907}-*bgaB*	This study
MK158	Strain MK72 derivative constructed using pGAM48-*PH1171sc*, *GK0707*::P_{gk704}-*PH1171sc*	This study
MK164	Strain MK72 derivative constructed using pGAM48-*PCAL1307*, *GK0707*::P_{gk704}-*PCAL1307*	This study
MK166	Strain MK72 derivative constructed using pGAM48-*amyE*, *GK0707*::P_{gk704}-*amyE*	This study
MK175	Strain MK72 harboring pSTE33T-*bgaB*	This study
MK194	Strain MK72 derivative constructed using pGAM48-*PH1171c*, *GK0707*::P_{gk704}-*PH1171c*	This study
MK204	Strain MK72 harboring pSTE33T-*PH1171c*	This study
MK205	Strain MK72 harboring pSTE33T-*GK3428*	This study
MK214	Strain MK72 harboring pSTE33T-*PCAL1307*	This study
MK217	Strain MK72 harboring pSTE33T-*amyE*	This study
MK248	Strain MK72 harboring pSTE33T-*ST0649*	This study
MK300	Strain MK72 harboring pSTE33T-*PH0380*	This study

to *G. kaustophilus* (24). *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium. Ampicillin (50 mg/liter), kanamycin (25 mg/liter), chloramphenicol (13 mg/liter), and tetracycline (6.5 mg/liter) were added when necessary. *G. kaustophilus* strains were grown at 60°C in LB, MM, or MY media. MM medium consisted of minimal medium elements (K_2SO_4, 0.3 g/liter; $Na_2HPO_4 \cdot 12H_2O$, 2.5 g/liter; NH_4Cl, 1 g/liter; 0.1% [vol/vol] trace element solution [26]; $MgSO_4$, 0.4 g/liter; $MnCl_2 \cdot 4H_2O$, 3 mg/liter; $CaCl_2 \cdot 2H_2O$, 5 mg/liter; $FeCl_3 \cdot 6H_2O$, 7 mg/liter; 10 mM Tris-HCl [pH 7.5]) and Casamino Acids (Difco) at 1 g/liter. MY medium consisted of minimal medium elements and 10 g of yeast extract (Difco)/liter. Kanamycin (5 mg/liter) and uracil (10 mg/liter) were added when necessary. The solid media contained 20 g of agar/liter. *E. coli-Geobacillus* shuttle plasmids, pGAM46 (to integrate in *GK0707* locus) and pSTE33T (capable of autonomous replication with 16 copies per chromosome), were constructed previously (23, 24). Plasmids pGAM46-*bgaB* and pGAM46-*amyE* (23) were used as sources for the *bgaB* and *amyE* genes, respectively. The *PH1171* and *PH0380* genes were derived from the *Pyrococcus horikoshii* OT3 (JCM 9974) chromosome and the *PCAL1307* and *ST0649* genes were derived from *Pyrobaculum calidifontis* JCM 11548 and *Sulfolobus tokodaii* strain 7 (JCM 10545) chromosomes, respectively.

Construction of *bgaB* reporter plasmids. Figure 1 shows the construction scheme of pGAM48-*bgaB*. A noncoding region (250 bp) upstream of a putative amylose-metabolizing gene cluster (P_{gk704}; Fig. 2A) was amplified by PCR using primers 0704F and 0704R. The amplified fragment was trimmed with HindIII and SphI and subcloned between the HindIII and SphI sites of pGAM46 to generate pGAM48. *bgaB* was excised with SphI and BamHI from pGAM46-*bgaB* and subcloned between the SphI and BamHI sites of pGAM48 to generate pGAM48-*bgaB*. Using this same strategy, putative promoter regions from the HTA426 genome were cloned upstream of the *bgaB* reporter gene in pGAM46 to generate pGAM49-*bgaB* (promoter region, P_{gk1859} upstream of the putative cellobiose-metabolizing genes *GK1856* to *GK1859*; promoter region length, 250 bp; primers used for PCR amplification, 1859F and 1859R), pGAM50-*bgaB* (P_{gk1894} upstream of *myo*-inositol-metabolizing genes *GK1887* to *GK1894* [27]; 300 bp; 1894F and 1894R), pGAM51-*bgaB* (P_{gk1899} upstream of *myo*-inositol-metabolizing genes *GK1896* to *GK1899* [27]; 260 bp; 1899F and 1899R), pGAM52-*bgaB* (P_{gk1907} upstream of putative L-arabinose-metabolizing genes *GK1904* to *GK1907*; 250 bp; 1907F and 1907R), and pGAM53-*bgaB* (P_{gk2150} upstream of putative D-galactose-metabolizing genes *GK2147* to *GK2150*; 250 bp; 2150F and 2150R).

Construction of pET-*PH0380*. The *PH0380* gene (Table 2) was amplified using primers 0380F and 0380R, trimmed with NdeI and BamHI,

TABLE 2 Thermophile genes used for expression in *Geobacillus kaustophilus*

Gene or locus tag	Product	Gene origin	GenBank ID	Reference(s)
bgaB	β-Galactosidase fused with His_6 tag at C terminus	*G. stearothermophilus*	AAA22262	52
amyE	α-Amylase (full length with the native secretion signal) fused with His_6 tag at C terminus	*G. stearothermophilus*	AAA22235	54
PH0380	Putative NTP transferase fused with His_{10} tag at N terminus	*P. horikoshii*	NP_142355	
PH1171c	Cellulase catalytic domain (codons 34 to 416 without the native secretion signal) fused with His_6 tag at C terminus	*P. horikoshii*	NP_143072	29, 50
PH1171sc	Cellulase catalytic domain (codons 1 to 416 with the native secretion signal) fused with His_6 tag at C terminus	*P. horikoshii*	NP_143072	29, 50
PCAL1307	Esterase fused with His_6 tag at C terminus	*P. calidifontis*	ABO08731	53
ST0649	D-Lactate dehydrogenase fused with His_6 tag at C terminus	*S. tokodaii*	BAB65648	55
GK3428	Putative azoreductase fused with His_6 tag at C terminus	*G. kaustophilus*	YP_149281	

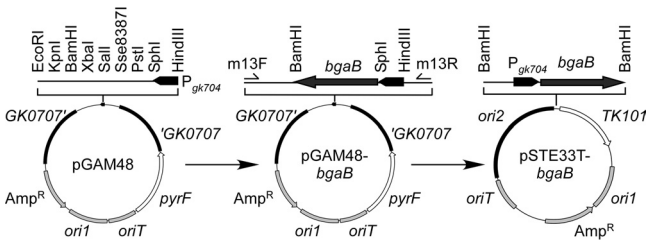

FIG 1 Construction of plasmids pGAM48-*bgaB* and pSTE33T-*bgaB*. The P_{gk704} region was cloned in pGAM46, which is an *E. coli-Geobacillus* plasmid to integrate the expression cassette to the *GK0707* locus (23), to give pGAM48. The *bgaB* gene was cloned in pGAM48, to give pGAM48-*bgaB*. The expression cassette was subcloned in pSTE33T, which is multicopy *E. coli-Geobacillus* shuttle plasmid capable of autonomous replication in *G. kaustophilus* (24) to give pSTE33T-*bgaB*. Amp^r, ampicillin resistance gene; *TK101*, kanamycin resistance gene; *ori1*, pUC replication origin for *E. coli*; *ori2*, pSTK1 replication origin for *Geobacillus*; *oriT*, conjugative transfer origin; *GK0707′*, *GK0707* upstream region; *′GK0707*, *GK0707* downstream region. The sites of primers of m13F and m13R (i.e., m13Rbam and m13Rbe) are also indicated.

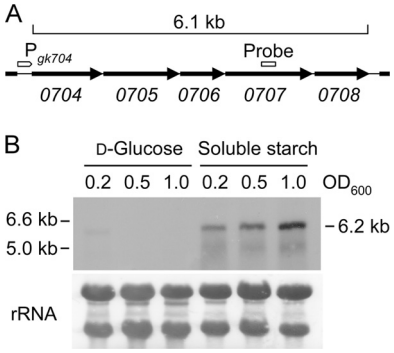

FIG 2 Amylose-inducible gene cluster in *G. kaustophilus* HTA426. (A) Organization of the gene cluster (*GK0704* to *GK0708*) and the P_{gk704} region. The *GK0704*, *GK0705*, and *GK0706* genes encode components of a sugar ABC transporter. The *GK0707* and *GK0708* genes encode α-amylase and a LacI family transcriptional repressor, respectively. The probe used for Northern blotting is also indicated. (B) Northern blotting of *GK0707* transcription. The cells were cultured in MM medium containing D-glucose or soluble starch until the OD_{600} reached 0.2, 0.5, or 1.0.

and subcloned between the NdeI and BamHI sites of pET-16b to generate pET-*PH0380*.

Construction of pGAM48 derivatives to integrate thermophile genes. Thermophile genes (Table 2) were amplified by PCR using the following primer pairs: *PH1171c* (codons 34 to 416), 1171F1 and 1171R; *PH1171sc* (codons 1 to 416), 1171F2 and 1171R; *PCAL1307* (full length), 1307F and 1307R; *amyE* (full length), amyF and amyR; *ST0649* (full length), 0649F and 0649R; and *GK3428* (full length), 3428F and 3428R. Amplified fragments were cloned in pCR4Blunt-TOPO, from which the *PH1171c*, *PH1171sc*, *PCAL1307*, and *ST0649* genes were excised with SphI and BamHI and subcloned between the SphI and BamHI sites of pGAM48 to give pGAM48-*PH1171c*, pGAM48-*PH1171sc*, pGAM48-*PCAL1307*, and pGAM48-*ST0649*, respectively. The *GK3428* gene was excised with PstI and BamHI and subcloned between the PstI and BamHI sites of pGAM48 to generate pGAM48-*GK3428*. *amyE* was excised with SphI and BglII and subcloned between the SphI and BamHI sites of pGAM48 to generate pGAM48-*amyE*. *PH0380* with His_{10} tag codons was amplified from pET-*PH0380* using primers t7sph and 0380R; the amplified fragment was trimmed with SphI and BamHI and subcloned between the SphI and BamHI sites of pGAM48 to generate pGAM48-*PH0380*.

Construction of pSTE33T derivatives to overexpress thermophile genes. Expression cassettes (P_{gk704}-gene) of pGAM48-*bgaB*, pGAM48-*PH1171c*, pGAM48-*PH1171sc*, pGAM48-*PCAL1307*, pGAM48-*ST0649*, pGAM48-*GK3428*, and pGAM48-*PH0380* were amplified using the primers m13Rbam and m13F, trimmed with BamHI, and subcloned into the BamHI site of pSTE33T with the given orientation, to obtain pSTE33T-*bgaB* (Fig. 1), pSTE33T-*PH1171c*, pSTE33T-*PH1171sc*, pSTE33T-*PCAL1307*, pSTE33T-*ST0649*, pSTE33T-*GK3428*, and pSTE33T-*PH0380*, respectively. The expression cassette of pGAM48-*amyE* was amplified using the primers m13Rbe and amyR, trimmed with BglII, and subcloned into the BamHI site of pSTE33T to generate pSTE33T-*amyE*.

Plasmid introduction and gene integration. Plasmids were introduced into *G. kaustophilus* by conjugative DNA transfer from *E. coli* BR408 (24). *E. coli* donors (10 ml) and *G. kaustophilus* recipients (100 ml) were grown in LB media to an optical density at 600 nm (OD_{600}) of 0.3. Cultures were mixed, centrifuged to concentrate cells, and spotted at high density on LB plates. After incubation at 37°C for 4 h, the resultant cells were collected and incubated at 60°C on appropriate media to isolate *G. kaustophilus* transconjugants. Gene expression cassettes in pGAM plasmids were integrated into the *GK0707* locus by *pyrF*-based counterselection, as described previously (23).

Transcription analysis. *G. kaustophilus* HTA426 was cultured in MM medium supplemented with either D-glucose or soluble starch. Total RNA was isolated from the cells and analyzed by Northern blotting using a digoxigenin-labeled probe to detect *GK0707* transcripts (Fig. 2A). The probe was synthesized using a DIG RNA labeling kit (Roche). The hybridized RNA was detected using a DIG luminescence detection kit (Roche).

Promoter assay using *bgaB* reporter strains. *G. kaustophilus* strains were cultured in MM liquid medium containing varied sugar until an OD_{600} of 1.0 (OD_{600} in stationary phase = 1.0 to 1.5). Harvested cells were sonicated in 50 mM sodium phosphate (pH 6.0) and centrifuged to obtain a clear lysate. The β-galactosidase activity in crude extracts was determined using *p*-nitrophenyl-β-D-galactopyranoside as the substrate. The reaction mixture (100 μl) contained 50 mM sodium phosphate (pH 6.0) and 2 mM *p*-nitrophenyl-β-D-galactopyranoside. After incubation at 60°C, reactions were terminated by the addition of ice-cold 2 M sodium carbonate (100 μl). *p*-Nitrophenol release was measured by the absorbance at 405 nm with reference to an experimentally derived standard curve. One unit was defined as the amount of enzyme required to generate 1 nmol of *p*-nitrophenol/min at 60°C.

Inducing gene expression using MY medium. *G. kaustophilus* strains were cultured in MY liquid medium until reaching an OD_{600} of 0.5. After supplementation with maltose to 1% (wt/vol), the cultures were further incubated for 20 h.

***PH0380* expression in *E. coli*.** *E. coli* BL21-CodonPlus(DE3)-RIL harboring pET-*PH0380* was cultured in LB medium until reaching an OD_{600} of 0.5. After IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM, cultures were further incubated for 4 h.

Enzyme purification. Cells were harvested by centrifugation (5,000 × *g* for 5 min) and resuspended in buffer (20 mM sodium phosphate [pH 7.4] and 0.5 M NaCl). After cell disruption by sonication, cell lysates were clarified by centrifugation and applied to a HisTrap HP column (1 ml; GE Healthcare) equilibrated with the buffer containing 40 mM imidazole at a flow rate of 1 ml/min using the ÄKTAprime system (GE Healthcare). Lysate-primed columns were then washed with 5 ml of the same buffer, followed by 10 ml of buffer containing 0.1 M imidazole. Proteins were eluted with a linear gradient of 0.1 to 0.5 M imidazole in the buffer over 10 ml. The protein concentration was determined using a protein assay kit (Bio-Rad) with bovine serum albumin as the standard. Protein purity was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A molecular weight marker (Bio-Rad) was used as the standard.

RESULTS

Identification of amylose-induced genes. Because the promoter region upstream of α-amylase gene serves as an efficient promoter

in *B. subtilis* (28), we explored amylose-metabolizing gene cluster in the HTA426 genome and identified genes *GK0704* to *GK0708* (Fig. 2A). Northern blotting showed that *GK0707* was transcribed in strain HTA426 when cultured in MM medium containing soluble starch but not in MM supplemented with D-glucose (Fig. 2B). *GK0707* transcripts were 6.2 kb, in good agreement with the length of *GK0704* to *GK0708*. These results suggested that the expression of genes *GK0704* to *GK0708* was amylose inducible and that the P_{gk704} region upstream of the genes contained an amylose-induced promoter.

Promoter assay of the P_{gk704} region. The promoter profile of the P_{gk704} region was analyzed using the *bgaB* reporter assay in strain MK93, which harbored the reporter cassette P_{gk704}-*bgaB* integrated in the chromosome. Cells were cultured in MM liquid medium supplemented with varied sugar until the late logarithmic phase. Although D-fructose, melibiose, and lactose were not effectively utilized by *G. kaustophilus*, cells grew to an OD_{600} of 1.0 for 4 to 6 h in all culture conditions due to Casamino Acids contained in the medium. As a reference, the profile of the P_{sigA} region upstream of housekeeping genes *rpoB* and *dnaG*, which was identified previously as a promoter region (23), was analyzed using strain MK95. The BgaB-specific activity in individual cells is summarized in Table 3. When strain MK93 was cultured in MM medium, the specific activity was 28 ± 1 U/mg protein. Because the activity of the negative control strain MK96 was <0.1 U/mg protein, the value indicated that the P_{gk704} region directed *bgaB* expression >280-fold higher than the background. The BgaB activity was further increased 4.5- and 12-fold by addition of either soluble starch or maltose to the culture medium, respectively. BgaB activity was decreased by addition of the pentose sugars D-xylose or L-arabinose, whereas D-glucose, D-galactose, sucrose, melibiose, lactose, *myo*-inositol, cellobiose, or fructose had negligible effects. The *bgaB* expression was also observed by culturing in LB medium. Overall, BgaB activity was higher in strain MK93 than in strain MK95. These results indicated that the P_{gk704} region contained an efficient promoter that functioned under multiple conditions and was substantially inducible by amylose and maltose.

Promoter assay of the P_{gk1859}, P_{gk1894}, P_{gk1899}, P_{gk1907}, and P_{gk2150} regions. We predicted putative promoter regions in the HTA426 genome outside the P_{gk704} region and analyzed their expression profiles using the *bgaB* reporter assay (Table 3). The P_{gk1899} region contained a strong but constitutive promoter, comparable in activity to P_{gk704}. Although the P_{gk1859}, P_{gk1894}, and P_{gk2150} regions contained promoters that were inducible by lactose, *myo*-inositol, or D-galactose, respectively, their maximal activity was less than the P_{gk704} region. The P_{gk1907}-driven expression was extremely low under all conditions examined. Because of its strength and inducibility, we chose the P_{gk704} region as the inducible promoter for further study.

Inducible BgaB activity in strain MK93. The inducibility of *bgaB* expression was examined using MY medium. Strain MK93 was first cultured in MY medium and then exposed to maltose for induction. After induction, BgaB activity increased with cultivation time (Fig. 3) and was 1,900 ± 990 U/mg protein after 20 h of maltose induction. Without induction, BgaB activity was 88 ± 37 U/mg protein.

Inducible BgaB activity in strain MK175. To achieve higher *bgaB* expression in *G. kaustophilus*, we constructed strain MK175 harboring the P_{gk704}-*bgaB* cassette on the multicopy plasmid

TABLE 3 β-Galactosidase activity in *bgaB* reporter strains cultured in MM medium supplemented with sugar additives[a]

Strain	Promoter	Possible inducer	Mean β-galactosidase activity (U/mg protein) ± SD with various sugar additives											
			None	D-Glucose	D-Galactose	D-Fructose	D-Xylose	L-Arabinose	*myo*-Inositol	Maltose	Cellobiose	Melibiose	Sucrose	Lactose	LB[b]
MK93	P_{gk704}	Maltose	28 ± 1	31 ± 13	23 ± 2	26 ± 2	3.3 ± 0.0	9.2 ± 0.6	25 ± 1	330 ± 10	30 ± 1	31 ± 3	27 ± 1	21 ± 2	15 ± 8
MK95	P_{sigA}		3.1 ± 0.1	4.2 ± 0.3	2.2 ± 0.7	3.4 ± 0.5	0.8 ± 0.1	1.8 ± 0.1	2.3 ± 0.6	16 ± 1	2.3 ± 0.4	3.5 ± 0.5	3.2 ± 0.2	2.8 ± 0.2	4.4 ± 1.1
MK96	None		<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
MK111	P_{gk1894}	*myo*-Inositol	32 ± 6	18 ± 5	19 ± 2	28 ± 3	1.2 ± 0.1	3.0 ± 0.5	120 ± 30	12 ± 1	26 ± 3	32 ± 9	16 ± 3	14 ± 1	0.4 ± 0.2
MK112	P_{gk1899}	*myo*-Inositol	180 ± 30	140 ± 7	140 ± 10	140 ± 20	16 ± 1	40 ± 1	440 ± 10	130 ± 20	200 ± 10	160 ± 30	160 ± 10	130 ± 10	41 ± 12
MK115	P_{gk2150}	D-Galactose	2.1 ± 0.2	1.6 ± 0.3	19 ± 4	1.9 ± 0.2	0.4 ± 0.0	1.7 ± 0.6	1.4 ± 0.2	1.7 ± 0.2	1.2 ± 0.1	3.7 ± 0.3	1.5 ± 0.2	6.8 ± 0.2	1.2 ± 0.3
MK116	P_{gk1859}	Cellobiose	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	1.8 ± 0.2	0.1 ± 0.0
MK118	P_{gk1907}	L-Arabinose	0.7 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.1

[a] *G. kaustophilus* strains were cultured in medium indicated until the late logarithmic phase and analyzed for β-galactosidase activity. The values are means ± the SD from four independent experiments.
[b] LB medium.

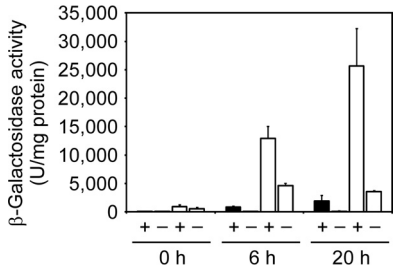

FIG 3 β-Galactosidase-specific activity in MK93 (solid bars) and MK175 (open bars) cells cultured with (+) or without (−) maltose. *G. kaustophilus* strains were cultured in MY medium and induced with maltose. Cells were further incubated for the periods indicated and analyzed for BgaB activity. The values are means ± the SD from four independent experiments.

pSTE33T. When MK175 was cultured in MM medium containing maltose, BgaB activity was 11,000 ± 1,900 U/mg protein. BgaB activity was further enhanced by maltose induction in MY medium, which resulted in 26,000 ± 6,600 U/mg protein after additional cultivation for 20 h (Fig. 3). BgaB protein was visible as a prominent band on SDS-PAGE analysis of crude extracts (Fig. 4A). Purification of BgaB using immobilized metal affinity chromatography resulted in a yield of 28 mg/liter of culture.

Expression of thermophile genes in *G. kaustophilus*. Several thermophile genes were cloned into pSTE33T along with the P_{gk704} region and expressed in *G. kaustophilus* under inducing conditions using MY medium (Fig. 4). The *PH1171c* gene encoded catalytic domain without the native secretion signal at N terminus or membrane anchor domain at C terminus (29). The *amyE* gene encoded full-length protein with a possible secretion signal at N terminus. The *bgaB*, *PCAL1307*, *ST0649*, and *GK3428* genes encoded full-length proteins, which did not contain possible secretion signals. All genes were fused with His_6 tag codons in the 3′ region. Although *PH1171sc* that encoded catalytic domain with the native secretion signal was successfully integrated in the *G. kaustophilus* chromosome to give strain MK158, we could not

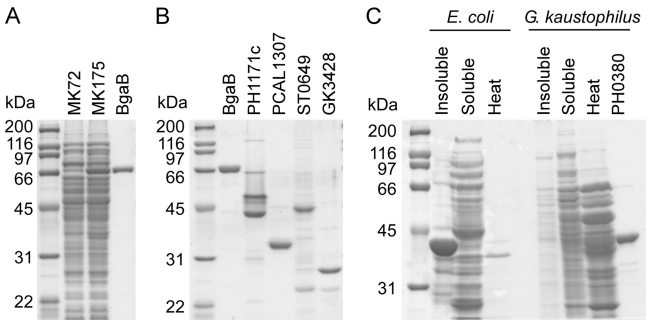

FIG 4 SDS-PAGE analysis of recombinant proteins produced in *G. kaustophilus*. (A) *bgaB* expression in strain MK175 under inducible conditions using MY medium. Proteins (10 μg) from MK72 and MK175 crude proteins and BgaB protein (10 μg) purified from MK175 crude proteins were analyzed. (B) Expression of thermophile genes in *G. kaustophilus*. Recombinant BagB (78 kDa), PH1171c (44 kDa), PCAL1307 (34 kDa), ST0649 (50 kDa), and GK3428 (24 kDa) proteins were purified from MK175, MK204, MK214, MK248, and MK205 cells, respectively. (C) *PH0380* expression in *E. coli* and *G. kaustophilus*. Insoluble and soluble proteins were prepared from *E. coli* cells harboring pET-*PH0380*. Soluble proteins were treated at 80°C for 20 min. Insoluble and soluble proteins were prepared from MK300 cells. Extracts were treated at 80°C for 20 min and used to purify PH0380 protein (37 kDa).

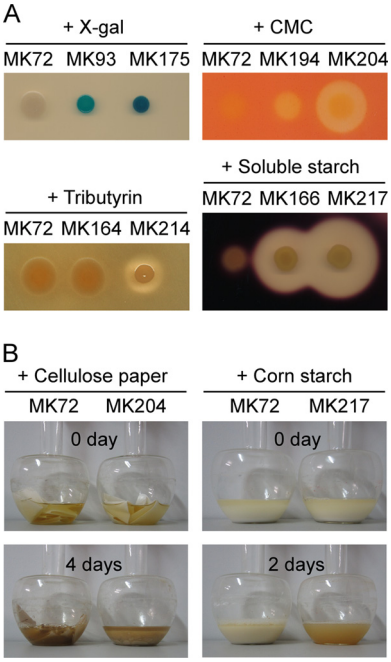

FIG 5 *In vivo* assay of heterologous gene expression in *G. kaustophilus* (A) and degradation of insoluble polysaccharides by recombinant *G. kaustophilus* cells (B). (A) The β-galactosidase activity in MK93 and MK175 cells was analyzed by 24 h of incubation on LB plates containing 200 mg of X-Gal/liter. Cellulase activity in MK194 and MK204 cells was analyzed by 48 h of incubation on LB plates containing 1% CMC. Residual CMC was stained with Congo red (51). Esterase activity in strains MK164 and MK214 was analyzed by 24 h of incubation on LB plates with 1% tributyrin. α-Amylase activity in MK166 and MK217 cells was analyzed by incubating on a LB plate with 1% soluble starch for 24 h. Residual starch was stained with I_2/KI solution. Strain MK72 was used as a negative control. (B) Strain MK204 was cultured for 4 days in MY medium with 0.3% (wt/vol) cellulose papers under inducing conditions. Strain MK217 was cultured for 2 days in LB medium containing 10% (wt/vol) corn starch. Strain MK72 was used as a negative control.

transform *G. kaustophilus* by pSTE33T-*PH1171sc*. The *PCAL1307*, *ST0649*, and *GK3428* genes were expressed as soluble proteins with yields of 0.88, 0.28, and 0.16 mg of purified protein per liter, respectively. The *PH1171c* gene was expressed with an apparent yield of 59 mg/liter, although the preparation contained unidentified contaminants with larger molecular weights than PH1171c. We also examined expression of *PH0380*, *PH1697* (GenBank ID: NP_143540), and *PH1219* (NP_143114) genes, which were expressed as insoluble proteins in *E. coli*. Of these, *PH0380* was successfully expressed in *G. kaustophilus* as a soluble protein, with a yield of 3.2 mg/liter (Fig. 4C). Strain MK217 cells lysed after maltose induction, resulting in neither intracellular nor extracellular production of AmyE. However, this strain produced AmyE extracellularly in LB medium (see below).

***In vivo* assay of heterologous gene expression.** *In vivo* functional expression of the *bgaB*, *PH1171c*, *PCAL1307*, and *amyE* genes in *G. kaustophilus* was confirmed using indicator plates (Fig. 5A). After incubation at 60°C, strains MK93 and MK175 both produced blue pigments on LB plates containing the BgaB substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Strain MK204 degraded carboxymethyl cellulose (CMC) and strain MK194 weakly degraded CMC. Strain MK214 but not MK164 degraded tributyrin. Strains MK166 and MK217 both de-

graded soluble starch. Overall, the pSTE33T expression plasmid was associated with stronger activity than expression cassettes integrated into the chromosome.

Comparison of PH1171sc and PH1171c production. Intracellular and extracellular production of PH1171sc and PH1171c were analyzed using strains MK158 and MK194, respectively (see Fig. S1 in the supplemental material). PH1171sc was produced predominantly in the culture supernatant. PH1171c was produced intracellularly and extracellularly at comparable levels.

Paper and starch degradation by *G. kaustophilus*. We examined cellulose paper and starch degradation by strains MK204 and MK217, respectively (Fig. 5B). Strain MK204 was cultured under inducing conditions in MY medium containing cellulose paper. After incubation for 4 days, the cellulose paper had been completely broken down. Because of cell lysis under inducing conditions (see above), strain MK217 was cultured in LB medium containing corn starch. Starch was clearly degraded after incubation for 2 days.

DISCUSSION

Although many different thermophiles have been successfully transformed with plasmids, efficient promoters for gene expression have been characterized for only a few thermophiles. In the thermophilic archaeon *S. solfataricus*, *tf55* and *araS* promoters have been used to express four homologous and heterologous genes (30, 31), with yields of 0.5 to 1.0 mg of purified protein per liter. In *S. acidocaldarius*, a heterologous gene from *S. solfataricus* is expressed from the *mal* promoter with a yield of 1 mg of purified protein per liter (32). Although the mass yields of recombinant proteins are not specified, thermophile genes are also functionally expressed from the *csg* and *gdh* promoters in *Thermococcus kodakarensis* (33–36), the *nar* and *slp* promoters in the Gram-negative bacterium *Thermus thermophilus* (37–42), and the *ldh* and *pfl* promoters in the Gram-positive bacterium *G. thermoglucosidasius* (5). The *pheA* promoter from *G. thermoglucosidasius* acts as an efficient promoter in *E. coli* (43), implying its functionality in *Geobacillus* spp. However, considering that gene expression in mesophiles provides typically more than 10 mg of recombinant protein per liter (44), there is considerable room for improvement in the efficiency of thermophile expression systems.

In the present study, we identified strong, inducible promoter activity in the P_{gk704} region upstream of the *GK0704* to *GK0708* genes of *G. kaustophilus* HTA426. Northern blotting indicated that the region contained an amylose-inducible promoter. The *bgaB* reporter assay showed that the region drove *bgaB* expression in LB and MM media. As expected, expression was substantially induced in MM medium containing amylose or maltose. BgaB activity after cultivation with maltose or D-xylose was 330 ± 10 and 3.3 ± 0.0 U/mg protein, respectively, indicating that the P_{gk704}-directed expression could be controlled over a range of ~100-fold. *bgaB* expression was further enhanced after optimization of the culture conditions. Using MY medium and maltose induction, BgaB protein was expressed with the specific activity of 1,900 ± 990 U/mg protein. Moreover, expression from a multicopy plasmid resulted in BgaB-specific activity of 26,000 ± 6,600 U/mg protein and a mass yield of 28 mg of purified protein per liter.

This system enabled expression of several thermophile genes as soluble proteins. In addition to BgaB protein, PH1171c protein was expressed with a yield of >20 mg/liter. The expression level is far higher than those previously reported for other thermophiles (see above). Possible reasons for these high yields include the efficiency of this expression system, the high cell density of the cultures, and the moderate GC content of the *G. kaustophilus* chromosome, which enables the expression of a wide range of genes. In addition to the high yields, expression analysis resulted in two important observations for the *G. kaustophilus* expression system. One is that the system produced soluble PH0380, which was insoluble when expressed in *E. coli* (Fig. 4C). This is attributable to high temperatures during *G. kaustophilus* cultivation, because some thermophile enzymes require high temperatures (>60°C) for correct protein folding into catalytically active forms (45–48). This result suggests that *G. kaustophilus* may be generally useful for expression of gene products that are insoluble when expressed in *E. coli*. The other is that AmyE and PH1171 proteins were produced extracellularly. Enzymatic assay showed that AmyE protein was present in culture supernatant of strain MK166 (data not shown) in agreement with our previous results (23). Strains MK158 produced PH1171sc protein predominantly in culture supernatant (see Fig. S1 in the supplemental material). These results suggest that bacterial and archaeal secretion signals can function in *G. kaustophilus*. Moreover, PH1171c was produced in the supernatant of strain MK194 in spite of lacking the secretion signal. Although this might arise from cell lysis, this result implies that nonsecretory proteins may become extracellular in this system through unidentified secretion mechanisms.

A few thermophiles have been used as host organisms for HTMPs involving overproduction of useful thermostable enzymes. Examples include protein degradation by *T. kodakarensis* cells genetically modified to overproduce thermostable proteases (35) and ethanol production by *G. thermoglucosidasius* cells that overproduce thermostable pyruvate dehydrogenase (5). The expression of heterologous genes in *G. kaustophilus* generated notable thermophiles capable of degrading plant biomass at high temperatures, e.g., strain MK204 to degrade cellulose paper (Fig. 5B) and MK217 to degrade insoluble corn starch. MK204 is particularly remarkable because it is the first thermophile capable of degrading cellulose paper, although *Clostridium thermocellum* can anaerobically utilize celluloses at 60°C (49). High temperature is important for this degradation because PH1171c can degrade crystal celluloses without involving other enzymes under high temperature conditions (50) and starch is more soluble at high temperatures. Thus, the combination of *G. kaustophilus*, its genetic tools, and useful thermostable enzymes provides great opportunities to generate new and practical HTMPs. The results presented here demonstrate that this expression system expands the potential uses for *G. kaustophilus* HTA426-derived strains as host organisms for HTMPs or a variety of other practical applications.

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